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# A role for differential Rubisco activase isoform expression in $C_4$ bioenergy grasses at high temperature

**Sang Yeol Kim**[1,2] | **Rebecca A. Slattery**[2] | **Donald R. Ort**[1,2,3]

[1]Department of Plant Biology, University of Illinois, Urbana, IL, USA

[2]Carl R. Woese Institute for Genomic Biology, University of Illinois, Urbana, IL, USA

[3]Department of Crop Sciences, University of Illinois, Urbana, IL, USA

**Correspondence**
Donald R. Ort, Department of Plant Biology, University of Illinois, Urbana, IL 61801, USA.
Email: d-ort@illinois.edu

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**Abstract**

Rubisco activase (Rca) facilitates the release of sugar-phosphate inhibitors at Rubisco catalytic sites during $CO_2$ fixation. Most plant species express two Rca isoforms, the larger Rca-α and the shorter Rca-β, either by alternative splicing from a single gene or expression from separate genes. The mechanism of Rubisco activation by Rca isoforms has been intensively studied in $C_3$ plants. However, the functional role of Rca in $C_4$ plants where Rubisco and Rca are located in a much higher [$CO_2$] compartment is less clear. In this study, we selected four $C_4$ bioenergy grasses and the model $C_4$ grass setaria (*Setaria viridis*) to investigate the role of Rca in $C_4$ photosynthesis. All five $C_4$ grass species contained two *Rca* genes, one encoding Rca-α and the other Rca-β, which were positioned closely together in the genomes. A variety of abiotic stress-related motifs were identified in the *Rca-α* promoter of each grass, and while the *Rca-β* gene was constantly highly expressed at ambient temperature, Rca-α isoforms were expressed only at high temperature but never surpassed 30% of Rca-β content. The pattern of Rca-α induction on transition to high temperature and reduction on return to ambient temperature was the same in all five $C_4$ grasses. In sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), and setaria, the induction rate of Rca-α was similar to the recovery rate of photosynthesis and Rubisco activation at high temperature. This association between Rca-α isoform expression and maintenance of Rubisco activation at high temperature suggests that Rca-α has a functional thermo-protective role in carbon fixation in $C_4$ grasses by sustaining Rubisco activation at high temperature.

**KEYWORDS**

$C_4$ bioenergy grass, carbon fixation, heat stress, photosynthesis, Rubisco, Rubisco activase, Rubisco activation

## 1 | INTRODUCTION

Rubisco requires carbamylation at a lysine residue (K201) within the catalytic site to allow $Mg^{2+}$ binding for activation and $CO_2$ fixation (Cleland et al., 1998). Rubisco incorporates a $CO_2$ molecule into ribulose-1,5-bisphosphate (RuBP) to generate two molecules of 3-phosphoglyceric acid, which are converted into carbohydrate using photosynthetically produced adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate. Sugar-phosphate

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molecules, such as xylulose-1,5-bisphosphate (XuBP) and 2-carboxy-D-arabinitol-1-phosphate (CA1P), can also bind to Rubisco catalytic sites and interfere with the process of carbamylation (Khan et al., 1999; Zhu & Jensen, 1991). XuBP does not bind tightly to carbamylated sites but does bind tightly to decarbamylated sites. CA1P is a naturally occurring analog of the carboxylase substrate and binds tightly to the active site of Rubisco, inhibiting activity of the enzyme (Berry et al., 1987; Gutteridge et al., 1986). RuBP can also be an inhibitor by binding decarbamylated Rubisco (Jordan & Chollet, 1983; Parry et al., 2008).

Rubisco activase (Rca) facilitates the removal of these inhibitory sugar-phosphates to allow Rubisco activation. Rca is an AAA+ family protein that hydrolyzes ATP to drive protein remodeling (Bhat et al., 2017; Mueller-Cajar, 2017; Portis et al., 2008). Rca binds Rubisco to induce a conformational change in Rubisco structure, thereby facilitating the release of tightly bound inhibitors and maintaining Rubisco activity. Recent work shows a hexamer of prokaryote Rca (*Rhodobacter sphaeroides*) positions the C-terminal tail of a Rubisco large subunit and transiently pulls it into the Rca hexamer pore, thereby enabling the release of inhibitory sugar-phosphates from the Rubisco catalytic site (Bhat et al., 2017; Bracher et al., 2017). Many plant species express both Rca-α and Rca-β isoforms by either alternative splicing from a single gene or from separate genes. The Rca-α isoforms are sensitive to redox regulation due to the C-terminal extension (CTE) that contains two conserved redox-sensing cysteine residues (Zhang & Portis, 1999). In Arabidopsis (*Arabidopsis thaliana*), phosphorylation of Rca at the Thr78 site also occurs under low light and darkness, which plays an additional regulatory role in photosynthesis in vivo (Kim et al., 2016, 2019).

Photosynthesis is susceptible to inhibition by moderate heat stress (Berry & Bjorkman, 1980). The inhibition of photosynthesis by high temperature is closely related to decreased Rubisco activation, which is caused by impairment in Rca function (Portis, 2003; Salvucci & Crafts-Brandner, 2004). Thus, the rate of Rubisco inactivation can exceed Rca restorative capacity at high temperature. Decades of research studying Rca function at high temperature in various plant species has led to a greater understanding but also revealed new questions (CraftsBrandner et al., 1997; Crafts-Brandner & Salvucci, 2002; Hendrickson et al., 2008; Kumar et al., 2016; Kurek et al., 2007; Law & Crafts-Brandner, 2001; Perdomo et al., 2017; Ristic et al., 2009; Scafaro et al., 2018; Sharkey et al., 2001; Yamori et al., 2012). In some plants, different sizes of Rca isoforms are produced at elevated growth temperature, suggesting high temperature regulates alternative splicing to increase *Rca* variants, which may affect Rubisco activity at high temperature (CraftsBrandner et al., 1997; Law & Crafts-Brandner, 2001). In Arabidopsis, increased thermostability of the Rca protein improves the photosynthetic rate and plant growth under heat stress (Kumar et al., 2009; Kurek et al., 2007). Moreover, transgenic rice (*Oryza sativa*) overexpressing maize (*Zea mays*) Rca-β show increased Rubisco activation and photosynthetic rates at high temperature (Yamori et al., 2012). These findings suggest that the expression of specific Rca isoforms can be a critical determinant of photosynthetic rate at high temperature. However, in some plants, electron transport capacity is the primary factor limiting photosynthetic rate during heat stress, rather than the direct impact on Rca activity (Sage & Kubien, 2007; Schrader et al., 2004; Sharkey, 2005; Wise et al., 2004). Therefore, in $C_3$ plants, the key limitations to photosynthesis at high temperature could be either Rca activity or electron transport, depending on plant species.

Unlike $C_3$ species, $C_4$ plants exhibit different limitations to photosynthetic rates in different environmental conditions (Yamori et al., 2014). In $C_4$ photosynthesis, phosphoenolpyruvate (PEP) carboxylase (PEPC) catalyzes the carboxylation of PEP in mesophyll cells to produce oxaloacetate, the four-carbon acid after which $C_4$ photosynthesis is named (Hatch & Slack, 1966). Therefore, photosynthetic rate at low $CO_2$ concentration is determined by $CO_2$ diffusion and activities of carbonic anhydrase and PEPC. Rubisco activity and RuBP regeneration become significant regulators of $C_4$ photosynthetic rate in high $CO_2$ environments (Caemmerer & Furbank, 2016). Although it is still unclear what limits $C_4$ photosynthesis at high temperature, electron transport rate and RuBP regeneration have been considered potential limitations during heat stress (Dwyer et al., 2007; Kubien et al., 2003; Pittermann & Sage, 2001; Sage, 2002). One study using RNAi transgenics of *Flaveria bidentis* (a $C_4$ dicot) with reduced levels of *Rca* transcript expression (Hendrickson et al., 2008) showed that reduction of Rubisco activity at high temperature (~40°C) was not related to the amount of Rca. However, another report concluded that intrinsic heat sensitivity of Rca is linked with decreased Rubisco activation in both $C_3$ and $C_4$ grasses (Perdomo et al., 2017). Thus, the basic response and functional role of $C_4$ Rca in sustaining Rubisco activation and photosynthetic rate at high temperature are unclear.

In this work, we analyzed gene structures and motifs in the promoters of *Rca* genes in four $C_4$ bioenergy grasses (sorghum [*Sorghum bicolor*], maize, sugarcane [*Saccharum officinarum*], and miscanthus [*Miscanthus sinensis*]) and the model $C_4$ grass setaria (*Setaria viridis*). All five $C_4$ plants contained two *Rca* genes, one encoding Rca-α and the other Rca-β, closely positioned in the genome. Due to a variety of stress-related motifs in *Rca-α* gene promoters, we examined Rca isoform expression under various stress conditions. At ambient growth temperature (~25°C), only Rca-β isoforms were expressed, whereas high temperature (~42°C) induced gradual Rca-α isoform accumulation which again decreased when temperature returned to the growth temperature. The

Rca-α induction profile was similar to the recovery profile of both $CO_2$ assimilation and Rubisco activation after a shift from ambient to high temperature. Our data suggest that Rca-α plays a central role in sustaining photosynthesis in $C_4$ grasses at high temperature by modulating either Rubisco activation activity and/or Rca stability.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and growth conditions

Seeds of five $C_4$ grasses (sorghum, setaria, maize, sugarcane, and miscanthus) were first sterilized for 5 min in 1% (v/v) bleach and rinsed with 70% EtOH and dried on filter paper. The seeds were then directly sown in pots (20 cm diameter) filled with soil (www.berger.ca, BM, custom blended) and germinated in a growth chamber (long-day photoperiod, 16/8 = light/dark, 25°C, photosynthetic photon flux density [PPFD] of 400 μmol $m^{-2}$ $s^{-1}$). Abiotic stress treatments were imposed 20 days after germination (DAG). The stress conditions were as follows: water deficiency for 7 and 14 days for drought stress, whereas control plants were supplied with water every 3 days; 100 mM NaCl solution for 6 and 24 hr for salt stress, 42°C for 6 and 24 hr for heat stress, and 4°C for 6 and 24 hr for cold stress. Arabidopsis Col-0 seeds were sterilized for 5 min in 1% (v/v) bleach and rinsed with 70% EtOH and dried on filter paper. The sterilized seeds were sown on MS (Murashige and Skoog basal salt mixture powder, Phyto Technology Laboratories, M524) agar (0.8%, w/v, A1296; Sigma-Aldrich) with 1% (w/v) sucrose. The plates were stored at 4°C for 2 days for cold stratification before transfer to a growth chamber. Seedlings were transferred into pots (5 cm diameter) filled with soil (www.berger.ca, BM, custom blended) before the third true leaf emerged, which was approximately 5–7 DAG. Plants were grown at either 22°C or 42°C in long-day photoperiod (16 hr light and 8 hr dark, 400 μmol $m^{-2}$ $s^{-1}$ PPFD).

### 2.2 | Genome database and promoter analyses

*Rca* genes and promoter sequences of five $C_4$ grasses (sorghum, setaria, maize, and miscanthus) were obtained from two databases (http://www.ncbi.nlm.nih.gov and https://phytozome.jgi.doe.gov) and sugarcane *Rca* data were acquired from https://sugarcane-genome.cirad.fr/. The 1.5 kb upstream promoter sequences of transcriptional start sites (TSSs) were analyzed using the PLANTCARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify critical motifs and *cis*-acting elements.

### 2.3 | RNA extraction

Total RNAs were extracted from the fifth to sixth fully expanded leaves following the stress treatments listed above as follows. Leaf discs were ground in liquid nitrogen into a fine powder using a pre-chilled mortar and pestle. Then 1.2 ml of extraction buffer (100 mM Tris-HCl pH 9.5, 150 mM NaCl, 1.0% sarkosyl, 5 mM dithiothreitol (DTT)) was added before the powder thawed. After mixing, the homogenate was transferred into a 2 ml tube and vortexed vigorously for 5 min and then centrifuged for 5 min at 11,000 × g. The resulting supernatant was transferred to a new 2 ml microfuge tube, and 0.5 volume of chloroform was added. This was then vortexed for 2 min, and 0.5 volume of acid phenol was added and vortexed for another 2 min followed by centrifugation at 11,000 × g for 15 min. The upper aqueous phase (avoiding the interface) was carefully removed and transferred to an RNase-free 1.5 ml microfuge tube, and ~90 μl 3 M sodium acetate (pH 5.2) and ~600 μl isopropanol were added and mixed. After incubation for 10 min and centrifugation at 11,000 × g for 10 min, the upper aqueous phase was removed and the pellet was washed with 1 ml 75% ethanol. This was then centrifuged at 11,000 × g for 10 min, and the supernatant was discarded. The pellet was then air-dried briefly. The pellet was resuspended in 1 ml TRIzol reagent and vortexed until the pellet was completely dissolved. Chloroform (200 μl) was added and mixed by shaking vigorously for 15 s. After incubation for 2–3 min, the mixture was centrifuged at 11,000 × g at 4°C for 15 min. The upper aqueous phase was transferred to an RNase-free 1.5 ml microfuge tube, and 500 μl isopropanol was added, followed by mixing and incubation for 10 min. After centrifugation at 11,000 × g at 4°C for 15 min, the pellet was washed with 1.2 ml 75% ethanol and spun at 11,000 × g at 4°C for 10 min. The supernatant was discarded and the pellet dried. The RNA pellet was then dissolved in 100 μl diethyl pyrocarbonate (DEPC)-treated distilled water, and 10 μl DEPC-treated 3 M sodium acetate (pH 5.2) and 250 μl RNase-free 100% ethanol were added and mixed. After incubation at −20°C for 20 min, the tubes were centrifuged at 11,000 × g at 4°C for 15 min. The pellet was washed with 1.2 ml 75% ethanol, followed by centrifugation at 11,000 × g at 4°C for 10 min. The supernatant was discarded and the pellet dried. The RNA pellet was then dissolved in 50 μl DEPC-treated distilled water.

### 2.4 | Quantitative reverse transcription polymerase chain reaction

One μg of total RNA was reverse transcribed into cDNA using superscript III (Invitrogen) in 20 μl total volume. Quantitative reverse transcription polymerase chain reaction amplification was carried out on a Light cycler 96 system thermal cycler (Roche) using FastStart Essential DNA Green Master (Roche). Each 10 μl reaction mixture included 5 μl of SYBR Green Master, 0.3 μM primers and 1 μL of diluted

cDNA. The PCR cycle conditions were 95°C for 10 min for pre-incubation, then 45 cycles of 95°C for 10 s, 52°C for 10 s, and 72°C for 20 s. The primer sequences used were as follows: *Sb-Rca-α* (forward: GTAACTACTTCCACGGCGG; reverse: TAGCGCTGACGGATCAGCTTC), *Sb-Rca-β* (forward: CTTCTCCTCCACCGTTGGAGCTC; reverse: CGCC ATCACCTTGAACCTGTTAAC).

## 2.5 | Protein extraction and immunoblotting assay

Total proteins were extracted from a frozen leaf disc (1 cm diameter) by grinding in 0.5 ml of sodium dodecyl sulfate (SDS)-sample buffer containing 62.5 mM Tris-HCl (pH 8.0), 2% SDS, 1 M urea, 10% glycerol, 2.1 M 2-mercaptoethanol, and 0.005% bromophenol blue. The ground samples were centrifuged (8,530 *g* for 0.5 hr at 4°C), and 5 µl of supernatant was then separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Gel-resolved proteins were electrophoretically transferred to a low fluorescence PVDF membrane (Immobilon-FL; from EMD Millipore) and treated with blocking solution (5% gelatin; Sigma) for 1 hr at room temperature. Primary antibody (anti-Rca, 1:4,000 dilution) was diluted into PBST solution and used as follows: anti-Rca, 1:4,000 dilution for 4 hr; anti-pT78, 1:4,000 dilution overnight. The anti-Rca antibodies were produced against the peptide antigen CELESGNAGEPAKLIR. Modification-specific antibody was generated against the phosphopeptide antigen RGLAYDpT78DDQQDC (Arabidopsis anti-pT78). For each peptide antigen, the Cys residue at the N- or C-terminus was added for coupling to keyhole limpet hemocyanin. All custom antibodies were produced by GenScript. Primary antibody reactions were performed at room temperature. Immunoblots involving fluorescent secondary goat anti-rabbit antibodies (IRDye 800CW; LI-COR Biosciences) were diluted 1:10,000 in PBST and incubated for 1 hr at room temperature and were then scanned using a LI-COR Odyssey Infrared Imaging System for visualization. All immunoblotting experiments were performed at least twice, and representative results were presented.

## 2.6 | Leaf photosynthetic measurements

All gas exchange measurements were performed using LI-COR 6400 portable gas exchange systems equipped with a Leaf Chamber Fluorometer (LI-COR p/n 6400-40). The measurement conditions in the LI-COR chambers were 25°C and 400 µmol $m^{-2}$ $s^{-1}$ PPFD. To test the gas exchange pattern during heat stress conditions, plants were stored at 42°C for 1, 2, 4, and 6 hr. The temperature of the LI-COR chamber was set to 25°C, and $CO_2$ assimilation rates were measured for 10 min following the indicated heat treatments. At least six plants were tested to determine means and *SE*s.

## 2.7 | In vivo Rubisco activation assay

Heat-treated sorghum leaf samples were collected at the end of the gas exchange measurements. Samples were immediately frozen in liquid nitrogen and stored at −80°C until used in the assays. Samples were ground with a glass tissue homogenizer, suspended in extraction buffer (50 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid-NaOH pH8.0, 1 mM ethylenediaminetetraacetic acid, 5 mM $MgCl_2$, 5 mM DTT, 1% polyvinylpolypyrrolidone, 1% protease inhibitor cocktail), and pelleted for 30 s. $^{14}$CABP was immediately added to an aliquot for the measurement of initial activation state (Butz & Sharkey, 1989). Aliquots for measurement of final activation were incubated for 30 min in elevated $CO_2$ and $Mg^{2+}$, after which an excess of $^{14}$CABP was added. The number of active Rubisco sites in the initial and total aliquots was measured by size exclusion chromatography, as previously described (Kubien et al., 2011). ANOVA was used to test for significant ($p < .05$) treatment effects, and Student's *t* tests were utilized to determine significant differences between individual heat treatments and the control.

# 3 | RESULTS

## 3.1 | All $C_4$ grasses examined contain *Rca-α* and *Rca-β* genes in tandem orientation

Three genomic databases (https://ncbi.nlm.nih.gov; https://phytozome.jgi.doe.gov; https://sugarcane-genome.cirad.fr/) were queried to identify *Rca* genes in the genomes of the $C_4$ grasses sorghum, setaria, maize, sugarcane, and miscanthus (Figure 1). Similar patterns of gene arrangement and transcriptional direction were found in the five $C_4$ plants. In all

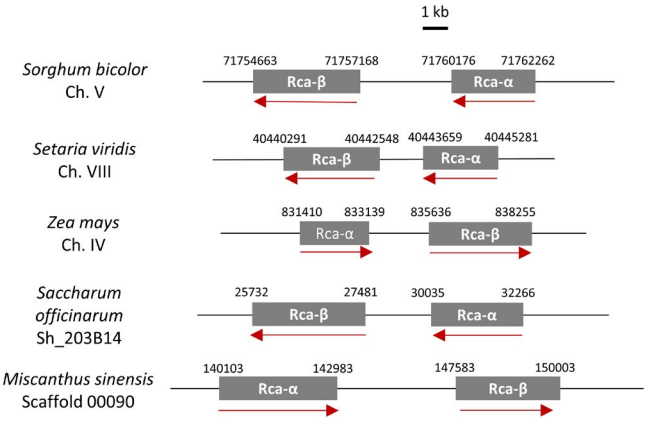



**FIGURE 1** *Rca* gene arrangement and location in the genome of five $C_4$ grasses. Gene information was extracted from three databases: http://www.ncbi.nlm.nih.gov, https://phytozome.jgi.doe.gov, and https://sugarcane-genome.cirad.fr/. The gray boxes represent *Rca* genes and the numbers above them represent physical distance in the chromosomes. The arrows indicate transcriptional direction

cases, the *Rca-α* gene was positioned before the *Rca-β* gene in the direction of transcription, and the gaps between *Rca-α* and *Rca-β* genes were narrow (1–5 kb), possibly indicating that the promoter of *Rca-β* may overlap with the 3′UTR of the *Rca-α* gene in some species. The two *Rca* genes in tandem orientation separated by a small intergenic region may have been generated by tandem gene duplication from a putative common grass ancestral *Rca* gene (Nagarajan & Gill, 2018), thereby indicating the *Rca* gene arrangement shown in the $C_4$ plants could be conserved across this $C_4$ grass subfamily.

## 3.2 | Key regulatory regions of Rca-α proteins are largely conserved in the five $C_4$ grasses

Rca isoforms can undergo post-translational modification by multiple mechanisms (Hartl et al., 2017; Kim et al., 2016; Vargas-Suarez et al., 2004). Threonine 78 (Thr78) is phosphorylated by cpCK2 in the dark or at low-light intensity in both isoforms of Arabidopsis, and the neighboring four aspartates surrounding Thr78 are critical for recruiting the kinase to phosphorylate Thr78 (Kim et al., 2016). Two cysteine residues in the C-terminal extension in Rca-α enable the formation of a disulfide bond that is responsive to redox signaling (Zhang et al., 2002). To examine whether these key regulatory regions are conserved in these five $C_4$ grasses, we compared their sequences with Arabidopsis Rca. The Thr78 residue was conserved in all of the Rca-α isoforms except for setaria, which contained isoleucine, and it was replaced in Rca-β isoforms of all five grasses by an isoleucine or valine substitution (Figure 2a). The four aspartates required for recruiting the kinase were conserved in all Rca isoforms regardless of the Thr78 conservation. The two cysteine residues in the CTE were conserved in all five Rca-α isoforms as well (Figure 2b).

## 3.3 | The Rca-α isoform is not expressed at ambient temperature and contains several stress-related elements in its gene promoter

*Rca* transcript abundance has a robust circadian rhythm, whereas Rca protein abundance is stable throughout the day in tomato (*Lycopersicon esculentum* Mill.) (Martinocatt & Ort, 1992). Rca-α and Rca-β isoform ratios are approximately equal in Arabidopsis (Liu et al., 1996), but the Rca-α isoform is often expressed at lower levels than Rca-β in other plant species (Salvucci et al., 1987). To examine the expression patterns of the isoforms in these five $C_4$ grasses, we extracted total proteins from the plants either in light or in dark conditions in long-day photoperiod (25°C and 400 μmol $m^{-2}$ $s^{-1}$ PPFD). Notably, only Rca-β proteins were detected in either condition (Figure 3) even though all five species contained the *Rca-α* genomic sequences (Figure 1).

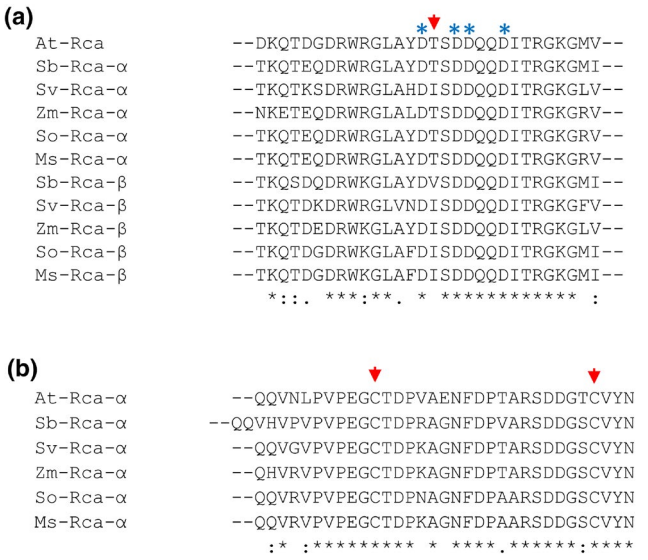



**FIGURE 2** Protein sequence comparison among Arabidopsis and $C_4$ Rca isoforms. (a) T78 and neighbor sequences of Arabidopsis and $C_4$ Rca-α and Rca-β isoforms. Arrow indicates T78 position in Arabidopsis and corresponding amino acid residues at that position in the other species and their isoforms. Asterisks indicate four aspartates that recruit cpCK2 in Arabidopsis and their conservation in all Rca isoforms regardless of the existence of T78. (b) Conserved cysteines responsive to redox signaling in C-terminal extension of all $C_4$ Rca-α isoforms (arrows). *At*, *Arabidopsis thaliana*; *Sb*, *Sorghum bicolor*; *Sv*, *Setaria viridis*, *Zm*, *Zea mays*; *Ms*, *Miscanthus sinensis*; *So*, *Saccharum officinarum*

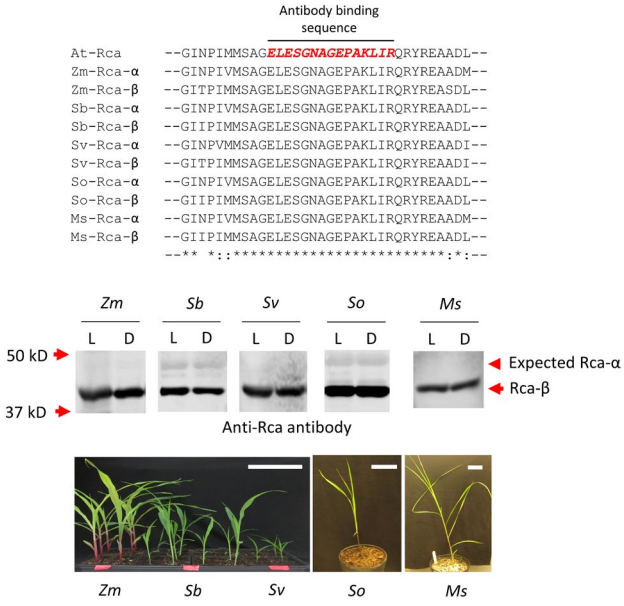



**FIGURE 3** Rca antibody peptide sequences and $C_4$ Rca protein expression in either light (L) or dark (D) conditions. Total proteins were extracted from plants grown at 25°C in long-day photoperiod. Only Rca-β isoforms were detected by immunoblot assays performed using Rca antibody. White scale bars in the plant pictures indicate 10 cm. *Sb*, *Sorghum bicolor*; *Sv*, *Setaria viridis*; *Zm*, *Zea mays*; *Ms*, *Miscanthus sinensis*; *So*, *Saccharum officinarum*

No expression of Rca-α indicated the promoters of *Rca-α* and *Rca-β* are distinct and that there may be unique motifs in each promoter. Accordingly, we examined promoter sequences 1.5 kb upstream from the TSS in the three genomic databases mentioned above and analyzed these using PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). In all five $C_4$ *Rca-α* promoters, *cis*-acting regulatory elements related to various abiotic stresses were identified; however, fewer of these were present in *Rca-β* promoters (Figure 4). The putative stress-related *cis*-acting regulatory elements identified were as follows: abscisic acid responsive element, ethylene responsive element, TGACG and CGTCA (methyl-jasmonic acid responsive element), TCA (salicylic acid responsive element), and TC-rich repeat (defense responsive element; Goldsbrough et al., 1993; Guo et al., 2008; Kaplan et al., 2006; Lin et al., 2007; Rouster et al., 1997).

## 3.4 | Rca-α isoforms are induced at moderate heat stress in all five $C_4$ grasses

In addition to the promoter analysis in Figure 4 showing abiotic stress-related motifs in *Rca-α* promoters, recent work suggests Rca protein expression responds to abiotic stresses in some plant species (Chen et al., 2015; Liu et al., 2015). To investigate if this also pertains to $C_4$ grasses, sorghum and setaria were exposed to drought, salt, heat, and cold stresses. Expression of *Rca-α* transcripts and proteins was induced by high temperature but not by the other stresses (Figure 5a,b), even though no heat shock motifs were observed in the $C_4$ *Rca* promoters. The temperature dependence profile of Rca-α expression in sorghum and setaria for a 6 hr treatment confirmed that Rca-α proteins were more strongly induced at 42°C than lower temperatures (Figure 5c). To examine if abundant Rca-β isoforms might also increase at high temperature, 10× diluted sorghum proteins were loaded on a PAGE gel, and no increase in Rca-β isoforms was observed after 6 hr of heat treatment (Figure 5d).

We also generated time course data at 42°C to examine the induction rate of Rca-α isoforms in response to heat stress (Figure 6). At least 4–6 hr were required to reach the maximum protein expression level. However, the maximum expression level of Rca-α compared to total Rca-β expression reached only 20%–30% in sorghum and only 15%–20% in setaria, and these ratios were maintained at a constant level during high temperature conditions. The ratio of Rca-α to Rca-β proteins at high temperature slowly decreased when

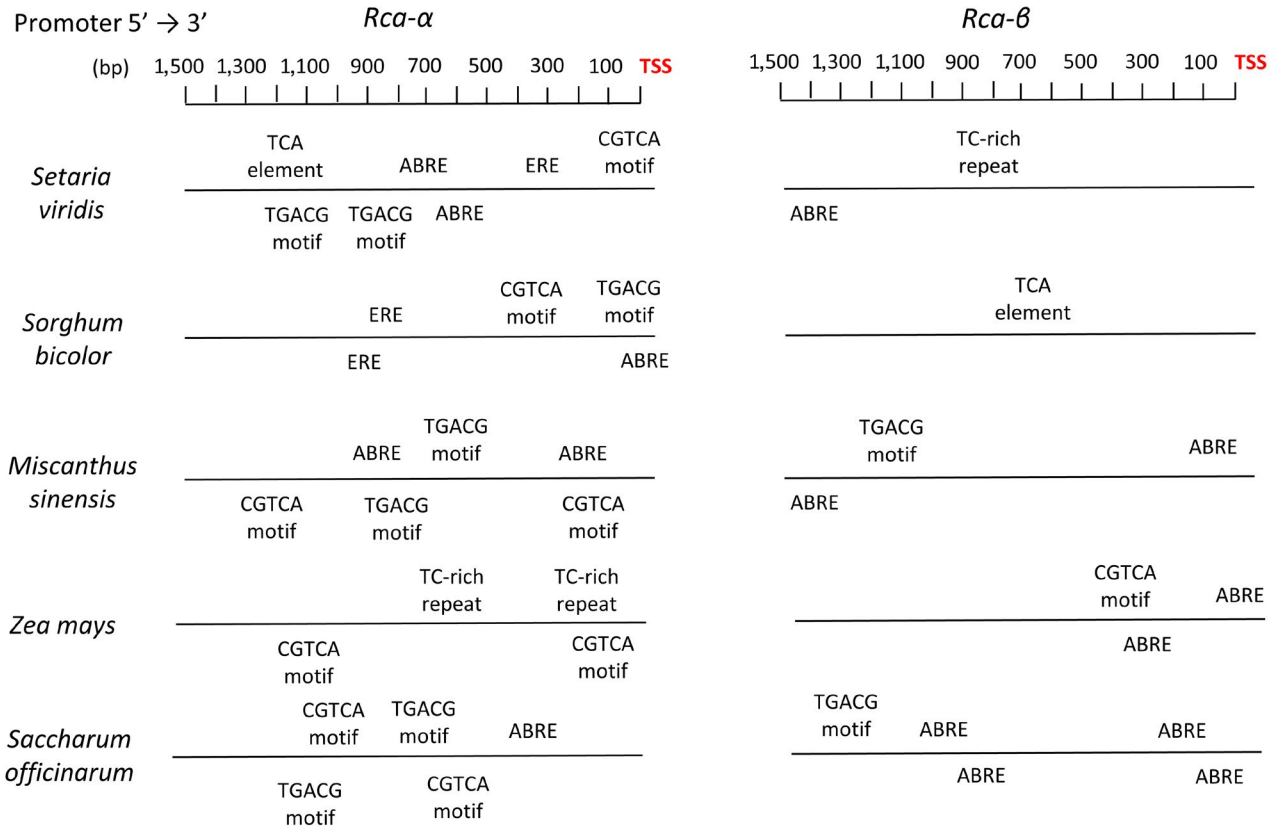



**FIGURE 4** Potential *cis*-acting regulatory elements identified in the *Rca* gene promoters in $C_4$ plants. Upstream promoters approximately 1.5 kb from transcriptional start sites were investigated for common *cis*-acting elements. ABRE, abscisic acid responsive element; ERE, ethylene responsive element; TC-rich repeat, defense responsive element; TCA, salicylic acid responsive element; TGACG and CGTCA, methyl-jasmonic acid responsive elements; TSS, transcriptional start site

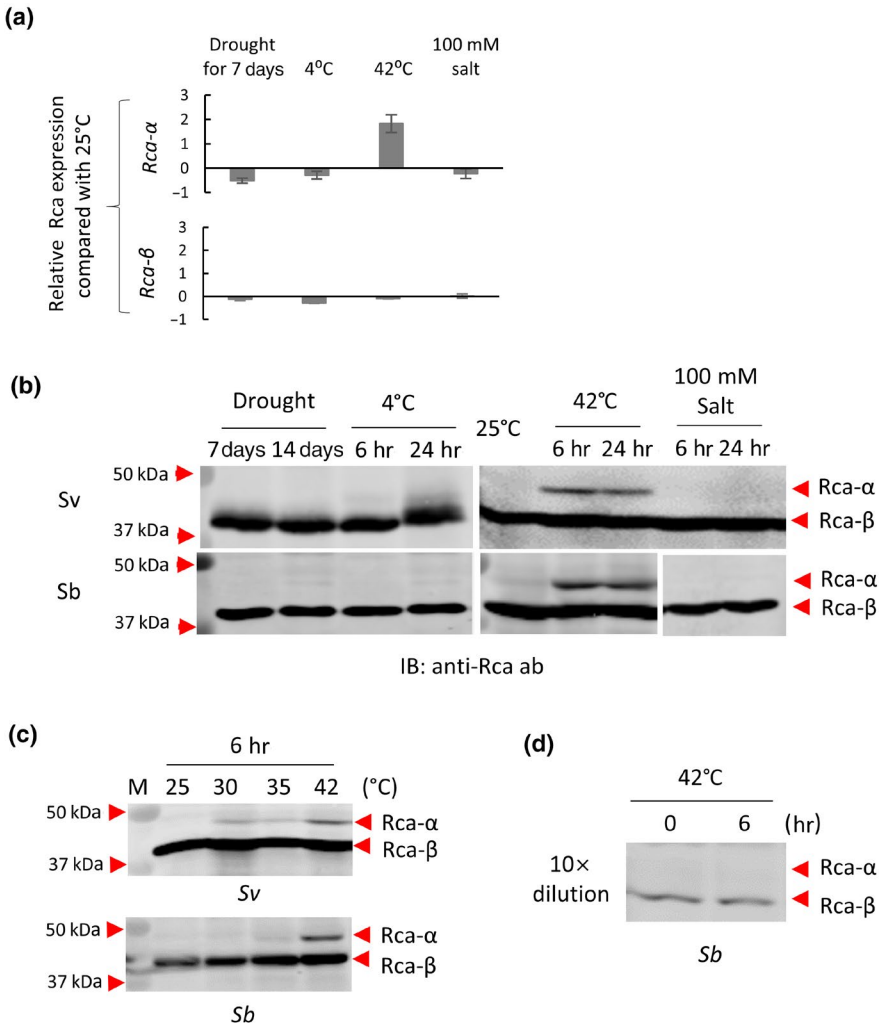



**FIGURE 5** *Rca-α* gene and protein expression in response to various stress conditions. (a) Relative expression levels of sorghum *Rca* genes following four abiotic stresses: drought for 7 days, 4°C for 6 hr for cold stress, 42°C for 6 hr for heat stress, 100 mM NaCl for 6 hr for salt stress. (b) Rca protein expression of sorghum (*Sb*) and setaria (*Sv*) following stress treatments. Two time points, 7/14 days for drought stress and 6/24 hr for the other stresses, were selected to test protein expression. (c) Total proteins extracted from plants stored at different temperatures for 6 hr. (d) Ten times diluted protein (10× dilution) samples were loaded to examine the expression of the Rca-β isoform at high temperature. *Sb*, *Sorghum bicolor*; *Sv*, *Setaria viridis*

the temperature returned to the starting ambient temperature (Figure 6).

The similar genomic structure of *Rca* genes and their promoters in these five $C_4$ grasses led us to expect that all would show a similar pattern in Rca-α expression at high temperature. Indeed, the other three $C_4$ grass species (maize, miscanthus, and sugarcane) showed a similar induction of Rca-α isoform upon transition from low to high temperature and reduction upon return to ambient temperature (Figure 7). The maximum levels of the larger Rca-α isoforms compared to Rca-β varied among species even though all showed a similar pattern of Rca-α expression at high temperature (Figure 7).

## 3.5 | Recovery of $CO_2$ assimilation and Rubisco activation mirrors Rca-α induction at high temperature

Three of the $C_4$ grass species (sorghum, sugarcane, and setaria) were tested to determine how $CO_2$ fixation rates aligned with Rca-α induction following transition from ambient to high temperature. As Rca-α protein levels gradually increased and reached highest levels ~6 hr after transition to high temperature, gas exchange measurements were performed for 10 min on the same leaf of each plant at 0, 1, 2, 4, and 6 hr after transitioning to 42°C (Figure 8). At ambient temperature, the $CO_2$ assimilation rate in sorghum was ~12 μmol $m^{-2}$ $s^{-1}$ and decreased to ~5 μmol $m^{-2}$ $s^{-1}$ after 1 hr of heat treatment. However, the photosynthetic rates slowly recovered over the period of 4–6 hr at high temperature to a rate similar to that measured prior to heat treatment. Stomatal conductance in the sorghum plants increased following the transition to high temperature but did not change with exposure time. Thus, stomatal conductance was not the cause of the decline or recovery of the $CO_2$ assimilation rate. Similar patterns were observed in sugarcane and setaria (Figure 8).

Rubisco activation initially decreased with heat stress but recovered to a normal level after 4 hr of heat exposure (Figure 9), which was similar to the recovery of the $CO_2$ assimilation rate (Figure 8) and to Rca-α accumulation (Figure 6). Significant treatment effects were confirmed by ANOVA (sorghum: *F*-factor = 18.71, *F*-critical

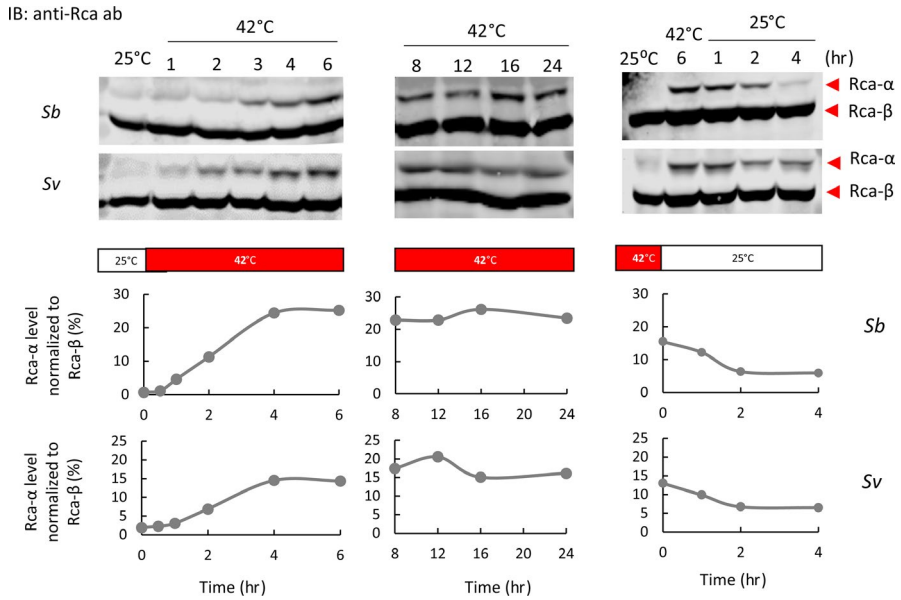



**FIGURE 6** Immunoblot analysis of the effect of temperature transitions on abundance of Rca isoforms in sorghum and setaria leaves. Left panels show expression levels as plants were grown in a growth chamber (light:dark = 16 hr:8 hr, 25°C, 400 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density) and then transferred to high temperature chamber for heat stress (42°C, red bar). Middle panels show isoform levels after sustained heat stress. Right panels show expression levels as growth temperature was lowered from heat stress conditions. Plants exposed to 42°C for 6 hr were transferred into normal growth chamber (25°C, white bar). Total proteins were extracted at the designated temperature and time points. Rca-α expression levels were normalized to Rca-β expression. All average values were generated with at least two biological replicates. *Sb*, sorghum; *Sv*, setaria

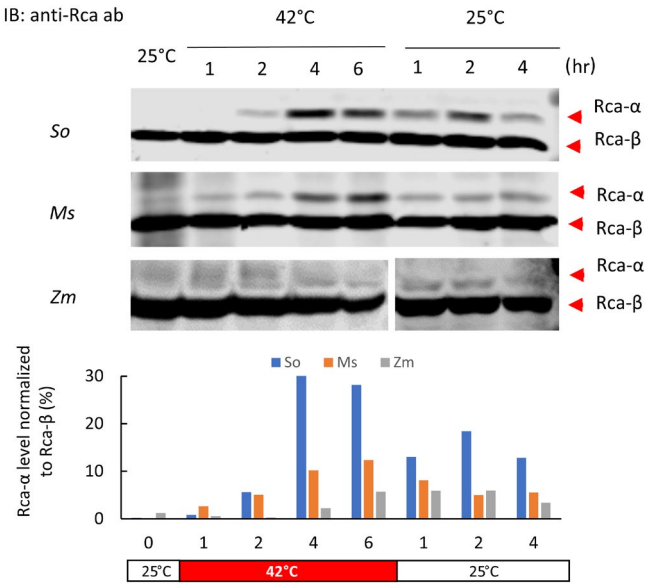



**FIGURE 7** Rca isoform protein expression at high temperature and normal temperature in sugarcane, miscanthus, and maize. Total proteins were extracted at the designated temperature and time points. Rca-α expression levels were normalized to Rca-β expression

cut-off = 5.98, $p$ = .0005; sugarcane: $F$-factor = 74.9, $F$-critical cut-off = 5.72, $p$ = .00001; setaria: $F$-factor = 19.39, $F$-critical cutoff = 5.98, $p$ = .00001). Only the 1 and 2 hr heat treatments were significantly different from the control, whereas full recovery of Rubisco activation was reached after 4 hr of heat treatment (Figure 9). These data show corresponding patterns for Rca-α induction, Rubisco activation, and the recovery of $CO_2$ assimilation following the heat stress treatment.

## 3.6 | $C_4$ Rca-α isoforms are not phosphorylated in the dark

We previously reported Arabidopsis Rca proteins containing the phospho-residue T78 are phosphorylated by cpCK2 in the dark or in low light [34]. T78 is conserved in Rca-α isoforms in four of the five $C_4$ grasses that we investigated (Figure 2a). Moreover, the four aspartates surrounding T78, key residues to recruit cpCK2 kinase, exist in all of the $C_4$ Rca isoforms (Figure 2a). We anticipated that the $C_4$ Rca-α isoforms containing T78 would be phosphorylated in the dark as is the case for Arabidopsis Rca (Kim et al., 2016). We extracted total proteins from sorghum stored in the dark at 42°C for 6 hr and performed immunoblot assays (Figure S1). Contrary to expectations, the heat-induced Rca-α proteins were not phosphorylated in the dark. It is possible cpCK2 proteins were degraded or inactivated at high temperature. We transferred sorghum plants that had been heat-treated for 6 hr in the dark to ambient temperature (25°C) in the dark for 2 hr to see if the kinase activity might be restored (Figure S1), but no phosphorylation of Rca-α was detected. On the contrary,

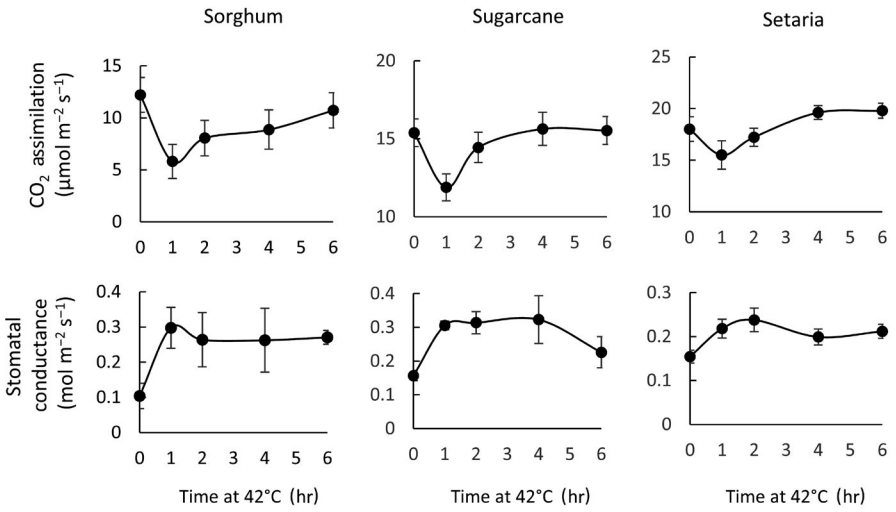



**FIGURE 8** Stomatal conductance and $CO_2$ assimilation rates in sorghum, sugarcane, and setaria following high temperature treatments. Plants grown in long-day photoperiod (25°C and 400 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density [PPFD]) were used for the measurement. Gas exchange rates were measured following the indicated periods of treatment at 42°C every 15 s over 10 min with the final measurement at 10 min shown. Temperature and light intensity for this measurement were 25°C and 400 μmol $m^{-2}$ $s^{-1}$ PPFD. Six plants were used to generate means and *SE*s

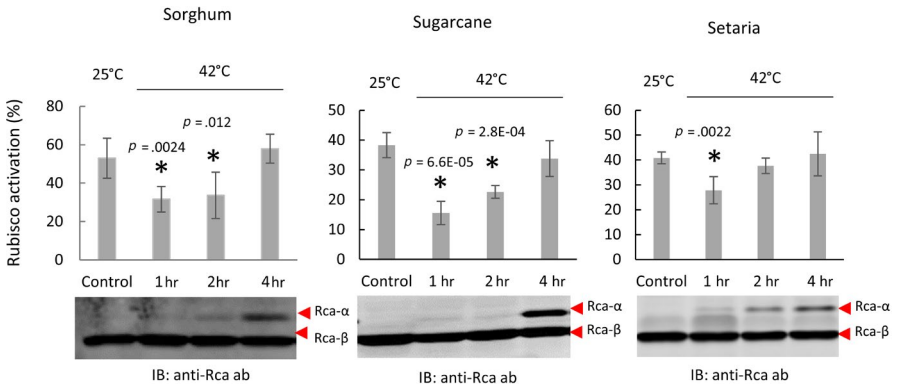



**FIGURE 9** Rubisco activation in three $C_4$ plant species at high temperature. Leaf discs were harvested from the plants in two different temperatures. At least four plants were used to generate means and standard errors. Following significant ANOVA results (sorghum: *F*-factor = 12.41, *F*-critical cut-off = 3.49, $p = .0005$; sugarcane: *F*-factor = 25.39, *F*-critical cut-off = 3.49, $p = .00001$), individual *t*tests were performed to test for significant differences between the control and the heated samples (*$p < .05$)

Arabidopsis Rca proteins were phosphorylated at 42°C in the dark (Figure S1).

### 3.7 | Rca-α protein expression is not increased by heat stress in Arabidopsis

The Arabidopsis *Rca-α* gene generates Rca-β isoforms by alternative splicing, and the ratio of Rca-α and Rca-β proteins is almost 1:1. We tested whether the ratio of Rca isoforms changes in Arabidopsis after exposure to 42°C for 1, 2, and 4 hr (Figure S2). However, no alteration in expression ratios of Rca-α to Rca-β isoforms was observed in heat-treated Arabidopsis plants.

## 4 | DISCUSSION

In this study, we investigated the role of Rca isoforms in regulating photosynthesis of five $C_4$ grasses. All five contained two *Rca* genes, one encoding Rca-α and the other encoding Rca-β, but these displayed differential expression with only the Rca-β isoform expression occurring at normally encountered growth temperatures and Rca-α isoform expression occurring only when the temperature exceeded 42°C. The high temperature-induced Rca-α profile corresponded to the recovery profiles of the high temperature-induced inhibition of $CO_2$ fixation and Rubisco activation, suggesting Rca-α maintains photosynthetic rates of $C_4$ grasses at high temperature, possibly by stabilizing the Rca oligomer responsible for sustaining Rubisco activation. While the ratio of Rca-α to Rca-β increased in $C_4$ plants upon heat stress, $C_3$ Arabidopsis expressed Rca-α and Rca-β in equal amounts at ambient and high temperatures, indicating that expression of the redox-regulated Rca-α isoform varies by species and may be adaptive in $C_4$ grasses under heat stress in sustaining photosynthesis.

### 4.1 | $C_4$ grasses show differential Rca isoform expression depending on growth temperature

Rca-α isoforms were induced at high temperatures despite the absence of known temperature-related motifs in the

promoters. Moreover, induction to maximum expression required ~6 hr, which is longer than the time required for expression of heat-responsive proteins in the $C_4$ model plant setaria as well as the $C_3$ model plant Arabidopsis (Singh et al., 2016; Swindell et al., 2007). Therefore, $C_4$ *Rca-α* gene expression may be directly induced by factors independent of temperature-responsive transcription factors.

An array of high temperature-induced effects on Rca expression and activity have been reported. Some species, such as wheat (*Triticum aestivum*) and cotton (*Gossypium hirsutum*), produce *Rca* variants at high temperature, and the different sizes of the variants can be the result of alternative splicing (Law & Crafts-Brandner, 2001; Law et al., 2001). There is also a considerable amount of literature documenting the high temperature sensitivity of Rca function (Busch & Sage, 2017; Crafts-Brandner & Salvucci, 2000; Perdomo et al., 2017; Sage et al., 2008). The degree of thermal stability of Rca is dependent on species and seemingly correlates with the climate in which a species has evolved such that Rca in temperate species is more heat labile compared to warm climate species (Carmo-Silva et al., 2011; Henderson et al., 2013; Scafaro et al., 2019). Among the three wheat Rca isoforms (TaRca1-β, TaRca2-α, and TaRca2), TaRca1-β, the most heat tolerant of the three, is induced in both spring and winter wheat cultivars when exposed to high temperature. That is, a Rca-β isoform appears to be adaptive under heat stress in wheat in sustaining photosynthesis, whereas we found the Rca-α isoform appears adaptive in $C_4$ grasses may be yet another example of the highly diverse regulation- and expression-by-environment interactions for Rca across species. Another example of this diversity is the phosphorylation of Rca T78 that occurs in Arabidopsis in the dark or in low light and downregulates photosynthesis (Kim et al., 2016, 2019) but does not occur in Rca-α of $C_4$ grasses that also have this phospho-residue (Figure S1). This result may indicate that the proper kinases for Rca phosphorylation do not exist in the bundle sheath cell in sorghum or that the phosphatase activity is so high that it negates the kinase activity.

## 4.2 | The higher ratio of Rca-α to Rca-β at high temperature may stabilize the interaction of Rca forms to maintain photosynthesis

While the in vitro interaction of plant Rcas is highly polydisperse, the existence of functional, stable oligomers (Blayney et al., 2011; Keown & Pearce, 2014; Stotz et al., 2011) suggests that an oligomer, possibly a hexamer, is the likely functional configuration. However, the Rca complex shows dynamic assembly and disassembly of forms occurring on a timescale of seconds (Wang et al., 2018). Recent works have also demonstrated several models of the oligomerization and thermotolerance of CAM, rice, and wheat Rca subunits at high temperature (Scafaro et al., 2019; Shivhare & Mueller-Cajar, 2017; Shivhare et al., 2019). Their in vitro assays revealed that Rca-β isoforms were related to heat tolerance. However, no functional coordination between Rca-α and Rca-β was shown in vitro yet, *in planta* data imply Rca-α isoforms are beneficial for activation of Rca and Rubisco under heat stress (Wang et al., 2010). The different observations in assays between in vitro and in vivo may be explained by cofactors recruited by the CTE of Rca-α, the regulation of which could be affected by changes in the redox state of the chloroplast at high temperature. Likewise, based on the data shown in our in vivo studies, heat-induced Rca-α isoforms in $C_4$ grasses may be more stable under heat stress or may stabilize the Rca interactions to maintain a higher Rubisco activation at high temperature than would otherwise be possible. Sorghum, sugarcane, and setaria photosynthetic rates initially decreased following high temperature treatment but gradually recovered as the heat stress was prolonged. Stomatal conductance increased at high temperature but did not recover during heat stress, demonstrating that neither the initial inhibition nor subsequent recovery of $CO_2$ fixation at high temperature was associated with stomatal conductance. The pattern of Rubisco activity recovery also corresponded to the patterns of both gas exchange and Rca-α induction following high temperature treatment, consistent with the notion that the presence of Rca-α proteins maintains high Rubisco activity to perform normal $CO_2$ fixation rates at high temperature.

## 4.3 | Rca-α induction with heat stress varies by species

Rca-β only-expressing Arabidopsis transgenic plants show higher rates of $CO_2$ fixation than Col-0 or transgenic plants expressing only Rca-α (Carmo-Silva & Salvucci, 2013; Zhang et al., 2002), suggesting a constraint by Rca-α on photosynthesis, at least in Arabidopsis under the conditions tested. Here we have shown that high temperature treatment does not further induce Rca-α expression in Arabidopsis and thus does not support a specific role for Rca-α in thermotolerance in this cool climate-adapted plant. However, our results here suggest Rca-α-induced thermotolerance is likely common across $C_4$ grasses. In the case of $C_4$ grasses, we found that although Rca-α expression increased during the first 4–6 hr of exposure to high temperature and remained stable for several hours, the maximum expression of Rca-α was only ~30% of total Rca-β expression, and that ratio was maintained at a constant level as long as the high temperature persisted, suggesting that regulation and maintenance of the specific level of Rca-α expression may be critical for optimal Rca activity at high temperature in these $C_4$ grasses. The maintenance of the ratio of Rca-α to Rca-β at high temperature seems to be transcriptionally and translationally regulated. Whether Rca-α only-expressing sorghum underperforms compared to

wild type at ambient temperatures and whether Rca-β only-expressing plants underperform compared to wild type at elevated temperatures will be directly tested once the transgenic plants have been generated.

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## AUTHORS' CONTRIBUTIONS

S.Y.K. and D.R.O. designed the research; S.Y.K. performed the research; S.Y.K., R.A.S., and D.R.O. analyzed the data and wrote the paper.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

*Rebecca A. Slattery* https://orcid.org/0000-0002-7165-906X

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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