C₃ photosynthesis in the desert plant *Rhazya stricta* is fully functional at high temperatures and light intensities

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**Summary**

- The C₃ plant *Rhazya stricta* is native to arid desert environment zones, where it experiences daily extremes of heat, light intensity (PAR) and high vapour pressure deficit (VPD). We measured the photosynthetic parameters in *R. stricta* in its native environment to assess the mechanisms that permit it to survive in these extreme conditions.
- Infrared gas exchange analysis examined diel changes in assimilation (A), stomatal conductance (gₛ), and transpiration (E) on mature leaves of *R. stricta*. A/E analysis was used to determine the effect of temperature on carboxylation capacity (*Vc, max*) and the light- and CO₂-saturated rate of photosynthesis (*Amax*). Combined chlorophyll fluorescence and gas exchange light response curve analysis at ambient and low oxygen showed that both carboxylation and oxygenation of Rubisco acted as the major sinks for the end products of electron transport.
- Physiological analysis in conjunction with gene expression analysis suggested that there are two isoforms of Rubisco activase which may provide an explanation for the ability of *R. stricta* to maintain Rubisco function at high temperatures.
- The potential to exploit this ability to cope with extreme temperatures is discussed in the context of future crop improvement.

**Introduction**

Global and regional climatic conditions are rapidly changing with increasing impact on plant growth and productivity through alterations in both abiotic and biotic stressors (Easterling *et al.*, 2000; Newton *et al.*, 2011). Critically, increasing temperatures and reduced water availability lead to heat and drought stress, both of which impact dramatically on crop yield (Lobell & Asner, 2003; Lobell & Field, 2007; Schlenker & Roberts, 2009). Predictive models for future climatic influences on grain production have proposed that heat stress rather than drought will cause significant yield losses in wheat across northern Europe (Semenova & Shewry, 2011). For example, Lobell & Field (2007) observed an approximate 25% annual yield loss in wheat as a result of heat stress. Further, this loss in yield is predicted to increase with rising global temperatures. The Intergovernmental Panel on Climate Change (IPCC, 2007) has predicted more frequent heat episodes with drought in some geographical regions in the future, with temperatures postulated to rise by between 0.6 and 4°C, depending on the scenario models followed. The optimum temperature for plant productivity varies between species. For example, temperate C₃ crops, such as wheat, generally prefer temperatures of between 15 and 25°C (Yamasaki *et al.*, 2002). This is c. 10°C lower than C₄ plants, which include crops such as maize and sorghum. In hot arid environments, Crassulacean acid metabolism (CAM) plants operate best at temperature ranges of 30–40°C. Exceptionally, some desert C₃ shrubs have also been found to achieve a high photosynthetic yield at 40°C or greater (Mooney *et al.*, 1978).

Physiological and metabolic processes decrease at temperatures higher or lower than the optimum (Sage *et al.*, 2008). The effect of heat stress on carbon metabolism is generally believed to be the primary limiting factor for photosynthesis at high temperatures (Kurek *et al.*, 2007) from both direct and indirect effects. Indirect effects of temperature on stomatal conductance and photosynthesis (to be described) reduce carbon assimilation, whereas temperature affects directly the activity of several Calvin cycle enzymes, with recent significant attention focused on Rubisco activase (RCA; Parry *et al.*, 2003; Galmés *et al.*, 2013). The ability of a plant to fix carbon is based on the catalytic capacity and activity of Rubisco to carry out carboxylation (Speitzer & Salvucci, 2002; Portis & Parry, 2007), which is regulated by carboxylation of the catalytic site (Whitney *et al.*, 1999; Parry *et al.*, 2008). Sugar phosphates (including ribulose bisphosphate (RuBP)) bind to Rubisco and prevent carbamoylation (or substrate binding if already carbamylated) (Parry *et al.*, 2008). RCA catalyses the removal of the sugar phosphates from the Rubisco catalytic sites (Portis *et al.*, 1986; Mate *et al.*, 1993; Speitzer &
Salvucci, 2002; Portis, 2003), and therefore RCA activity plays an important regulatory role in photosynthetic performance. Although Rubisco itself has a relatively high temperature tolerance (up to 50°C) (Crafts-Brandner & Salvucci, 2000), the thermal instability of RCA is the major limitation to photosynthesis at high temperatures (Laws & Crafts-Brandner, 1999; Crafts-Brandner & Salvucci, 2000; Salvucci & Crafts-Brandner, 2004a,b; Feller et al., 1998), and therefore of photosynthetic capacity. However, the effects of high temperatures on photosynthesis are multiple and often interacting, and are not restricted to reduced RCA activity alone. Photosynthetic carboxylation is also decreased as temperatures increase because of restricted CO₂ diffusion driven by stomatal closure (Feller, 2006), as well as alterations in gas diffusion coefficients (Brooks & Farquhar, 1985) and Rubisco specificity (Hall & Keys, 1983; Jordan & Ogren, 1984). At higher temperatures, Rubisco specificity for CO₂ decreases and the water solubility of O₂ relative to CO₂ increases (Bunce, 1998), increasing the metabolic loss through photorespiration. Increasing temperature also reduces electron transport (Bjorkman et al., 1980), with thylakoid reactions postulated as a site of injury at high temperatures (Wise et al., 2004). Specifically, damage may occur to photosystem II (PSII) (Berry & Bjorkman, 1980) or thylakoid membranes, with a downstream effect on RuBP regeneration via a reduction in ATP synthesis, and thus photosynthetic productivity and yield.

Depending on their growth environment, plants have adaptive strategies to deal with the impacts of heat stress, including the expression of heat shock proteins (HSPs) (Scharf et al., 2011) and heat stable enzymes (such as RCA isoforms; see the Discussion section), and rapid induction of non-photochemical quenching (NPQ) of excess excitation energy (Brestic et al., 1995). Here, we present a physiological analysis of the leaves of the perennial shrub *Rhazya stricta* carried out in its native habitat in a desert site near Jeddah, Saudi Arabia (Emad El-Deen, 2005; Marwat et al., 2012). This is a typical arid environment with low rainfall (60–250 mm; Noy-Meir, 1973). A preliminary field study in September 2011 (S. A. Yates et al., unpublished) identified exceptionally high photosynthetic rates in *R. stricta*, despite high ambient temperatures and the lack of any direct precipitation in the preceding 10 months. Crucially, it was also established using photosynthetic gas exchange that *R. stricta* performed C₃ photosynthetic metabolism (see the Results Section). This strongly indicated that *R. stricta* has distinct physiological adaptations enabling it to thrive where most C₃ plants could not grow. The adaptive potential of plants to grow in such environments depends on the level of genetic variation (Flood et al., 2011), which provides the underlying basis for plant distribution. Contrary to the lack of rainfall, the relatively high stomatal conductances observed suggested that the plant was not severely water stressed (S. A. Yates et al., unpublished). This agrees with the observation that *R. stricta* is often found growing in wadis (Emad El-Deen, 2005) and has a deep root system, typical of other arid zone perennial species (Perry & Goodall, 2009).

Climatic change in temperate zones may lead to conditions, such as extreme temperatures, akin to those already experienced by plants in arid geographical areas. Desert plants, like *R. stricta*, are highly likely to possess alleles that would benefit C₃ crops in temperate regions in the future. Exploitation of this natural variation first requires the identification and quantification of important regulatory components of photosynthesis (Lawson et al., 2012). The aim of this study was to determine the key physiological mechanisms that enable *R. stricta* to sustain high primary productivity at the temperatures encountered in this environment. All measurements were performed *in situ* in the field, enabling a confident evaluation of the plant’s physiological responses and its overall performance. In an earlier field campaign, we collected mature and apical leaf samples at three time points throughout the day at dawn, midday and dusk. These were used to construct the transcriptome of *R. stricta* and to examine gene expression changes to assess the significance of genes responsive to abiotic stress. These findings (S. A. Yates et al., unpublished), together with the physiological analysis presented here, have allowed us to probe the mechanisms that enable this plant to thrive in such an extreme environment.

### Materials and Methods

#### Site description

The study on *R. stricta* Decne was conducted between 23 and 27 September 2012 at a field site in Bahrah, c. 30 km south-east of Jeddah, Saudi Arabia (at 21°26.456′N, 39°31.847′E, 200 m above sea level). The site was undeveloped, uncultivated and unirrigated, and was dominated by the species *R. stricta* and *Acacia* spp. The area has been classified as arid by the Köppen-Geiger climate system (Peel et al., 2007). Average annual rainfall in the Jeddah area is 45 mm, with most precipitation falling between November and January (Weatherbase, Canty and Associates LLC, Great Falls, VA, USA).

#### Photosynthetic measurements

Photosynthetic gas exchange measurements were made using a portable open gas analysis system (LI-6400, Li-Cor, Lincoln, NE, USA). The analyser was calibrated before use for CO₂ using a standard gas (±2.5% tolerance) (BOC, Guildford, Surrey, UK) and for H₂O using a dewpoint generator (LI-610, Li-Cor). All measurements were taken on the youngest fully expanded leaves, whilst still attached to the plant.

#### Diurnal photosynthesis

The diurnal response of net CO₂ assimilation (*A*) and stomatal conductance (*g*) was determined from pre-dawn (06:00 h) to post-dusk (18:00 h) at 2-h intervals. Methodology minimized alteration to the *in situ* incident irradiance (photosynthetically active radiation, PAR), leaf temperature and vapour pressure deficit (VPD). The gas exchange system used a transparent cuvette, and only leaves oriented towards direct sunlight were selected. In an attempt to minimize perturbations in PAR, the leaf orientation and inclination remained unchanged during each individual measurement. Air temperature was measured immediately before
each time interval, and the cuvette was actively controlled to this temperature using an integrated Peltier cooler (Li-Cor). The ambient water vapour concentration remained unchanged. Each individual measurement took < 60 s.

In addition, at 08:00, 10:00, 12:00 and 16:00 h, the response of A to 20 mmol mol\(^{-1}\) \([O_2]\) was measured to quantify photorespiration. After an initial measurement of A at normal atmospheric \([O_2]\) (210 mmol mol\(^{-1}\)), the concentration was immediately decreased to 20 mmol mol\(^{-1}\) and the measurement was repeated. The percentage increase in \(A_{max}\) at 20 mmol mol\(^{-1}\) \([O_2]\) was calculated from paired measurements. The 20 mmol mol\(^{-1}\) \([O_2]\) was supplied by a gas cylinder (AHG, Jeddah, Saudi Arabia) and was humidified to match ambient conditions by passing the air through a 100-ml closed vessel containing damp tissue paper before flowing into the cuvette. In parallel, but on different plants to the photosynthetic gas exchange measurements, leaves were dark adapted for 20 min, and the maximum operating capacity of PSII (\(F_{v’}/F_m’\)) was measured at multiple time points until 16:00 h using a chlorophyll \(a\) fluorimeter (Li6400-40, Li-Cor).

Response of \(V_{c,max}\) and \(J_{max}\) to field temperatures and VPD

The maximum rate of carboxylation (\(V_{c,max}\)) and the rate of RubP regeneration (\(J_{max}\)) were determined at a range of temperatures and VPDs typical of a diel period by measuring the response of A to changes in \(c_i\) at multiple times between post-dawn and solar midday (08:00–13:00 h). Measurements were made under saturating light using integrated red/blue LEDs (Li-Cor). Leaf temperature and VPD varied with ambient air temperature and humidity, and typically ranged from 28 to 44°C and 2 to 5 kPa, respectively. However, to avoid large variation (> 1°C) in leaf temperature during each individual \(A/c_i\) response, air temperature was measured at the start of each measurement, and the cuvette was actively controlled to this temperature. For each \(A/c_i\) response, photosynthesis reached a steady state at 400 \(\mu\)mol mol\(^{-1}\), decreased stepwise to a lower limit of 50 \(\mu\)mol mol\(^{-1}\) and then increased stepwise to an upper limit of 1200 \(\mu\)mol mol\(^{-1}\). Each stepwise measurement was completed within 1–2 min so as to minimize alteration to the activation state of Rubisco (Long & Bernacchi, 2003). The equations of von Caemmerer & Farquhar (1981) were used to calculate values of A and \(c_i\) (LI-6400 software, Li-Cor). The key \(A/c_i\) parameters \(V_{c,max}\) and \(J_{max}\) were calculated separately, from different phases of the \(A/c_i\) response; \(V_{c,max}\) was determined from points at low \(c_i\) visually judged to be below the inflexion of the \(A/c_i\) plot, and \(J_{max}\) from values judged to be above the inflexion. The method of Sharkey et al. (2007) was used to correct all \(V_{c,max}\) and \(J_{max}\) values to 25°C.

Response of \(F_{v’}/F_m’/\Phi CO_2\) to field temperatures and VPD

Parallel to the above \(A/c_i\) measurements, the responses of the maximum quantum efficiency of \(CO_2\) fixation (\(\Phi CO_2\)) and \(F_{v’}/F_m’\) to PAR were also determined at a range of temperatures. Diel timing of measurement, temperature control and VPD were the same as for the \(A/c_i\) response measurements. Chlorophyll \(a\) fluorescence was measured with an integrated fluorimeter (Li6400-40, Li-Cor). To eliminate photorespiration, all measurements were made at 20 mmol mol\(^{-1}\) \([O_2]\), as described above. Photosynthesis reached steady state at 2000 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR, and then decreased stepwise to zero. All measurements were made at 400 \(\mu\)mol mol\(^{-1}\) \([CO_2]\).

The quantum efficiency of \(CO_2\) fixation (\(\Phi CO_2\)) was calculated as \((A + Rd)/(PARz)\), where \(Rd\) is the rate of \(CO_2\) evolution after 5 min in the dark and \(z\) is the leaf absorbance between 400 and 700 nm determined by an Ulbricht integrating sphere (University of Essex, Colchester, Essex, UK). The relationship between \(F_{v’}/F_m’\) and \(\Phi CO_2\) at 20 mmol mol\(^{-1}\) \([O_2]\) was used to quantify the capacity of sinks other than carbon fixation for electron transport. This was calculated as the slope of the linear section of the \(F_{v’}/F_m’/\Phi CO_2\) response, as described previously (Oehuber et al., 1993).

Water relations

On the same day and time points to the diel photosynthetic response, leaf relative water content (RWC) and leaf water potential (\(\Psi_p\)) were measured. Six leaves per time point were sampled, immediately weighed on site and then dried in an oven at 70°C to constant weight. RWC was calculated using the method of Smart & Bingham (1974). Leaf water potential was measured using a pressure chamber (SKPM 1400, Skye Instruments, Llandrindod Wells, UK).

Sequence retrieval of RCA transcripts and alignment

For the alignment of RCA genes, both \(R.\) stricta 8598 and 16796 were retrieved from transcriptome shotgun assembly (TSA) BioProject PRJNA214091. The longest open reading frame (ORF) was then found using BioPerl tools (Stajich et al., 2002). For the alignment, both nucleotides and amino acids (predicted) were aligned using MAFFT (Katoh & Standley, 2013).

Phylogenetic analysis

For broad phylogenetic analysis of \(R.\) stricta genes, the following were used, selected on the basis of being members of conserved gene families in plants (Vandepoele & Van de Peer, 2005): \(R.\) stricta 17002 (transketolase), \(R.\) stricta 24653 (ribosomal L5P), \(R.\) stricta 4458 (Rubisco small chain subunit), \(R.\) stricta 6131 (chloroplast sedoheptulose-1,7-bisphosphatase), \(R.\) stricta 368 (Rieske (2Fe-2S)) . To find homologues of these genes, the predicted ORFs were extracted and verified using basic local alignment search tool proteins (BLASTP, Altschul et al., 1990) against the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) database. To identify these conserved homologues, the \(R.\) stricta sequences were compared using BLASTP against canonical protein sequences downloaded from Ensembl (\(e\)-value > 1e-20 criteria) using the Perl API tool (Hubbard et al., 2009; McLaren et al., 2010; Flicek et al., 2012). All sequences were concatenated to form a super gene alignment.
(Gadagkar et al., 2005) and phylogenetic analysis was conducted using www.phylogeny.fr ‘one-click’, and again the alignment was manually inspected for errors. Briefly, sequences were aligned with MUSCLEv3.7 (Edgar, 2004). After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) (Castresana, 2000) using the following parameters: minimum block length after gap cleaning of 10, no gap positions were allowed in the final alignment, all segments with contiguous non-conserved positions > 8 were rejected and a minimum number of sequences for a flank position was 85%. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT; Guindon & Gascuel, 2003). The WAG (Whelan & Goldman, 2001) substitution model was selected assuming an estimated proportion of invariant sites and four gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data. Reliability for internal branch was assessed using the aLRT test (SH-Like; Anisimova & Gascuel, 2006). Graphical representation and editing of the phylogenetic tree were performed with TreeDyn (v198.3; Chevenet et al., 2006). Branches were collapsed when branch support values were ≤70%. Finally, the alignment was manually inspected for errors.

For the analysis of RCA homologues in R. stricta, two sequences were annotated by S. A. Yates et al. (unpublished) as encoding genes based on the longest ORF. These are R. stricta:8598 and 16796, encoding a short and long isoform of RCA, respectively. Both ORFs were analysed by BLASTP against the NCBI database and the top 100 blast hits were retrieved. Both results were then concatenated and redundant sequences were removed; each species was manually checked when multiple sequences were returned. If the sequences were identical, except for a C-terminal addition, the sequences were called ‘long’ and ‘short’ based on length. If multiple sequences were returned that showed amino acid differences throughout the sequence, these were labelled A,B or 1,2 depending on the sequence description. The phylogenetic analysis was repeated as described above using www.phylogeny.fr ‘one-click’. All sequence identifiers and their assigned names in this work are shown in Supporting Information Table S1.

Statistical analysis

To test the dependence of each parameter relating to photosynthetic capacity (Figs 3, 4), a two-tailed linear regression was used with temperature as the independent variable (SX-stat, University of Essex). To test the difference between means, a post-hoc Tukey analysis was carried out using SPSS (IBM SPSS Statistics 19, IBM, Portsmouth, UK).

Results

The A/lc response of R. stricta (Fig. 1) is typical for a C3 plant (von Caemmerer & Farquhar, 1981), illustrated by the relatively high lc concentration required to saturate A, compared with C4 plants, which saturate at CO2 concentrations well below the current atmospheric value (lc, c. 200 µmol mol⁻¹). The CO2 compensation point of c. 50 µmol mol⁻¹ (Tolbert et al., 1995) is also indicative of C3 photosynthesis, which is substantially greater than the <20 µmol mol⁻¹ typically observed for C4 plants (Rajendrudu et al., 1986).

Micro-meteorological conditions experienced by R. stricta in its native habitat exhibited extreme changes over the diel period (Fig. 2a). Within 4 h of dawn, PAR had increased from zero to >1500 µmol m⁻² s⁻¹, after which it decreased rapidly to zero by 18:00 h. This high level of irradiance was maintained until 14:00 h, after which it decreased rapidly to 18:00 h. This typical diel trend illustrates that R. stricta was exposed to saturating irradiance for photosynthesis of >1000 µmol m⁻² s⁻¹ for at least 8 h of the diurnal period. Leaf temperature followed a similar trend to PAR, and reached a maximum of 43°C at 14:00 h, thereafter remaining above 36°C until 18:00 h. VPD remained constant at 2 kPa until 10:00 h, and then rapidly increased concurrently with the rise in temperature, to 5.5 kPa at 14:00 h, and stayed above 4 kPa for the remainder of the diel period (Fig. 2a). From dawn until 10:00 h, photosynthetic carbon assimilation (A) increased rapidly with increasing PAR, reaching a maximum of 22.9 µmol m⁻² s⁻¹ (Fig. 2b). At 12:00 h, A declined rapidly to 4.86 µmol m⁻² s⁻¹, before increasing again to 10.0 µmol m⁻² s⁻¹ at 16:00 h (Fig. 2b). Stomatal conductance (g) (Fig. 2c) and leaf transpiration (E) (Fig. 2d) both reached their maximum values at 08:00 h: 0.449 and 3.77 mol m⁻² s⁻¹, respectively. Although they exhibited a similar direction of response during the day, the magnitude of response was different. By 14:00 h, compared with the maximum at 08:00 h, g had decreased by 88% and E by 63%. This
Divergence was also apparent at 16:00 h, where \( g_s \) had decreased by 63%, but \( E \) only by 18% from the maximum. It is also noteworthy that \( g_s \) decreased between 10:00 and 12:00 h without causing a reduction in \( A \) (Fig. 2b,c). Calculations of water use efficiency (WUE) based on the ratio of photosynthetic carbon gain to water lost via transpiration followed a similar pattern to photosynthesis (Fig. 2e). The maximum WUE value of \( 6.77 \) \( \mu \text{mol} \cdot \text{mol}^{-1} \text{C} \) was recorded at 10:00 h. After 10:00 h, WUE stabilized at a slightly lower value of \( 4.01 \) \( \mu \text{mol} \cdot \text{mol}^{-1} \text{C} \) before dropping to negative at 18:00 h (Fig. 2e).

Measurements of leaf water potential over the same period were highly variable and showed an average of \(-2.5\) kPa with a distinct trough at 10:00 h where values reached \(-2.0\) kPa (Fig. 2f). Leaves were collected from the same plants over the diurnal period to determine RWC, which showed no distinct pattern over the measurement period (Fig. 2g), and maintained values between 80% and 90% of the first pre-dawn sample. Again, no distinct pattern was apparent in the maximum PSII efficiency parameter \( (F_v/F_m) \) (Baker, 2008) between 06:00 and 14:00 h, conserving values between 0.748 and 0.812. Photorespiration was quantified by comparing \( A \) measured at \( 210 \) mmol \( \text{mol}^{-1} \text{C} \) with that at \( 20 \) mmol \( \text{mol}^{-1} \text{C} \), at four diurnal time points. Photorespiration increased by 50% between 08:00 and 10:00 h, reaching a maximum of 60% for the remaining measurement period up to 16:00 h (Fig. 2i). Overall, these data illustrate that the diel pattern of photosynthetic rate in \( R. \) stricta was dictated by the incident light, as well as stomatal behaviour, with the photosynthetic rate following PAR until 10:00 h, after which decreased stomatal conductance restricted \( \text{CO}_2 \) diffusion and limited carbon assimilation (Fig. 2). However, this response was not co-ordinated with that of \( g_s \), which dropped 2 h before any decrease in \( A \).
In order to determine the ability of *R. stricta* to maintain photosynthetic performance at high temperatures, photosynthesis was analysed as a function of light (A/IPAR) and internal CO₂ concentration (A/lₚ) at a range of temperatures during the morning period. The light- and CO₂-saturated maximum photosynthetic rate (Aₘₐₓ) increased significantly (t,df = 2.12,21; P < 0.05) with increasing temperature (Fig. 3a). An increase in temperature from 28 to 37°C resulted in a 15 µmol m⁻² s⁻¹ increase in Aₘₐₓ. Both the maximum rate of carboxylation (Vₐₘₐₓ) and the rate of RuBP regeneration (Jₘₐₓ) did not respond significantly to temperature, exhibiting typical values of 80 and 140 µmol m⁻² s⁻¹, respectively (Fig. 3b,c). As expected CO₂ evolution in the dark (Rd) showed a steady significant increase with temperature (t,df = 2.67,16; P < 0.05) up to c. 39°C, when it stabilized (Fig. 3e). Rd more than doubled when the temperature increased from 28 to 44°C. By contrast, there was a significant negative effect of temperature on light-saturated photosynthesis (Aₛᵃᵗ; t,df = -5.21,16; P < 0.01). Aₛᵃᵗ showed a relatively high rate of 15 µmol m⁻² s⁻¹ up to 35°C, after which further increases in leaf temperature resulted in a significant reduction in Aₛᵃᵗ (Fig. 3d) of 5 µmol m⁻² s⁻¹ (Fₐₛᵃᵗ = 10.75,18; P < 0.001). As temperature approached 40°C, Aₛᵃᵗ decreased from c. 15 to 8 µmol m⁻² s⁻¹.

*Post-hoc* (Tukey) analysis revealed highly significant differences between measurements obtained at average temperatures of 25–35°C and 40–45°C (P < 0.01; Fig. 3d).

Figure 4(a) shows the effect of temperature on the maximum quantum efficiency (Φ(CO₂)) determined from the slope of the light response curves carried out under non-photorespiratory conditions (20 mmol mol⁻¹ O₂). This provides a measure of the efficiency of light utilization for CO₂ fixation. Non-photorespiratory conditions were used for the light response curves conducted to assess the relationship between the photosynthetic operating efficiency (Fₐ/q/Fₐₘₐₓ) and CO₂ fixation corrected for leaf fractional light absorptance (Φ(CO₂)). The relationship between the photosynthetic operating efficiency and yield of CO₂ assimilation enables an assessment of possible alternative electron sinks to Rubisco activity. Neither the maximum quantum efficiency of CO₂ fixation (Φ(CO₂)) nor its ratio with the operating efficiency of PSII (Fₐ/q/Fₐₘₐₓ/Φ(CO₂)) was affected significantly by temperature (Fig. 4). Regression analysis gave t,df values of 0.498,11 and 0.227,14 (P > 0.05), respectively, for Φ(CO₂) and Fₐ/q/Fₐₘₐₓ. Values of Φ(CO₂) typically ranged between 0.03 and 0.06, and Fₐ/q/Fₐₘₐₓ was highly conserved above 8.00.

The analysis of genes coding for components essential for photosynthesis confirmed that *R. stricta* is typical of many plant species in this respect. The phylogenetic history of five photosynthesis components identified from the *R. stricta* transcriptome (transketolase; ribosomal L5P; Rubisco small chain subunit; chloroplast sedoheptulose-1,7-bisphosphatase; Rieske B6f (2Fe-2S)) (S. A. Yates et al., unpublished), compared with all plant species available from Ensembl, illustrated that the relatedness of these *R. stricta* proteins fell within a classification as a member of the Asteraceae, related to two solanacaeous species: tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) (Fig. S1).

In the work by S. A. Yates et al. (unpublished), transcripts encoding two RCA isoforms were found: *R. stricta*.8598 and...
16796. The alignment of these two sequences showed that one (R. stricta.16796) encodes a regulatory C-terminal domain, whereas the other (R. stricta.8598) does not (Table S2), termed long and short, respectively, hereafter. At the protein level, BLASTP analysis revealed an 80.2% similarity between the two sequences, suggesting that the two contiguous transcripts reported by S. A. Yates et al. (unpublished) are encoded by different genes. For further investigation, a phylogenetic-based approach was used to estimate the divergence of these proteins against other plant species. First, the retrieved sequences were, on average, 82.4% and 78.4% similar to the other species used for long and short R. stricta RCA, respectively. This analysis (Fig. 5) revealed a number of insights. Broadly, the expected RCA phylogeny is conserved, for example within the Asteraceae, Brassicaceae and Fabaceae families and also in monocots (Fig. 5). Also, when both short and long sequences were identified, they tended to share a terminal branching point (Fig. 5). However, there are aberrations in this phylogeny, most notably for R. stricta, where the two proteins appear to be more distantly related (as highlighted by the alignments in Table S2). Assuming no

Fig. 4 The response to temperature in Rhazya stricta of: (a) the ratio of the operating efficiency of photosystem II to the maximum quantum efficiency of net CO2 assimilation (Fv’/Fm’/\(\Delta F_{\text{CO}_2}\)); (b) the maximum quantum efficiency of net CO2 assimilation (\(\phi_{\text{CO}_2}\)). Circles represent individual measurements. All measurements of R. stricta were taken from dawn to 14:00 h at the temperature incident at that point in time, between 23 and 27 September 2012.

Fig. 5 Phylogenetic analysis of protein sequences of Rubisco activase (RCA). Branch support values are shown in red at branch points. The scale at the bottom shows substitutions per amino acid. For each species (including Rhazya stricta), up to two RCA isoforms are shown: ‘long’, which includes a C-terminal regulatory subunit, and ‘short’, without the regulatory subunit. Where multiple isoforms were found, but no distinction could be made regarding long or short, the isoforms were called A or B. Refer to Supporting Information Table S1 for relevant National Center for Biotechnology Information (NCBI) identifiers.
evolutionary pressure, these two RCA genes would be expected to share ancestry with other Asteraceae species. However, this is not evident; therefore, RCA isoforms for *R. stricta* can be considered as outliers (Fig. 5). BLASTP analysis of *Nicotiana tabacum* proteins against other Asteraceae species showed an average 89.3% similarity (excluding *R. stricta*). By contrast, *R. stricta* RCA proteins are, on average, 77.6% similar to those of other Asteraceae species, suggesting the increased divergence of the *R. stricta* protein. The most similar RCAs based on sequence similarity are those of *Gossypium hirsutum*, *Vitis vinifera* and *Theobroma cacao* for the long isoform, and *Ricinus communis*, *Zantedeschia aethiopica* and *Datisca glomerata* for the short isoform.

The phylogenetic analysis (Fig. 5) suggests that the short isoform of RCA in *R. stricta* is related to the Gymnosperm *Picea sitchensis*. However, the branch lengths of *R. stricta* and *P. sitchensis* (Fig. 5) reflect a large number of sequence differences between them. Therefore, we interpret this observation to indicate that both *R. stricta* and *P. sitchensis* are outliers compared with all the other Angiosperm species, and have been grouped together simply because of their relatively large sequence differences, and this does not imply that the short form RCA from *R. stricta* is closely related to the Gymnosperm sequences.

**Discussion**

Although the high temperature, high PAR and high VPD in the desert are extreme for many C3 plants (Feller *et al.*, 1998), *R. stricta* appears to be well adapted to them, with no obvious adverse physiological response and a highly dynamic transcriptome profile throughout the day (S. A. Yates *et al.*, unpublished). Indeed, the high temperature tolerance of *R. stricta* is remarkable, with no decrease in photosynthetic capacity observed up to leaf temperatures of 48°C (Fig. 3), when the majority of C3 species would be unable to survive (Feller *et al.*, 1998; Sage & Kubien, 2007). The thermal stability of photosynthesis in *R. stricta* with rising temperature over the diel period was confirmed by the lack of a temperature effect on the light- and CO2-saturated rate of photosynthesis (*A*max) and photosynthetic efficiency (*F*O′/Fm′). Further evidence for temperature tolerance was shown by the maximum *in vivo* carboxylation capacity of Rubisco (Vc,max), which was unaffected by temperatures up to 45°C (Fig. 3b). Similar photosynthetic capabilities have been observed in the C3 desert plants *Calitropis procerus* and *Calitropis gigantean* (Tezara *et al.*, 2011). The relatively high stomatal conductance values (Fig. 2c; compared with those from Willmer & Fricker (1996)) and stable leaf RWC (Fig. 2g) throughout the diel period suggest that *R. stricta* does not experience severe water stress. This confirms findings from a differential transcriptional analysis, which showed no up-regulation of common water stress transcripts over the day in *R. stricta* (S. A. Yates *et al.*, unpublished), and could be explained by the deep root system that has been observed in *R. stricta* (Emad El-Deen, 2005).

With the exception of dawn and dusk, values of WUE (A/E) typically ranged between 4 and 6 μmol mol⁻¹. These values were generally higher than those of common C3 crop species, such as cotton, wheat and sunflower grown under well-watered conditions, which ranged between 2.3 and 3.7 μmol mol⁻¹ (Rawson *et al.*, 1977; Constable & Rawson, 1980).

Photosynthesis is recognized as one of the most temperature-sensitive processes in plants (Yamori *et al.*, 2013), limiting plant productivity and determining plant distribution (Mittler, 2006). The cumulative photosynthesis over the diel period and the growing season dictates plant biomass (Sage *et al.*, 2008; Parry *et al.*, 2012). Therefore, the quantification of the impact of temperature on productivity requires an analysis of integral photosynthesis over at least a day. We compared the cumulative photosynthetic carbon fixed by *R. stricta* over a diel period (A) relative to that of C3 temperate crop plants routinely experiencing temperatures >30°C (Table 1). Although photosynthesis was slightly lower in *R. stricta*, none of these crops experienced such an extreme combination of high temperature and VPD. For example, cotton (Pima B375) showed a comparable A to *R. stricta* despite the latter coping with temperatures 7.5°C higher than those experienced by cotton, and a VPD that was more than double that of cotton (Table 1).

**Table 1** The daily integral net CO2 assimilation (A) of C3 crop species and *Rhazya stricta* at maximum leaf temperatures of >30°C

<table>
<thead>
<tr>
<th>Species</th>
<th>Diurnal photosynthesis A′ (mmol m⁻²)</th>
<th>Maximum temperature (°C)</th>
<th>Maximum VPD (kPa)</th>
<th>Irrigated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gossypium barbadense</em> (Cotton) Pima 32</td>
<td>540</td>
<td>35</td>
<td>2.6</td>
<td>Yes</td>
<td>Cornish <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>Cotton B375</td>
<td>526</td>
<td>35</td>
<td>2.6</td>
<td>Yes</td>
<td>Cornish <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>Cotton Pima S-6</td>
<td>619</td>
<td>35</td>
<td>2.6</td>
<td>Yes</td>
<td>Cornish <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>Cotton P-70</td>
<td>694</td>
<td>35</td>
<td>2.6</td>
<td>Yes</td>
<td>Cornish <em>et al.</em> (1991)</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> (Wheat)</td>
<td>590</td>
<td>35</td>
<td>4.9</td>
<td>Yes</td>
<td>Garcia <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>Glycine max</em> (Soybean)</td>
<td>644</td>
<td>35</td>
<td>2.21</td>
<td>No</td>
<td>Rogers <em>et al.</em> (2004)</td>
</tr>
<tr>
<td><em>Vitis vinifera</em> (Grape)</td>
<td>322</td>
<td>31</td>
<td>2.7</td>
<td>No</td>
<td>Downton <em>et al.</em> (1987)</td>
</tr>
<tr>
<td><em>Olea europaea</em> (Olive)</td>
<td>649</td>
<td>31</td>
<td>3.0</td>
<td>Yes</td>
<td>Angelopoulou <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>R. stricta</em></td>
<td>516</td>
<td>43</td>
<td>5.7</td>
<td>No</td>
<td>This study</td>
</tr>
</tbody>
</table>

If calculated A′ was not published, graphs were scanned and data were acquired using image processing software (Image). National Institutes of Health, Bethesda, MD, USA) and A′ was estimated using graphical analysis software (SigmaPlot 11.2, Systat Software Inc., Point Richmond, CA, USA). Where pre-dawn and post-dusk data were omitted, A was given a default value of −1 μmol m⁻² s⁻¹ at these time points. VPD, vapour pressure deficit.
Elevated temperature and heat stress are often coupled with water stress (Lobell et al., 2012), driven by increased evaporative cooling and transpiration as VPD increases (Lobell et al., 2013). In order for plants to use water efficiently, stomata must balance demands for CO₂ uptake with transpirational water loss (Wong et al., 1979; Peary, 1990; Lawson et al., 2010). The uncoupling of A and gₚ observed in R. stricta at particular time points during the day (Fig. 2) is not uncommon. The early morning peak in stomatal conductance (Fig. 2b), which is considerably higher than necessary for photosynthesis at that incident PAR, is mostly driven by the plant’s need for evaporative cooling to maintain a lower leaf temperature (Lobell et al., 2013), confirming that, under early diel conditions, R. stricta is not water stressed (S. A. Yates et al., unpublished). However, later in the day, when water becomes limiting and gₚ decreases, stomata close to avoid dehydration, thereby restricting photosynthesis through CO₂ diffusion (Cornic, 2000; Tezara et al., 2011). This control of gₚ on A is exemplified by the late afternoon re-opening of the stomata when gₚ has recovered and VPD has dropped, and A increases (Fig. 2). Stomatal closure is often driven by changes in abscisic acid (ABA) content (Schroeder et al., 2001; Seo & Koshiha, 2002) and increased expression of NCED (9-CIS EPOXYCAROTENOID DIOXYGENASE) and ZEP (ZEAAXANTHIN EPOXIDASE) genes, which code for key enzymes of ABA biosynthesis (Nambara & Marion-Poll, 2005), and was observed through the day in R. stricta (S. A. Yates et al., unpublished). Later in the day, stomatal conductance was uncoupled from transpiration (E), which is typical of plants experiencing high VPDs (Motzer et al., 2005) when a local equilibrium of humidity at the leaf surface is encountered (Meinzer et al., 1993). The high transpiration rates do not lead to a drop in leaf RWC, suggesting that changes in gₚ are driven by altered osmotic potential, for example, by the accumulation of compatible solutes, such as proline, commonly found in desert plants (Laurie et al., 1994). WUE (Fig. 2e), reducing stomatal conductance to maintain leaf water content, can induce other stresses, including damage to the photosystems (photoinhibition) when PAR is greatly in excess of that used for photosynthesis or safely dissipated as heat, and when sinks for the end products of electron transport are severely restricted (Valladares & Pearcy, 1997). Photoinhibition is apparent as a decrease in the maximum quantum efficiency of PSII photochemistry (Fₚ/Fₘ) (Baker, 2008). Rhazya stricta showed no signs of photoinhibition throughout the day with Fₚ/Fₘ maintained above 0.7, irrespective of temperature (Fig. 2). The photosystems are protected by mechanisms such as NPQ, which dissipates excess excitation energy when PAR is high or when photochemical quenching is reduced by stomatal closure (Daley et al., 1989; Brestic et al., 1995; Osmond et al., 1998). Rhazya stricta showed a typical increase in NPQ with increasing PAR and temperature up to c. 1.5 by 08:00 h, and then remained stable over midday (data not shown).

Previous studies examining the impact of high temperatures have shown effects on both photosynthetic electron transport and carbon fixation (Sage et al., 2008), both of which were unaffected in R. stricta. Thermotolerance has often been associated with the induction of HSPs and heat shock transcription factors (HSFs) (Scharf et al., 2011), or with hormones, such as salicylic acid (Dat et al., 1998), which decrease susceptibility to photoinhibition (Karpinski et al., 2003; Bechtold et al., 2005; Mateo et al., 2006). Transcriptional expression analysis in R. stricta revealed an increase in both HSP and HSF, as well as genes involved in the production of salicylic acid, throughout the day, with peak expression values observed at dusk (S. A. Yates et al., unpublished).

Rhazya stricta also has the capacity to increase photorespiration as temperatures rise throughout the day (Fig. 2i), which acts as a major sink for the end products of the electron transport chain, providing a mechanism for the dissipation of excess excitation energy at low gₚ (Fig. 2c) (Osmond & Bjorkman, 1972; Cornic & Briantais, 1991; Murchie & Niyogi, 2011). Protection via photorespiration is demonstrated by the fact that gₛ/Fₘ and the ratio of Fₚ/₁/Fₘ/₁/PCO₂ (measured under 20 mmol mol⁻¹ [O₂]) is unaffected at temperatures > 36°C (Fig. 3). This lack of decreased photosynthetic capacity suggests that the lower Aₚ at higher temperatures is driven by gₛ and/or photorespiration (Fig. 2). The increase in photorespiration validates the expression of many of the genes encoding enzymes of the photosynthetic electron transport cycle in R. stricta (S. A. Yates et al., unpublished). In addition to protection by photorespiration, there are a number of additional well-developed processes for the dissipation of excitation energy present in R. stricta, such as PLASTID TERMINAL OXIDASE (PTOX) (S. A. Yates et al., unpublished). Collectively, these most likely underpin the high resistance of R. stricta leaves to photoinhibition under high PAR (Fig. 1h). However, the ratio of Fₚ/₁/Fₘ/₁/pcO₂ in R. stricta was unresponsive to temperature and typically remained below 10, suggesting that both photosynthesis and photorespiration are the major sinks for the end products of photosynthetic electron transport (Genty et al., 1989).

Previous studies have shown a correlation between a reduction in RCA content at elevated temperatures (and associated drop in Rubisco activation state) and lower photosynthetic rates (Henrickson et al., 2008), especially in C₃ crops with thermolabile RCAs (Crafts-Brandner & Salvucci, 2000) which typically denature irreversibly at > 42°C (Gen & Sage, 2005). In many species, RCA exists in two distinct isoforms (Ristic et al., 2009) that may be expressed by different genes (Salvucci et al., 2003) or formed by alternative splicing of a single pre-mRNA (Werneke et al., 1989; Rundle & Zielinski, 1991; To et al., 1999). The effects of heat stress in these species included differential expression of endogenous RCA isoforms or the appearance of novel putative RCAs (Sanchez de Jimenez et al., 1995; Ristic et al., 2009). Expression of a distinct RCA isoform in cotton, maize and wheat has been shown to require prolonged exposure to heat stress and in cotton, this isoform has been proven to be extremely thermostable in vitro (Kurek et al., 2007). One or both of the R. stricta putative RCA isoforms may be thermally stable. This suggestion is supported by the ability of R. stricta to maintain photosynthesis and/or photorespiration under these extreme temperatures. That the RCA(s) of R. stricta may have unique properties is supported by their aberrant position in the phylogenetic analysis and their divergence from one another and from those of other Asteraceae species (Fig. 5). We suggest, based on the
sequence alignment (Table S2), that RCA has undergone a duplication event in which one copy \((R.\ stricta.8595)\) has lost the ability to generate an isoform that contains the regulatory subunit as found in other species (Salvucci et al., 2003), whereas the other, \(R.\ stricta.16796\), has the regulatory subunit. The discordant position of the two isoforms of \(R.\ stricta\) on the phylogenetic tree suggests that these two genes are under functional divergence (Fig. 5) (de Vienne et al., 2012), where positive selection for changes in amino acids could modulate enzyme activity or stability. Further work is required to assess the functionality of the two \(R.\ stricta\) RCA homologues. The feasibility and positive impact on the photosynthetic rate of RCA by genetic manipulation have been successfully demonstrated by the genetic manipulation of Arabidopsis using gene shuffling techniques to produce a more thermostable isoform (Kurek et al., 2007). However, further investigation to determine the species dependences of Rubisco–RCA interactions is required before such approaches can be used to manipulate RCAs in crop species (Wang et al., 1992).

Acknowledgements

This project was funded by the Deanship of Scientific Research (DSR), King Abdul Aziz University (KAU), Jeddah, Saudi Arabia, under grant number (D 008/431). The authors therefore acknowledge with thanks DSR and KAU’s Vice President for Educational affairs, Professor A. O. Alyoubi, for technical and financial support. Professor M. Parry and Dr J. Andralojc are acknowledged for their assistance in our failed attempts to determine Rubisco activity in \(R.\ stricta\). Dr T. Hofmann is thanked for useful comments.

References


Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phylogenetic analysis of species from common plant proteins.

Table S1 Rubisco activase (RCA) homologues in plant species from BLASTP against National Center for Biotechnology Information (NCBI) database

Table S2 Amino acid sequence alignment of Rubisco activase (RCA) genes in *Rhazya stricta*

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