Rubisco activities, properties, and regulation in three different C_4 grasses under drought

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Abstract

In C_4 plants, water deficit may decrease photosynthetic CO_2 assimilation independently of changes in stomatal conductance, suggesting decreased turnover by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The activity and biochemistry of Rubisco was studied in three different C_4 grasses: Paspalum dilatatum, Cynodon dactylon, and Zoysia japonica. The objectives were to characterize the C_4 Rubisco in these species and to identify factors associated with decreased photosynthetic rates caused by drought. Rubisco isolated from each of the three C_4 grasses was characterized by smaller specificity factors (S_{C/O}), larger Michaelis–Menten constants for CO_2 (K_c) and O_2 (K_o), and larger maximum carboxylation velocities (V_c) than Rubisco from wheat, which can be rationalized in terms of the CO_2-rich environment of C_4 Rubisco in the bundle sheath. During leaf dehydration the quantity and maximum activity of Rubisco remained unchanged but the initial and total activities declined slightly, possibly due to increased inhibition. Tight-binding inhibitors were present in the light but were more abundant in the dark, especially in Z. japonica, and increased in quantity with drought stress. The inhibitor from darkened leaves of Z. japonica was identified as 2-carboxyarabinitol-1-phosphate (CA1P). Consistent with the presence of CA1P, the total activity of Rubisco was decreased after 12 h darkness in Z. japonica. Ribulose-1,5-bisphosphate (RuBP) in the leaves decreased with drought stress, to quantities approximating those of Rubisco catalytic sites. The magnitude of the decrease in RuBP suggested that, at least in C. dactylon and Z. japonica, it could contribute to the drought-induced decrease in photosynthesis.

Key words: CA1P, Cynodon dactylon, kinetic constants, Paspalum dilatatum, Rubisco, RuBP, water deficit, Zoysia japonica.

Introduction

The presence of a CO_2-concentrating mechanism in the leaves of C_4 plants results in improved water use efficiency compared to C_3 plants (Long, 1999) and is generally seen as a potential advantage for areas with increased aridity. Stomatal closure is amongst the earliest plant responses to decreasing water availability and, in C_3 plants, constitutes a major limitation to photosynthetic CO_2 assimilation under such conditions. In those plants, metabolic or non-stomatal limitations to photosynthesis may also be observed under mild to moderate water-deficit conditions, concomitantly with stomatal limitation, but their relative contribution to decreased CO_2 assimilation increases with the severity of stress (Lawlor, 2002). C_4 photosynthesis saturates at much lower CO_2 concentrations and is therefore unlikely to be affected by stomatal closure in the same fashion as C_3 photosynthesis. Increased leakiness of the bundle sheath may
cause decreased CO₂ concentrations at the site of carboxylation (Saliendra et al., 1996; Williams et al., 2001). However, in several C₄ grasses subjected to water deficit, raising CO₂ concentrations to physiologically high levels—which would suppress the effect of decreased stomatal conductance and increased leakiness—did not result in the recovery of net photosynthetic rates to those of well-watered plants (Carmo-Silva et al., 2008; Ghanoum, 2009), supporting the presence of non-stomatal limitations of photosynthesis. The limited number of studies with C₄ species suggests higher susceptibility of the C₃ than the C₄ cycle enzymes to impairment by water deficit (reviewed by Ghanoum, 2009).

Ribulose-1,5-bisphosphate (RuBP) carboxyylase/oxygenase (Rubisco) is crucial in the regulation of photosynthesis in fully-hydrated leaves of the C₄ species *Flaveria bidentis* (Furbank et al., 1997), but the importance of this most abundant of leaf proteins in the limitation of C₄ photosynthesis under drought conditions is not well understood. Flexas and Medrano (2002) suggested that, in C₃ plants, Rubisco capacity was of little importance in the drought-induced limitation of photosynthesis, whilst decreased capacity for RuBP regeneration—possibly as a result of decreased ATP synthesis or impaired Calvin cycle enzyme activities (Lawlor, 2002)—would constitute a major metabolic limitation. However, decreased activity and/or quantity of Rubisco has been observed in several different C₃ and C₄ species exposed to water deficit (Majumdar et al., 1991; Du et al., 1996; Lal and Edwards, 1996; Parry et al., 2002; Tezara et al., 2002; Bota et al., 2004; Marques da Silva and Arrabaça, 2004; Carmo-Silva et al., 2007; Soares-Cordeiro et al., 2009).

Rubisco activity is regulated by the extent of carboxylation of a lysyl residue within the catalytic site (Lorimer and Miziorko, 1980) and is affected by various chloroplast metabolites (Hatch and Jensen, 1980; Badger and Lorimer, 1981; Jordan et al., 1983) and by naturally occurring tight-binding inhibitors (Pearce and Andrews, 2003; Kim and Portis, 2004). At night, Rubisco may be inhibited by a specific tight-binding inhibitor, 2-carboxyarabinitol-1-phosphate (Gutteridge et al., 1986; Berry et al., 1987; Moore et al., 1992), which is present in some species (Vu et al., 1984; Seemann et al., 1985; Servaites et al., 1984, 1986; Holbrook et al., 1992; Sage and Seemann, 1993). The main contender for the inhibition of Rubisco in the light (Kane et al., 1998), 3-d-glycerido-2,3-diulose-1,5-bisphosphate (PDBP), is too labile for detailed study, and the occurrence of inhibitors during daytime (Keys et al., 1995) has become associated with misfire products formed during enolization and oxygenation of RuBP catalysed by Rubisco (Pearce and Andrews, 2003; Kim and Portis, 2004). It is generally accepted that removal of all these inhibitors requires the ATP-dependent action of Rubisco activase. Tight-binding inhibitors such as CAIP protect Rubisco from proteolytic breakdown (Khan et al., 1999) and may play an important role in regulating the enzyme under stress conditions (Parry et al., 2008), as well as during darkness and at low irradiance (Kobza and Seemann, 1989a, b).

Photorespiration is initiated by the oxygenase activity of Rubisco and decreases both the rate and efficiency of CO₂ assimilation in C₃ plants. Because CO₂ and O₂ are competing substrates (Bowes and Ogren, 1972; Laing et al., 1974), the high CO₂ concentration in the bundle sheath of C₄ plants means that the oxygenase activity is much decreased. This situation could be compromised under conditions of water deficit since the concomitant reduction in stomatal aperture leads to decreased CO₂ availability. However, photorespiration has been shown neither to increase nor contribute to the limitation of photosynthesis in C₄ grasses under drought stress (Carmo-Silva et al., 2008).

From a consideration of the kinetics of the carboxylation and oxygenation of RuBP catalysed by Rubisco, the specificity factor (Sₕₒ) defines the relative reactivity towards the two gaseous substrates and is given by $V_c/K_c$ over $V_o/K_o$, where $V_c$ and $V_o$ represent the maximum velocities of the carboxylase and oxygenase reactions, respectively, and $K_c$ and $K_o$ the Michaelis–Menten constants for CO₂ and O₂. The individual kinetic constants are known for relatively few types of Rubisco, mostly from C₃ species. Michaelis–Menten constants of Rubisco from C₃ and C₄ grasses for CO₂ and RuBP have been reported by Yeoh et al. (1980, 1981) and the Rubisco kinetic parameters have recently been compared in eudicot plants with the C₃ and C₄ photosynthetic pathways (Kubien et al., 2008). In the sequence of studies described by Carmo-Silva et al. (2007, 2008, 2009) and Soares-Cordeiro et al. (2009) on the responses of three different C₄ grasses to water deficit, it became important to add details of the kinetic constants for the Rubiscos peculiar to these three species.

The objectives of this investigation were to study the biochemistry of Rubisco and to determine the effects of water deficit on the regulation of the enzyme in three C₄ grasses of different metabolic subtypes: *Paspalum dilatatum* Poir. (NADP-malic enzyme, NADP-ME), *Cynodon dactylon* (L.) Pers (NAD-malic enzyme, NAD-ME), and *Zozysia japonica* Steudel (phosphoenol/pyruvate carboxykinase, PEPC). The previous studies showed that net photosynthesis was decreased in these three grasses by both slowly and rapidly induced drought stress (Carmo-Silva et al., 2007, 2008). The purpose of the present study was to investigate the relationship to drought of decreased carboxylation of RuBP (photosynthesis), the activation state of Rubisco due to carboxylation, the presence of inhibitors, and the quantity of RuBP in the leaves.

**Materials and methods**

**General methods**

Rubisco was purified from wheat as described by Keys and Parry (1990). RuBP prepared as in Wong et al. (1980), 2-carboxyarabinitol-1,5-bisphosphate (CABP) prepared from RuBP by reaction with KCN or [¹⁴C]-KCN followed by hydrolysis of the cyanohydrins, and CAIP made from CABP by partial dephosphorylation using alkaline phosphatase (Gutteridge et al., 1989) were all purified by anion-exchange chromatography. Radioactivity of [¹⁴C] labelled compounds, typically in a volume of 0.4–0.45 ml of aqueous solution, was measured after mixing with 3.6 ml Ultima Gold Scintillation cocktail in a Liquid Scintillation Analyser (Perkin-Elmer, USA). Specific mixtures of N₂ and O₂ were
prepared using a gas divider (Signal Group, UK) and concentrations of O₂ in solution were calculated by taking the solubility at 25 °C in water as 257.5 μM in a standard atmosphere at 100% relative humidity and correcting for the atmospheric pressure during measurements [using the formula 257.5 × (P/11589) (101 325–11589), where P is the observed pressure, 11589 Pa is the saturated H₂O vapour pressure at 25 °C, and 101325 Pa is the standard atmospheric pressure]. Concentrations of CO₂ in solution in equilibrium with HCO₃⁻ were calculated assuming a pK₅ for carbonic acid at 25 °C of 6.11 and using accurate measures of the pH of each buffer solution. Values of Michaelis–Menten constants and maximum velocities were estimated using EnzFitter ( Biosoft: Software for Science, UK).

Plant growth and drought stress induction

The C₄ grasses Paspalum dilatatum Poir. cv. Raki (NADP-ME), Cynodon dactylon (L.) Pers var. Shangri-Lá (NAD-ME), and Zopisia japonica Steudel ‘Jacklin Sunrise Brand’ (produced by Jacklin Seed Company, USA) (PEPCCK) were grown from seeds in trays or pots with peat-free compost in a greenhouse, as previously described by Carmo-Silva et al. (2008, 2009). Artificial light was provided whenever the natural light was below a photosynthetic photon flux density (PPFD) of 500 μmol m⁻² s⁻¹ during a 16 h photoperiod. The temperature was set not to fall below 25 °C during the day or below 18 °C during the night. Pots, containing five seedlings each, were well-watered until the beginning of the drought stress treatment and then placed according to a rectangular split-plot design, where each column of pots was a main plot of the different measurements. Because the growing conditions were not duplicated and organized in two blocks per pot corresponded to one independent sample. When appropriate, a lar split-plot design, where each column of pots was a main plot of five seedlings each, were well-watered until the beginning of the photoperiod, or in the dark, after placing the growth environment under fully illuminated conditions, 4 h after the addition of PEG4000 to a final concentration of 25% and the addition of 20 mM MgCl₂ and the incubation for 5 min to hydrolyse lactones of CA1P and allow the binding to Rubisco. Incubating 25 μl of the same extract for 3 min with all the components except RuBP, to allow carbamylation of all available Rubisco catalytic sites, and then starting the reaction by adding RuBP. Maximal activity was measured after the removal of Rubisco tight-binding inhibitors by incubating 250 μl of crude extract with 200 mM Na₂SO₄, 10 mM NaHCO₃, and 20 mM MgCl₂ for 30 min at 4 °C. Protein was precipitated with 20% (w/v) PEG4000 and 20 mM MgCl₂ and then washed three times with 20% PEG6000 in 100 mM Bicine-NaOH pH 8.2, 20 mM MgCl₂, 10 mM NaHCO₃, and 50 mM 2-mercaptoethanol. The final precipitate was dissolved in extraction buffer and assayed for total activity. The quenched reaction mixtures were completely dried at 100 °C and the residues rehydrated before ¹³C determination.

Quantities of RuBP and Rubisco tight-binding inhibitors

RuBP and Rubisco tight-binding inhibitors were extracted by grinding frozen leaf samples (0.1–0.4 g FW) to a fine powder in LN₂ and then adding 0.45 M trifluoroacetic acid (TFA; 0.25 ml per 0.1 g FW). The mixture was ground further during thawing. Duplicate subsamples (20 μl) were taken for chlorophyll determination as phaeophytin and the remaining homogenate was centrifuged for 5 min at 16000 g and 4 °C.

For the estimation of RuBP, an aliquot (50 μl) of the acid extract was dried in a glass vial under high vacuum over anhydrous CaCl₂ and NaOH pellets. The residue was dissolved in 50 μl H₂O, dried down, and redissolved in 50 μl H₂O again. The RuBP contained in each vial was converted to [¹³C]phosphoglycerate by incubating at room temperature for 45 min in a reaction mixture (0.5 ml) containing 100 mM Bicine-NaOH pH 8.2, 20 mM MgCl₂, 8 mM NaH¹³CO₃ (18.5 kBq μmol⁻¹), and 20 μg of pure, activated wheat Rubisco. The reaction was quenched with 0.1 ml 10 M HCOOH. The mixture was dried down and the residue rehydrated for liquid scintillation counting.

Rubisco inhibitors in 20 μl of the acid extracts were measured by comparison to inhibition of the enzyme by known quantities of CA1P (in 20 μl of 0.45 M TFA). Each standard and sample extract solution was mixed with 230 μl of (final concentrations) 100 mM Bicine-NaOH pH 8.2, 20 mM MgCl₂, 10 mM NaH¹³CO₃, and 10 μg of activated wheat Rubisco, and incubated for 5 min to hydrolyse lactones of CA1P and allow the binding to Rubisco. Rubisco activity was measured by adding 250 μl of 100 mM Bicine-NaOH pH 8.2, 20 mM MgCl₂, 10 mM NaH¹³CO₃ (18.5 kBq μmol⁻¹) and 0.4 mM RuBP, and the reaction was quenched after 2.5 min with 0.1 ml 10 M HCOOH. The mixture was dried down and the residue rehydrated before scintillation counting.

Idenification of CA1P by HPLC

Frozen leaf samples (0.1–0.2 g FW) were ground to a fine powder in LN₂ and extracted with 1.0 ml of 0.45 M TFA after the addition of a an...
of 490 Bq (240 pmol) [14C]-CA1P. After centrifugation, the extracts were purified by passage through 0.5 g Solid Phase Extraction columns (C18-E, Phenomenex, USA). The eluate was evaporated to dryness in vacuo over anhydrous CaCl2 and NaOH pellets. The residue was resuspended in H2O, passed through a column of Dowex 50 H+ (0.5 ml), and the eluate evaporated to dryness as before. The residue was dissolved in 0.25 ml H2O and mixed with 50 μl 1 M TRIS base. The resulting, mildly alkaline, solution was stored at –20 °C before fractionation by HPLC. The fractionation was conducted on a Dionex PA1 column (4×250 mm) using a DX500 chromatography system operated at 1 ml min⁻¹. The eluent was 100 mM CO2-free NaOH containing varying concentrations of sodium acetate: 100 mM from 0 to 5 min increasing to 800 mM at 30 min, to 900 mM at 35 min, and decreasing back to 100 mM at 40 min. In this system the retention times (min) were 8.9 for glucose-1-phosphate, 15.1 for fructose-6-phosphate, 16.0±0.2 for CA1P, and 20.9 for fructose-1,6-bisphosphate. Fractions of 0.5 ml were collected and each mixed with 75 μl of 0.2 M Bicine-NaOH pH 8.0 and 1 M HCl. Fractions containing CA1P were identified by the presence of [14C] and frozen at –20 °C.

The CAIP was restricted to two HPLC fractions and the total in each sample was obtained by adding the quantities estimated for each fraction. CAIP in 100 μl aliquots was measured by the inhibition of Rubisco activity by comparison to CAIP standards, making appropriate allowance for the presence of the small quantity of added [14C]-CAIP. Reaction mixtures (0.5 ml) contained 100 mM Bicine-NaOH pH 8.0, 20 mM MgCl2, 10 mM NaH14CO3 (18.5 kBq μmol⁻¹), 0.4 mM RuBP, and 10 μg of activated Rubisco. Samples and standard solutions were preincubated with the reaction mixture without RuBP for 7 min before adding the RuBP to start the reaction. The reaction was stopped after a further 7 min by adding 0.1 ml 10 M HCOOH. The acidified reaction mixtures were dried and 14C measured in the residues after rehydration. Corrections for losses of CAIP during the isolation process were made, based upon the recovery of the added [14C]-CAIP.

**Total chlorophyll determination**

The total chlorophyll content in the leaf homogenates was determined spectrophotometrically, as either chlorophyll or phaeophytin, after extraction in ethanol (Wintermans and de Mots, 1965). The total chlorophyll content (relative to the leaf area and turgid mass) remained unchanged with water deficit in the three C4 grasses (data not shown) and provided a good basis to normalize the data of the different measurements.

**Rubisco kinetic constants**

Seedlings of *Triticum aestivum* L. cv. Riband (C3 species used as the reference) and of the three C4 grasses were grown in trays for the determination of Rubisco kinetic parameters using young leaves. Leaf samples (0.5 g FW) were quickly frozen in LN2 and used within one day. Rubisco was extracted by grinding the leaves in a cold mortar with quartz sand, 2.5% (w/v) insoluble PVP, and 5 vols of ice-cold extraction medium containing 100 mM Bicine-KOH pH 8.2, 0.1 mM EDTA, 6% (w/v) PEG4000, 10 mM DTT, 50 mM 2-mercaptoethanol, 2 mM MgCl2, 10 mM NaHCO3, 1 mM benzanilide, 1 mM ε-aminocaproic acid, and 1% (v/v) protease inhibitor cocktail (Sigma, USA). After grinding to produce a fine suspension, the homogenate was centrifuged for 4 min at 16000 g and 4 °C. Low molecular weight proteins and salts present in the leaf crude extracts were removed by passage of supernatant (1 ml) through a Sephadex G-200 (GE Healthcare, USA) column (20 ml bed volume, 1.5×11.5 cm) pre-equilibrated and eluted with desalt buffer (100 mM Bicine-KOH pH 8.2, 0.5 mM EDTA, 1 mM KH2PO4, 20 mM MgCl2, 10 mM NaHCO3, 1 mM benzanilide, and 1 mM ε-aminocaproic acid). This treatment was introduced to remove potentially interfering enzymes of mass ≤150 kDa.

Fractions of 0.5 ml were collected and, after measuring the soluble protein content (Bradford, 1976), the three fractions containing most protein were combined and the protease inhibitor cocktail added to a final concentration of 2.5%/v/v. The mixture was divided into different aliquots, some of which were immediately frozen in LN2 (for later measurement of Rubisco quantity and for appropriate control assays).

All measurements for the determination of kinetic parameters were conducted at 25 °C. The Michaelis–Menten constant (Km) for CO2 (Kc) was measured essentially as described by Bird et al. (1982), but Km for O2 (K0) was estimated by measuring Kc apparent at several O2 concentrations (0, 21, 60, and 100%, balanced with N2). The carboxylation activity of Rubisco was determined at several CO2 concentrations for each gas mixture. The assay buffer was pre-treated by sparging with the appropriate gas mixture. Septum-sealed vials with stirring magnets were connected in series through the septa using butyl rubber transfer tubes fitted at each end with hypodermic needles and flushed with the appropriate CO2-free gas mixtures at 20 ml min⁻¹ for at least 30 min. Flushing of the vials was discontinued after the addition of the appropriate buffer but before the addition of NaH14CO3. The reaction mixtures (1 ml) contained (final concentrations) 100 mM Bicine-NaOH pH 8.2, 20 mM MgCl2, 10 μg ml⁻¹ carbonic anhydrase (freshly dissolved), 0.4 mM RuBP, and six different concentrations of NaH14CO3 (0–10 mM; 37 kBq μmol⁻¹). The reactions were started at 30 s intervals by adding 20 μl of the partially purified leaf extract previously activated by incubation with 10 mM NaH14CO3 (37 kBq μmol⁻¹) for 30 min. The reactions were quenched after 2 min with 0.1 ml 10 M HCOOH. Changes in the activity of Rubisco through the course of the assays were monitored by the use of replicates of the same vial at staggered time points. The acidified mixtures were dried down and the residues rehydrated for scintillation counting.

Several control assays were performed in order to ensure that 14C incorporation by Rubisco occurred in the conditions of the assay at saturating CO2 and low O2 concentrations (positive control), and that 14C incorporation did not occur (a) in the absence of the substrate RuBP; (b) when RuBP had been replaced by phosphoglycerate; or (c) when the Rubisco preparation had been preincubated with CABB to block the catalytic sites (negative controls). The quantity of Rubisco in the partially purified extracts was determined essentially as described above, using [14C]-CABB.

**Rubisco specificity factor**

Rubisco was purified from fresh and young leaves of each of the three C4 grasses to determine the specificity factor (Fspec) by total consumption of RuBP in the oxygen electrode (Hansatech Instruments, UK) as described by Parry et al. (1989). The purification was essentially as described by Haslam et al. (2005) and consisted of precipitation of Rubisco from the leaf extracts with 20% PEG4000, step-elution from an anion-exchange column, and desalting.

**Statistical analysis**

All the analyses were made using GenStat® 9.2, 2005 (Lawes Agricultural Trust, UK). Regression analysis was applied to model the variation of the different measurements with RWC. Non-significantly different parameters (t tests, P >0.05) in the significant model terms of the regression (F-tests, P<0.05) were amalgamated in order to attain parsimony. The residuals were checked and found to conform to the assumptions of the analysis. The resulting best regression models were plotted and the parameter estimates (intercepts and slopes) with their respective standard errors (se), the percentage of variance accounted for by the model (R2), the residuals mean square (s²), and the degrees of freedom (df) are given with the figures.
Results

Rubisco activities

The initial and total activities of Rubisco declined slightly with decreasing RWC but maximal activities of the enzyme were not significantly affected (P >0.05) by leaf dehydration (Fig. 1). Initial activities were approximately 60% of maximal activities for P. dilatatum and about 40% of maximal activities for C. dactylon and Z. japonica, indicating that Rubisco activity was down-regulated by the lack of carboxylation of many catalytic sites under the growing conditions. Total activities were also less than maximal activities, especially at decreased RWC, consistent with further down-regulation by the presence of inhibitors in all three species.

Quantities of Rubisco and RuBP

The concentration of Rubisco catalytic sites mg⁻¹ chlorophyll in leaves of the three species was not significantly affected (P >0.05) by water deficit and the average values for P. dilatatum, C. dactylon, and Z. japonica were 26.6±0.8, 24.4±0.8, and 21.0±0.8 nmol mg⁻¹ Chl, respectively. In all three species, as the RWC fell, the quantities of RuBP decreased to values approaching the catalytic site concentration (Fig. 2), suggesting that the rate of carboxylation could become increasingly limited by the availability of the CO₂ acceptor substrate as the leaves suffered water deficit. The quantities of RuBP present in the fully-hydrated leaves of C. dactylon and Z. japonica were greater, but the decrease with water deficit was more marked than in P. dilatatum.

In order to compare the effects of water deficit on RuBP quantities (this study) and on net CO₂ assimilation (Carmo-Silva et al., 2008), values obtained for drought-stressed plants of each of the three species were expressed as a percentage of average values for the corresponding control plants. Both the relative rates of net CO₂ assimilation and RuBP quantities declined with decreasing RWC in the leaves of the three species (Fig. 3). Notably, in C. dactylon and Z. japonica, but not in P. dilatatum, the decrease in RuBP was apparently more marked than the decrease in the net photosynthetic rates.

Evidence for tight-binding inhibitors

Tight-binding inhibitors were quantified by the inhibition of purified Rubisco by acid extracts (Fig. 4). In illuminated leaves these inhibitors were present in very low quantities and appeared to increase only marginally with leaf dehydration (Fig. 4B), up to the equivalent of ~5% of the Rubisco catalytic sites (Fig. 2). Due to a non-significant

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**Fig. 1.** Rubisco initial, total and maximal carboxylation activities (µmol CO₂ min⁻¹ mg⁻¹ chlorophyll) as a function of the relative water content (RWC, %) in the leaves of Paspalum dilatatum (A, D), Cynodon dactylon (B, E), and Zoysia japonica (C, F). Initial activities (A–C, black symbols) were determined immediately after extraction, total activities (A–C, grey symbols) after activation in the presence of CO₂ and Mg²⁺, and maximal activities (D–F, open symbols) after removal of tight-binding inhibitors with sulphate. Each data point corresponds to one sample, with seven (P. dilatatum and Z. japonica) or eight (C. dactylon) control (well-watered) and 12 non-watered samples per species. Regression lines were fitted when the RWC effect was significant (F-test; Initials: R²=50.5%, s²=0.033, df=55, P <0.001; Totals: R²=32.5%, s²=0.052, df=56, P <0.001; Maximals: no regression on RWC, P >0.05).
interaction between species and treatment ($P>0.05$), the same regression with $RWC$ fitted the data for the three C4 grasses in the light (Fig. 4B). Larger quantities of tight-binding inhibitors were found in dark-adapted than in illuminated leaves, especially in *Z. japonica* (Fig. 4A).

Because $RWC$ was partly re-established during the night, through decreased evaporation from the leaves and continued water absorption from the soil, samples collected from non-watered pots at the end of the 12 h dark period had higher $RWC$ values, and corresponded to a narrower $RWC$ range, than samples collected in the light period (compare Fig. 4A with Fig. 4B). Even so, and despite the large scatter, a significant ($P<0.001$) increase in the quantity of inhibitor with increasing water deficit was observed. In the dark, the inhibitor content increased with decreasing $RWC$ to the equivalent of $\approx 8\%$ of the Rubisco catalytic sites in *P. dilatatum* and *C. dactylon* and to $\approx 28\%$ of the catalytic sites in *Z. japonica*.

Further studies attempted to characterize the inhibitor in *Z. japonica*. A component was isolated from acid extracts of the dark-adapted leaves of this species, which inhibited purified Rubisco and had a retention time in HPLC identical to that of authentic CA1P. Quantification of this inhibitory component utilized a trace of $[^{14}C]$-CA1P—added at the time of tissue homogenization—to correct for losses during extraction and HPLC
separation. Leaves of plants not subjected to drought were used to establish the occurrence of CA1P after a prolonged period of darkness. The quantities of CA1P estimated after HPLC (Table 1) were in the range of values obtained by the direct estimates of inhibitor in crude acid extracts shown in Fig. 4. Therefore, it is likely that CA1P is the predominant inhibitor present in the dark in Z. japonica.

In agreement with the presence of a tight-binding inhibitor in the dark in Z. japonica, the total activities of Rubisco in dark-adapted leaves of this species (after the 12 h dark period) were ~74% of the total activities in leaves harvested in the light (Table 2), meaning that inhibitors were bound to ~26% of the catalytic sites in the dark. This result was independent of water deficit and was observed only for Z. japonica. No significant (P > 0.05) differences in Rubisco total activities were observed between the light- and dark-adapted leaves of P. dilatatum and C. dactylon and therefore no evidence was shown of a bound inhibitor for these two species. In neither light nor dark was there evidence of an increase in tight-binding inhibitor with drought stress, so these data did not support the slight trend shown in Figs 1 and 4.

Rubisco kinetic parameters

Rubisco purified from each of the three C4 grasses, P. dilatatum, C. dactylon, and Z. japonica, was characterized by lower S_C/O than in wheat (C3 species used as reference) (Table 3). The Michaelis–Menten constants of Rubisco for CO2 and O2 (K_c and K_o) estimated for each of the C4 species were in the same range and were higher than the corresponding values estimated for wheat. The maximum Rubisco carboxylation activity (V_c) was also higher in the C4 than in the C3 species, and among the grasses V_c was higher in Z. japonica than in C. dactylon and lowest in P. dilatatum.

Attempts were made to measure both V_c and V_o by an HPLC method based on that of Yaguchi et al. (1996) using partially purified extracts of leaves from wheat and each of the three C4 grasses. These attempts failed and V_o was subsequently determined indirectly by solving the equation S_c/O = V_c/K_o/V_o/K_c. However, the variation in the measurements of S_c/O is too high to allow much precision in the

Table 1. Quantity of CA1P (nmol mg⁻¹ chlorophyll) in light- and dark-adapted leaves of Zoysia japonica

Samples were taken from well-watered plants after a period of 12 h in darkness (Dark) or 4 h after the beginning of the photoperiod (Light). The inhibitor was extracted with trifluoroacetic acid and measured after HPLC separation. Extracts were spiked with tracer quantities of [¹⁴C]-CA1P to allow correction for losses in processing the samples (average 56.4%). The retention time in the chromatographic system was the same as authentic CA1P and consistent with the radioactive spike. Values are means of four biological replicates ± standard errors.

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Z. japonica CA1P (nmol mg⁻¹ Chl)</th>
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<tbody>
<tr>
<td>Dark</td>
<td>5.97 ± 1.76</td>
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<tr>
<td>Light</td>
<td>0.53 ± 0.39</td>
</tr>
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Table 2. Total activity of Rubisco (µmol min⁻¹ mg⁻¹ Rubisco) extracted from light- or dark-adapted leaves of well watered (control, C) and non-watered (drought stress, S) plants of Paspalum dilatatum, Cynodon dactylon, and Zoysia japonica

Samples were taken after a period of 12 h in darkness (Dark) or 4 h after the beginning of the photoperiod (Light). Corresponding leaf relative water contents (RWC, %) are also given. Values are means of three biological replicates ± standard errors.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P. dilatatum</th>
<th>C. dactylon</th>
<th>Z. japonica</th>
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<tbody>
<tr>
<td>Rubisco total activity (µmol min⁻¹ mg⁻¹ Rubisco)</td>
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<td></td>
</tr>
<tr>
<td>Dark</td>
<td>2.03 ± 0.06</td>
<td>1.83 ± 0.10</td>
<td>1.74 ± 0.07</td>
</tr>
<tr>
<td>S</td>
<td>1.96 ± 0.08</td>
<td>1.81 ± 0.10</td>
<td>1.74 ± 0.03</td>
</tr>
<tr>
<td>Light</td>
<td>2.11 ± 0.02</td>
<td>1.73 ± 0.25</td>
<td>2.36 ± 0.04</td>
</tr>
<tr>
<td>S</td>
<td>2.14 ± 0.16</td>
<td>1.79 ± 0.07</td>
<td>2.39 ± 0.05</td>
</tr>
<tr>
<td>RWC (%)</td>
<td>98.2 ± 0.4</td>
<td>97.1 ± 0.3</td>
<td>97.5 ± 0.4</td>
</tr>
<tr>
<td>Dark</td>
<td>96.4 ± 0.4</td>
<td>95.7 ± 0.6</td>
<td>93.8 ± 3.2</td>
</tr>
<tr>
<td>Light</td>
<td>98.6 ± 0.1</td>
<td>97.0 ± 1.2</td>
<td>96.9 ± 1.5</td>
</tr>
<tr>
<td>S</td>
<td>80.2 ± 4.6</td>
<td>87.9 ± 4.1</td>
<td>76.1 ± 0.3</td>
</tr>
</tbody>
</table>
estimation of \( V_o \). The estimated \( V_o \) was highest in \textit{Z. japonica}, the species with lowest specificity towards CO2.

### Discussion

In the three \( C_4 \) grasses, \textit{Paspalum dilatatum}, \textit{Cynodon dactylon}, and \textit{Zoysia japonica}, Rubisco initial and total activities decreased slightly with water deficit, but maximal activity and Rubisco quantity remained unchanged, consistent with commonly observed drought effects (Ghaonnou 2009; Lawlor and Tezara, 2009). In all three species, Rubisco initial activity was lower than total activity and both initial and total activities were lower than maximal activity, and suggested increasing down-regulation with leaf dehydration (decreasing \( RWC \)). Rubisco down-regulation can be explained by a combination of decreased carbamylation of the active site lysine and the presence of competitive and tight-binding inhibitors. Carbamylation may be impeded by negative effectors (Hatch and Jensen, 1980; Badger and Lorimer, 1981; Jordan et al., 1983) or by RuBP bound into the non-carbamylated sites (Brooks and Portis, 1988), but these effects will not necessarily affect the activity in extracts because of dilution. The proportion of initial to total activity was not affected by leaf dehydration (see Supplementary Fig. S1 at \textit{JXB} online), whereas both initial and total activities showed a similar decrease in relation to maximal activity with increasing water deficit, suggesting a possible increase in Rubisco tight-binding inhibitors.

Parry et al. (2002) showed that Rubisco activity was inhibited in tobacco under drought stress. In that study, the inhibitory component in acid extracts of illuminated leaves was assumed to be \( \Delta \)-glycerol-2,3-diulose-1,5-bisphosphatase (PDBP). Figure 4 shows that the quantities of tight-binding inhibitors in the leaves of \textit{P. dilatatum}, \textit{C. dactylon}, and \textit{Z. japonica} were only marginally increased with water deficit. The maximum quantities in the light were estimated to be, at the most, \(~5\%\) of the Rubisco catalytic sites and, therefore, insufficient to explain entirely the decrease in total activity with respect to maximal activity. It can be argued that some Rubisco inhibitors are unstable in the acid extracts and this merits further investigation.

The quantity of Rubisco inhibitor in the acid extracts from leaves after 12 h in the dark was most in \textit{Z. japonica} and least in \textit{P. dilatatum}. The first evidence of CA1P in leaves was the observed modulation of Rubisco activity by light and dark transitions (Vu et al., 1983, 1984; Besford, 1984; Servaites et al., 1984). Sage and Seemann (1993) reported regulation of Rubisco in \textit{Z. japonica} by darkness and considered that this might be due to the accumulation of CA1P. The removal of CA1P from Rubisco catalytic sites in the light depends on the action of Rubisco activase and the inhibitor is then degraded by CA1P phosphatase (Robinson and Portis, 1988). The effects of CA1P may continue at the beginning of the light period, at low light intensities, and in long periods of low light intensity due either to dense cloud coverage (Sage et al., 1993) or to shading by neighbouring leaves (Kobza and Seemann, 1989a, b).

The concentration of inhibitor in acid extracts from dark-adapted leaves was approximately 28\%, in \textit{Z. japonica}, and 5\% in \textit{P. dilatatum} and \textit{C. dactylon}, of the quantity of Rubisco catalytic sites in each species. For \textit{Z. japonica}, the difference in the total Rubisco activity between extracts of leaves harvested in the light and after 12 h in the dark was consistent with the quantity of inhibitor measured, but there were no consistent differences between total Rubisco activity in the light- and dark-adapted leaves for \textit{P. dilatatum} and \textit{C. dactylon}. These results corroborate the observations of Sage and Seemann (1993), suggesting the presence of CA1P in \textit{Z. japonica}, but are not conclusive about the presence/absence of this inhibitor in \textit{P. dilatatum} and \textit{C. dactylon}. To confirm the identity of the inhibitor occurring in \textit{Z. japonica} in the dark, the putative CA1P was analysed by HPLC after isolation from light- and dark-adapted (12 h dark period) leaves. Rubisco activity was inhibited by components in the acid extracts that were recovered in the same HPLC fractions as authentic CA1P and the quantities of inhibitor estimated by this method were in the range found by the direct estimates on crude leaf acid extracts, thus providing definitive evidence for the presence of CA1P in the leaves of \textit{Z. japonica}. The presence of inhibitors is likely to protect Rubisco from proteolysis, especially in stressed leaves (Parry et al., 2008).

The RuBP content was greater in fully hydrated leaves of \textit{C. dactylon} and \textit{Z. japonica} than in \textit{P. dilatatum}. In vivo, this potential disadvantage for \textit{P. dilatatum} may be partly counter-balanced by a greater Rubisco activation state, as indicated by the higher initial activity in this species than in \textit{C. dactylon} or \textit{Z. japonica}. Leaf dehydration caused a much
larger decrease in the quantity of RuBP in *C. dactylon* and *Z. japonica* than in *P. dilatatum*. Decreased RuBP availability with water deficit has been observed in several studies (Gimenez et al., 1992; Gunasekera and Berkowitz, 1993; Tezara et al., 1999; Wingler et al., 1999; Parry et al., 2002; Bota et al., 2004). Because RuBP consumption and production are interdependent, it is difficult to assess whether decreased RuBP availability may constitute a limitation to or be a consequence of decreased CO₂ assimilation. Comparison of relative effects of drought stress revealed a more marked decrease in RuBP quantities than in net photosynthetic rates by *C. dactylon* and *Z. japonica*, suggesting that the decline in substrate availability could constitute a factor contributing to limit carboxylation in these two C₄ grasses. In *P. dilatatum* the moderate relative decrease in RuBP with water deficit is more likely to represent a consequence of decreased CO₂ assimilation (and thus decreased 3-phosphoglycerate production), rather than constitute a limiting factor per se.

Decreased capacity for RuBP regeneration may be a consequence of impairment of ATP synthesis, of Calvin cycle enzymes activity, or both. Whilst some studies on tobacco and barley indicate decreased activity of Calvin cycle enzymes (Gunasekera and Berkowitz, 1993; Wingler et al., 1999), in sunflower, although the decrease in ATP was less dramatic than the decrease in RuBP, it was concluded that the latter was a consequence of decreased ATP synthesis through its effect on RuBP regeneration (Tezara et al., 1999; Lawlor, 2002). Decreased ATP synthesis, due to impaired photophosphorylation by chloroplastic ATP synthase, in association with increased concentrations of Mg²⁺, has been suggested as a major metabolic limitation to photosynthesis under water deficit (Lawlor, 2002; Lawlor and Tezara, 2009). An alternative view is that ATP availability may decrease as a result of increased consumption by alternative sinks under drought stress. For instance, increased bundle sheath leakage, as observed in sorghum and sugarcane when subjected to drought (Saliendra et al., 1996; Williams et al., 2001), decreases the efficiency of the C₄ cycle, and accumulation of compounds associated with drought tolerance, such as proline, as observed for the three C₄ grasses under study (Carmo-Silva et al., 2009), would increase the usage of ATP.

The quantities, on a chlorophyll basis, of both RuBP (Rᵢ) and Rubisco catalytic sites (Eᵢ) were included in Fig. 2 to allow easy comparison. In *P. dilatatum*, Rᵢ was only marginally above Eᵢ, even in well-watered plants. Even though only some 60–70% of the catalytic sites were active in this species, the ratio Rᵢ/Eᵢ was less than 2 and so approaching the range where RuBP becomes limiting in C₃ species (below ~1.5–1.8; von Caemmerer, 2000; Lawlor, 2002). The modelling of the situation where the substrate concentration is similar to the catalytic site concentration is described by von Caemmerer (2000). The Rᵢ in *C. dactylon* and *Z. japonica* was much higher in control plants, corresponding to Rᵢ/Eᵢ ratios of 3.5 and 2.9, respectively, but the values fell steeply as RWC decreased, giving ratios similar to those in *P. dilatatum* at about 85% RWC. Thus, the quantities of RuBP in the leaves of all three species under drought stress were close to the critical value for RuBP-dependence of C₃ photosynthesis (Lawlor, 2002).

Rubisco activase removes inhibitors from carbamylated catalytic sites and facilitates the removal of RuBP bound to non-carbamylated sites. Rubisco activase requires ATP for activation of Rubisco but is inhibited by ADP (Portis, 2003). If the decreased capacity for regeneration of RuBP under drought is a consequence of decreased ATP synthesis, a further consequence could be more Rubisco-bound inhibitors and fewer carbamylated Rubisco catalytic sites (Lawlor, 2002; Lawlor and Tezara, 2009) – both resulting from the decline in activase activity caused by a fall in the ratio ATP/ADP. Inactivation of Rubisco catalytic sites with increasing water deficit will decrease both carboxylation and oxygenation and, therefore, photosynthesis and photorespiration, as previously predicted by the modelling of carbon assimilation under drought (Carmo-Silva et al., 2008).

Rubiscos isolated from leaves of the three C₄ grasses had lower specificity factors (S<sub>CO₂</sub>) and higher Michaelis–Menten constants for CO₂ (K<sub>c</sub>) and higher maximum carboxylase activities (V<sub>c</sub>) than wheat Rubisco, which is in agreement with previous reports on the kinetics of Rubisco from C₃ and C₄ species (Yeoh et al., 1980, 1981; Tcherkez et al., 2006; Kubien et al., 2008). The lower specificity for CO₂ of Rubisco from C₄ compared with C₃ monocots is associated with specific differences in the large subunits (Christin et al., 2008) and may be a consequence of diminished selection pressure resulting from increased CO₂ concentrations in the vicinity of the enzyme (Jordan and Ogren, 1981). Tcherkez et al. (2006) suggested evolutionary optimization of Rubiscos from different species to the subcellular environment in which the enzyme functions. In the present study, the relatively high V<sub>c</sub> would support rapid assimilation in the CO₂-rich environment of the bundle sheath, consistent with the high photosynthetic performance of the three C₄ grasses (Carmo-Silva et al., 2008).

The Michaelis–Menten constant for oxygen (K<sub>O</sub>) of Rubisco from different photosynthetic organisms is largely unknown. The few values published (see von Caemmerer, 2000; Kubien et al., 2008) suggest that K<sub>O</sub> may be highly variable between different species, even when they are closely related and represent the same photosynthetic pathway. The need to determine K<sub>O</sub> for each species is therefore justified. The estimates of K<sub>O</sub> obtained here for the three C₄ grasses were similar and provide a valuable contribution for future modelling of photosynthesis.

In view of the effects of drought on the quantities of RuBP in the leaves, a measure of the Michaelis–Menten constant for RuBP (K<sub>RuBP</sub>) would be a valuable addition to the kinetic constants determined for the three grasses. Values for K<sub>RuBP</sub> for several C₄ species have been published (Yeoh et al., 1981) and ranged from 15 μM to 100 μM which also suggests that measurements are needed for each species for which the objective is the mechanistic modelling of photosynthesis. The much lower concentration of RuBP in fully-hydrated leaves of *P. dilatatum* (NADP-ME) than in *C. dactylon* (NAD-ME) may be associated with a lower average K<sub>RuBP</sub> in NADP-ME than in NAD-ME C₄ grasses.
(calculated from data in Yeoh et al., 1981). Estimation of the effective concentration of RuBP at the catalytic site of Rubisco is problematic because of the presence of competitive inhibitors and the formation of complexes with Mg$^{2+}$ (von Caemmerer, 2000).

The results presented here show that the decline in the quantity of RuBP in the leaves as water deficit increased was of the same order of magnitude as the decline in photosynthetic rates reported previously (Carmo-Silva et al., 2008). Concomitant measurements of ATP and potential competitive inhibitors of RuBP binding to Rubisco, including intermediate metabolites of the Calvin cycle, particularly 3-phosphoglycerate, would improve understanding of factors limiting C$_4$ photosynthesis under water deficit. The marginal increase in Rubisco tight-binding inhibitors under water deficit is unlikely to have a relevant contribution to the decreased photosynthetic rates. The presence of CAIP in dark-adapted leaves of Z. japonica may confer protection against degradation of Rubisco by proteolysis, but the mechanism by which Rubisco is down-regulated in the three C$_4$ grasses under water deficit merits further study.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Rubisco activation state in relation to $RWC$ in the three C$_4$ grasses

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