RESEARCH PAPER

Improved analysis of C₄ and C₃ photosynthesis via refined in vitro assays of their carbon fixation biochemistry

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Abstract

Plants operating C₃ and C₄ photosynthetic pathways exhibit differences in leaf anatomy and photosynthetic carbon fixation biochemistry. Fully understanding this underpinning biochemical variation is requisite to identifying solutions for improving photosynthetic efficiency and growth. Here we refine assay methods for accurately measuring the carboxylase and decarboxylase activities in C₃ and C₄ plant soluble protein. We show that differences in plant extract preparation and assay conditions are required to measure NADP-malic enzyme and phosphoenolpyruvate carboxylase (pH 8, Mg²⁺, 22 °C) and phosphoenolpyruvate carboxykinase (pH 7, >2 mM Mn²⁺, no Mg²⁺) maximal activities accurately. We validate how the omission of MgCl₂ during leaf protein extraction, lengthy (>1 min) centrifugation times, and the use of non-pure ribulose-1,5-bisphosphate (RuBP) significantly underestimate Rubisco activation status. We show how Rubisco activation status varies with leaf ontogeny and is generally lower in mature C₄ monocot leaves (45–60% activation) relative to C₃ monocots (55–90% activation). Consistent with their >3-fold lower Rubisco contents, full Rubisco activation in soluble protein from C₄ leaves (<5 min) was faster than in C₃ plant samples (<10 min), with addition of Rubisco activase not required for full activation. We conclude that Rubisco inactivation in illuminated leaves primarily stems from RuBP binding to non-carbamylated enzyme, a state readily reversible by dilution during cellular protein extraction.

Key words: Carbamylation, carbon fixation, CO₂-concentrating mechanism, photosynthesis, Rubisco, Rubisco activase.

Introduction

Plants operating the C₃ and C₄ pathways contain differing biochemical and anatomical features that facilitate their climatic adaptation to cool-temperate and warm-tropical environments, respectively (Edwards et al., 2010). The rate-limiting CO₂ fixation step common to both pathways is catalysed by the photosynthetic enzyme ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco, EC 4.1.1.39). Fixation of CO₂ to RuBP by Rubisco produces two molecules of 3-phosphoglycerate (PGA) that are cycled through the photosynthetic carbon reduction (PCR) cycle to produce triose-phosphates, the building blocks of carbohydrates needed for plant growth (Raines, 2003).

Rubisco is an imperfect catalyst that is remarkably slow (completing only 2–4 cycles s⁻¹ in leaves) and can fix O₂
instead of CO\textsubscript{2}, with oxygenation of RuBP leading to the production of 2-phosphoglycolate (PGly) whose recycling back to PGA by the photorespiratory pathway spans three cellular compartments and undesirably consumes energy and releases fixed CO\textsubscript{2} (Bauwe et al., 2010). Overcoming the catalytic limitations of Rubisco by directed changes to the enzyme or concentrating CO\textsubscript{2} around Rubisco to reduce the costs of photorespiration are ongoing bioengineering challenges (Parry et al., 2013; Price et al., 2013; Long et al., 2015). Current efforts to generate or discover plant Rubisco isoforms with joint improvements in specificity for CO\textsubscript{2} as opposed to O\textsubscript{2} (S\textsubscript{2,CO}) and carboxylation efficiency (defined as the maximum carboxylation rate (k\textsubscript{cat}) divided by the K\texttextsubscript{m} for CO\textsubscript{2} under ambient O\textsubscript{2}; K\textsubscript{c}\textsubscript{21/o2}) have yet to yield success (Sharwood and Whitney, 2014), despite such Rubiscos existing in some non-green algae (Andrews and Whitney, 2003).

The evolution of C\textsubscript{4} photosynthesis ~35–40 million years ago provided a natural solution to remedy the inefficiency of Rubisco (Sage et al., 2012). The anatomical separation of phosphoenolpyruvate carboxylase (PEPC) in mesophyll cells (MCs) and Rubisco in the bundle sheath cell (BSC) chloroplasts was accompanied by adaptation of biochemical CO\textsubscript{2} concentrating mechanisms (CCMs) (Hatch, 1987; Kanai and Edwards, 1999). The C\textsubscript{4} pathway involves the hydration by carbonic anhydrase of CO\textsubscript{2} to HCO\textsubscript{3} which is combined with phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxylase (PEPC) into the 4C acid oxaloacetate (OAA) that is converted into malate or aspartate before diffusing into the BSCs where they are decarboxylated, raising the CO\textsubscript{2} around Rubisco >4-fold higher than ambient CO\textsubscript{2} (von Caemmerer, 2000; Sage, 2004). The 3C decarboxylation product, pyruvate, returns to the MCs for PEP regeneration by pyruvate phosphate dikinase (PPDK) at the cost of two ATP equivalents.

The ratio of the activity between the main carboxylases is a key determinant of the efficiency of the CCM and can be an indication of the CO\textsubscript{2} supply to BSCs (von Caemmerer et al., 2014). On the one hand, PEPC activity is much higher than that of Rubisco (2- to 10-fold depending on plant species and environmental conditions) to enable the C\textsubscript{4} acid gradient to build and facilitate the diffusion of the C\textsubscript{4} acids into the BSCs. On the other hand, the PEPC:Rubisco ratio must be optimized to minimize CO\textsubscript{2} leakage from the BSCs, leading to futile cycling involving the CCM (Kromdijk et al., 2008). F utile cycling of the CCM is energetically wasteful for the plant through use of ATP to regenerate PEP.

Based on the main decarboxylating enzymes, C\textsubscript{4} plants can be grouped into three biochemical subtypes: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK) (Hatch, 1987; Kanai and Edwards, 1999). In addition, there is flexibility among some NADP-ME (e.g maize, sorghum, and Panicum antidotale) and NAD-ME (e.g Cleome angustifolia, Bienertia sinuspersici, and Panicum coloratum) species which also harbour PECK (Furbank, 2011; Pinto et al., 2014; Koteyeva et al., 2015). The significance of the dual decarboxylation pathways is not yet fully understood, but evidence suggests that PECK allows flexibility to the decarboxylation pathway(s) that may be dependent on environmental cues (Bellasio and Griffiths, 2014; Sharwood et al., 2014).

Assaying for PEPCK is often difficult because pure enzyme is required for assaying in the decarboxylase direction due to interference from other C\textsubscript{4} pathway enzymes (Ashton et al., 1990). However, assaying for PECK activity in the carboxylase direction is also troublesome as PECK can interfere with this assay, although variations in PECK activities can be corroborated by western blot analysis of PECK content (Sharwood et al., 2014). Accurately determining the level of maximum PECK activity is requisite to determine the level of flexibility in the decarboxylation pathways that may exist.

A conserved feature of Rubisco catalysis is the required priming (activation) of each catalytic site (E) located at the interface of adjoining paired 50 kDa subunits (L\textsubscript{2}) that arrange as (L\textsubscript{2})\textsubscript{4} tetrad cores and are capped at either end with tetrads of 15 kDa small (S) subunits to form an ~520 kDa L\textsubscript{2}S\textsubscript{8} complex (Andersson and Backlund, 2008). Activation proceeds via the slow and reversible binding of non-substrate CO\textsubscript{2} to the ε- amino group of a conserved L-subunit Lys201 producing a carbamate (EC) that is rapidly stabilized by Mg\textsuperscript{2+} to produce a tertiary complex (ECM) capable of RubBP binding and enolization and its subsequent carboxylation or oxygenation (Andersson, 2008). In vivo, the pool of inactive Rubisco comprises decarboxamylated catalytic sites (E) and ECM complexes binding inhibitory sugar-phosphate molecules (ECMI) (Parry et al., 2008) (Fig. 1). Examples of these inhibitors include the catalytic misfire product xylulose 1,5-bisphosphate (XuBP) and the ‘nocturnal’ or ‘shade’ inhibitor 2-carboxy-d-arabinitol 1-phosphate (CA1P) produced under low light and darkness (Gutteridge et al., 1986; Moore and Seemann, 1992; Pearce, 2006; Andralojc et al., 2012). Binding of RubBP to E also leads to the production of catalytically stalled ER complexes (Laing and Christeller, 1976; Jordan and Chollet, 1983). Release of these sugar-phosphate molecules is catalysed by Rubisco activase (RCA) via ATP hydrolysis (Parry et al., 2008; Mueller-Cajar et al., 2014). Following their RCA-facilitated release, the rebinding of XuBP and CA1P is prevented by the enzymes XuBPase (Bracher et al., 2015) and CA1Pase (Salvucci and Holbrook, 1989; Moore and Seemann, 1992). The enzyme CA1Pase is
also able to metabolize the inhibitor pentadiulose-1,5-bisphosphate (PDBP; Andralojc et al., 2012), a relatively labile oxygenation byproduct whose inhibitory relevance in vivo remains indeterminate but is a significant contaminant of non-pure RuBP (Kane et al., 1998). Conditions that stimulate Rubisco inactivation include increasing temperature (increased XuBP and PDBP production), low illumination (stimulated C1AP synthesis), and elevated CO2 (possibly increases ER levels) (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004; Kim and Portis, 2005; Parry et al., 2008).

Extrapolating aspects of cellular biochemistry from leaf gas exchange measurements using available C3 and C4 photosynthesis models is highly reliant on accurately knowing the content and catalytic properties of the carboxylation and decarboxylation enzyme activities (Farquhar et al., 1980; Salvucci and Crafts-Brandner, 2004; Sharkey et al., 2007). Here we appraise and refine the methods for assaying PEPC, PEPCk, and Rubisco activity and activation status using NADH-linked spectrometric assays. We apply these refined assay methods to leaf extracts from C3 and C4 grasses to demonstrate their applicability in accurately measuring variations in the carboxylation/decarboxylation biochemistries of leaves of differing ontogeny.

Materials and methods

Plant seeds and growth conditions

Seeds for Panicum bisulcatum and Megathyrsus maximum were obtained from the Australian Plant Genetic Resources Information System (QLD, Australia) and Queensland Agricultural Seeds Pty. Ltd (Toowoomba, Australia), while the seeds for tobacco (Nicotiana tabacum, cv Petit havana), maize (Zea mays cv Kelvedon Glory), and wheat (Triticum aestivum cv Y70) were sourced locally. The seeds were sown in 2–5 litre pots of commercial self-fertilizing potting mix at 5–7 d intervals to obtain plants of different ages to sample simultaneously. The plants were grown in a glasshouse at set 28/22 °C day/night temperatures under natural illumination during November and December in Canberra, Australia. Plants were watered regularly, with the addition of Hoaglands nutrients to mature plants every 2 d.

Leaf harvesting, protein extraction, and protein assay

Samples of known area (0.3 or 0.5 cm2) were harvested using brass cork borers (Met-App Metalware, Melbourne) from different aged leaves on the same day, 5–7 h into the light period. The samples were rapidly frozen in liquid nitrogen before storing at −80 °C. For assays of Rubisco activity and content as well as the activity of PEPC and NADP-ME, the soluble leaf protein was extracted using ice-cold 2 ml glass homogenizers (Wheaton) into 0.5–1 ml of ice cold, N2-sparged extraction buffer [50 mM EPPS-NaOH, pH 8.0, 0.5 mM EDTA, 2 mM DTT, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), and 1% (w/v) polyvinylpyrrolidone (PVPP)] containing 0.2, 5, or 10 mM MgCl2. The lysate was rapidly centrifuged for 0.5, 2, or 5 min (16 000 g, 4 °C), and 10 µl of the soluble leaf protein was assayed for initial and total Rubisco activities (see below) and 50 µl to measure Rubisco content by [14C]CABP (2-C-carboxyribabinitol 1,5-bisphosphate) binding as described (Whitney et al., 2001). Protein content was measured against BSA standards using a Coomassie dye binding assay (Pierce). The leaf area extracted in 1 ml of buffer was generally 0.3 cm2 (T. aestivum), 0.5 cm2 (N. tabacum), 0.6 cm2 (P. bisulcatum), and 0.9 cm2 (P. bisulcatum, M. maximus, and Z. mays).

Phosphoenolpyruvate carboxylase assay

Maximal PEPC activities were measured using an NADH-coupled assay as previously described (Ashton et al., 1990). Extraction was performed using the same extraction buffer as described for Rubisco containing 5 mM MgCl2, and samples were incubated at room temperature or on ice for 0, 5, 10, and 20 min before adding 10 µl of extract to initiate assays.

NADP-malic enzyme assay

Maximal NADP-ME activity was determined in a coupled NADP assay as previously described (Ashton et al., 1990; Pengelly et al., 2012). Briefly, NADP-ME activity in leaf extracts prepared as described above was assayed in 50 mM Tricine-KOH pH 8.3, 5 mM malic acid, initiating with 10 mM MgCl2.

Phosphoenolpyruvate carboxykinase assay

The maximal activity of PEPCk was measured in the carboxylase direction using a method adapted from Chen et al. (2002) and Walker et al. (2002) as described by Sharwood et al. (2014) in an NADP-coupled assay as depicted in Fig. 2A. To remove interference with PEPC, MgCl2 was excluded from the extraction and assay buffers. PEPC background activity was determined by assaying for PEPC activity as above with and without MgCl2, with no PEPC activity observed when MgCl2 is omitted from the extraction and assay buffer (data not shown). For PEPCk assay, leaf discs were extracted in 50 mM HEPES pH 7.0, 5 mM DTT, 1% (v/v) PVP, 2 mM EDTA, 2 mM MnCl2, and 0.05% Triton. PEPCk activity from leaf extract was measured in assay buffer [50 mM HEPES, pH 7.0, 4% mercaptoethanol (w/v), 100 mM KCl, 90 mM NaHCO3, 1 mM ADP, 2 mM MnCl2, 0.14 mM NADH, and malate dehydrogenase (MDH; 6 U; 3.7 µl)] after the addition of 15 mM PEP. An optimal PEP concentration was determined in separate assays titrated with 2.5–20 mM PEP.

Rubisco activity assays

Rubisco activity was measured at 25 °C using an NADPH-coupled enzyme assay (Kubien et al., 2011; Supplementary Fig. S1 at JXB online) with the rate of NADPH oxidation monitored at 340 nm using a diode array spectrophotometer (Agilent model 8453). The RuBP used in the assays was either that synthesized and purified as previously described (Kane et al., 1998) or commercially supplied (Sigma; R0878). Assays were performed in 1 ml cuvettes containing 0.48 ml of assay buffer [100 mM EPPS-NaOH, pH 8.0, 10 mM MgCl2, 0.2 mM NADH, 20 mM NaHCO3, 1 mM ATP, pH 7.0, 5 mM phosphocreatine, pH 7.0, and 4% (v/v) coupling enzymes] (see Supplementary Table S1). RuBP (0.4 mM) was included in the cuvettes used to measure initial Rubisco activity, with 20 µl of soluble leaf protein sample added to start the assays. To measure total Rubisco activities, 20 µl of leaf protein was first activated for 10–15 min in RuBP-free assay buffer before initiating Rubisco activity measurements by adding RuBP to 4 mM. The Rubisco carboxylation rate was determined using the equation:

$$\text{mol RuBP consumed min}^{-1} = \frac{\Delta OD_{340}}{4(6.22 \times 10^{-3} \text{M}^{-1})}$$  \(1\)

which uses the extinction coefficient of NADH (6.22 × 104 M−1 cm−1), the rate of change of absorbance at 340 nm per minute (ΔOD340), and accounts for the four NADH molecules oxidized per RuBP carboxylated by Rubisco in the coupled assay (Supplementary Fig. S1). Substrate-saturated Rubisco carboxylase activity in the same leaf soluble protein was measured by 14CO2 fixation assays as described (Sharwood et al., 2008). The carboxylation turnover rate (kcat) was determined from the RuBP activity measured by either the
NADH-coupled enzyme assay or the \(^{13}\text{CO}_2\) fixation assay divided by the Rubisco active site content in the assay as quantified by \(^{14}\text{C}\) C4BP binding (Ruuska et al., 1998). Time course measurements of Rubisco activity over 30 min at 25 °C were undertaken to confirm the functional integrity of Rubisco in the leaf protein extracts.

**Rubisco activase purification and assay**

Rubisco RCA was expressed and purified from *Escherichia coli* as described (Baker et al., 2005). Two-stage assays similar to that described by Barta et al. (2011) were used to assess if sugar-phosphate Rubisco inhibition in tobacco leaf protein extracts influenced measurements of total Rubisco activity. In the first assay stage, 50 μl of leaf extract was incubated in a 0.5 ml final volume with 80 μg ml\(^{-1}\) RCA (or BSA in the RCA-free controls) for 2 min at 25 °C in ATPase assay buffer [100 mM EPPS, pH 8.0, 20 mM KCl, 5 mM MgCl\(_2\), 6% (w/v) polyethylene glycol (PEG) (mol. wt 3350Da)], 2 mM PEP, 0.2 mM NADH, 2 mM ATP, 1% (v/v) of a pyruvate kinase/lactate dehydrogenase mixture (PK, 745 U ml\(^{-1}\); LDH, 906 U ml\(^{-1}\); Sigma-Aldrich). In the second assay stage, 100 μl of the RCA leaf or BSA leaf protein reactions were added to the NADH-linked Rubisco assays and the total activities compared. Control assays examining tobacco RCA activation of purified tobacco Rubisco ER complexes are described in Supplementary Fig. S2.

**Results and Discussion**

*Optimizing the assay of PEPC and PEPCK activities in total soluble leaf protein*

Measuring PEPC and PEPCK activities separately in soluble leaf protein extracts using NADH-linked spectrophotometric assays is complicated by their common requirement for substrate PEP (Fig. 2A) particularly in assays containing Mg\(^{2+}\) and Mn\(^{2+}\) at physiological concentrations (Muhaidat and McKown, 2013). Determining maximal PEPC activity in soluble leaf protein extracts is typically achieved by assaying the decarboxylation reaction of PEPCK, requiring purified protein free of other C\(_4\) enzymes such as PEPC (Ashton et al., 1990; Chen et al., 2002). In contrast, measures of PEPC rates free of PEPCK activity can easily be made by omission of ADP from the assay (Fig. 2A). As *in vitro* measures of PEPC are sensitive to low temperature storage (Hatch and Oliver, 1978), we examined the PEPC activity in soluble leaf protein from *Z. mays* and *M. maximus* stored either at room temperature (22 °C) or on ice (0 °C) (Fig. 2B). When incubated at 22 °C for 20 min, there was little or no loss of PEPC activity evident in replica leaf samples from either *M. maximus* or *Z. mays*. In contrast, storage of the leaf protein extracts on ice significantly reduced PEPC activities (measured at 25 °C), particularly in *M. maximus* where >65% of PEPC activity was lost after 5 min at 0 °C (Fig. 2B). As a result of its sensitivity to low temperature, all assays of PEPC activity were performed on rapidly extracted (homogenized in <0.5 min and centrifuged for 0.5 min at 4 °C) leaf soluble protein without storage on ice.

In *C₄* plants with phosphoenolpyruvate carboxykinase (PCK) physiology (e.g. *M. maximus*), PEPC is the dominant decarboxylase enzyme that utilizes ATP hydrolysis during its reversible decarboxylation of OAA (Fig. 2A). In contrast to the alkali pH 8 preference of PEPC (Greenway et al., 1978), the activity of PEPC is optimal at pH 7 and 80% lower at pH 8.0 (Ray and Black, 1976; Pierre et al., 2004). To minimize, possibly preclude, PEPC activity, the extraction and measurement of PEPC carboxylase activity was undertaken at pH 7.0 with Mg\(^{2+}\) (required for PEPC activity) omitted and replaced with 2 mM Mn\(^{2+}\), a PEPC cofactor (Fig. 2A). Under these conditions, stable rates of PEPC were obtained in assays initiated by the addition of PEP (solid line, Fig. 2C) (Sharwood et al., 2014).
Alternative PEPCK analyses were undertaken where the assays were initiated with ADP, not PEP (Fig. 2C, black dashed line). Omission of ADP in control assays produced background rates of apparent PEPC activity (Fig. 2C, lower black dashed line); however, the addition of 5 mM aspartate (Fig. 2C, grey dashed line) or 5 mM glucose-6-phosphate (and MgCl₂) which inhibit and stimulate PEP activity, respectively (Fig. 2A), had a negligible effect on the measured activities. This suggests that the background ‘no ADP’ PEP carboxylase activities observed arise from PEPCK activity that is utilizing residual ADP in the soluble protein extract. Consequently, we propose that extracting leaf protein in pH 7.0 buffer with no MgCl₂ and assaying in an MnCl₂-containing buffer at the same pH is sufficient to measure PEPCK activity with little or no contaminating PEP activity.

Assays containing 2.5–25 mM PEP were used to determine that a saturating concentration of 15 mM PEP was required for maximal PEPCK activity in soluble leaf protein from 1.3 mm² of *M. maximus* leaf tissue (Fig. 2D). This saturating PEP concentration is 7-fold higher than the *K*m for PEP measured for *M. maximus* PEPCK (Chen et al., 2002) and was the concentration used in all subsequent PEPCK assays.

**Measuring Rubisco carbamylation status**

The carboxylase-limiting component of the C₃ photosynthesis models stemming from those derived by Farquhar et al. (1980) are typically used to derive estimates of *V*ₗₜₜ [in units of μmol CO₂ fixed m⁻² s⁻¹], that equate to the product of Rubisco sites (μmol CO₂ fixed m⁻²) and *K*ₚₖ [s⁻¹]). This measure is extrapolated from the response of the CO₂ assimilation rate (A) with increasing CO₂ measured by leaf gas exchange, and relies heavily on the temperature response measurements of *v*ₗₜₜ, *K*ₚₖ, the *K*ₚₖ for O₂ (*K*ₚₖ), and CO₂/O₂ specificity (*S*ₚₖₕ) made for tobacco Rubisco (Sharkey et al., 2007). The universal suitability of these parameters is now in question given the substantial variation observed in the temperature response of these parameters among plant Rubiscos (Walker et al., 2013; Boyd et al., 2015; Perdomo et al., 2015). Moreover, differences in *V*ₗₜₜₜ are primarily attributed to variations in Rubisco content and generally overlook differences in the activation status of Rubisco in vitro, despite its critical influence on estimates of *v*ₗₜₜₜ and in vivo determined *k*ₚₖ (Bernacchi et al., 2001; Salvucci and Crafts-Brandner, 2004).

As indicated in Fig. 1, within an illuminated leaf chloroplast the catalytic sites of Rubisco (shown as E) are primarily CO₂−Mg²⁺ activated (ECM) and capable of RuBP catalysis. Binding of inhibitory XuBP or, in darkened leaves, CA1P to ECM produces catalytically inactive ECMI complexes whose activation involves RCA-catalysed dissociation of the sugar-phosphate ligands which are then degraded by substrate-specific enzymes (Jordan and Chollet, 1983; Gutteridge et al., 1986; Edmondson et al., 1990; Bracher et al., 2015). Thus the most abundant form of Rubisco inhibition in illuminated chloroplasts is probably the binding of RuBP to non-carbamylated Rubisco (ER) that renders the catalytic site inactive (Jordan and Chollet, 1983).

The NADH-linked spectrophotometric and ¹⁴CO₂ fixation in vitro assays typically used to measure Rubisco activation status involve three stages (Fig. 1). The first stage involves the rapid measurement of Rubisco activity in rapidly extracted soluble leaf protein. This measures the ‘initial’ Rubisco activity. Saturating amounts of RuBP are included to prevent ECM formation from the ER complexes and ensure that the assays are not RuBP limited (Laing and Christeller, 1976). Replica samples of the leaf protein extracts are then allowed to activate fully by incubating in buffer lacking RuBP but containing saturating CO₂ and Mg²⁺. During the second stage, the inconsequential RuBP levels in the extract enable its dissociation from the ER complexes to allow ECM formation. The third stage measures the ‘total’ Rubisco activity rate. In samples from darkened or stressed leaves, the formation of ECMI complexes can cause significant underestimation of the ‘total’ activities (Parry et al., 1997, 2002; Carmo-Silva et al., 2010) a consideration we sought to avoid in this study by sampling healthy, naturally illuminated glasshouse-grown plant material 6–7 h into the photoperiod.

**Optimizing leaf protein extraction for measuring Rubisco activation status**

Analyses undertaken using tobacco leaves highlighted the requirement for speed and inclusion of ~5 mM Mg²⁺ during leaf protein extraction for accurate measurements of initial Rubisco activity (Fig. 3A). As shown in Fig. 1, formation of ECM is initiated by the slow and reversible carbamylation of Lys201 in the catalytic site of E followed by the rapid binding of Mg²⁺. To avoid Rubisco carbamylation occurring during extraction, the leaf soluble protein is extracted in N₂-sparged (i.e. CO₂-free) buffer. It was hypothesized that the exclusion of MgCl₂ during extraction would undesirably produce EC complexes from which the activating CO₂ would dissociate to form inactive E. Indeed, the omission of MgCl₂ in the CO₂-free extraction buffer led to significantly lower measurements of initial Rubisco activity (70% of maximum activity) after just 0.5 min relative to those extracted with 2–10 mM MgCl₂ (82–85% of maximum activity; Fig. 3A). Extending the extraction (centrifugation) period to 5 min reduced all initial Rubisco activity measurements, more so in those extracted without MgCl₂ (35% of maximum activity) compared with those extracted with MgCl₂ (55–60% of maximum activity). In all treatments, very similar total activity rates were attained, indicating that the varying extraction conditions did not compromise Rubisco integrity (Fig. 3A, circles). These findings caution against the omission of MgCl₂ when assaying for initial activities. Furthermore, potential inaccuracies are incurred with extended centrifugation times of >0.5–1 min following extraction, emphasizing the need for rapid leaf protein extraction and assay.

**The importance of using purified RuBP**

Using pure RuBP devoid of inhibitory impurities such as PDBP is critical for accurately measuring Rubisco catalysis in vitro (Kane et al., 1998; Andralojc et al., 2012). As observed by Scales et al. (2014), by using pure RuBP the measured rates of initial and total activity remain relatively linear over a 4 min assay period (Fig. 3B). This (i) indicates that insignificant levels
Fig. 3. Evaluating the experimental methodology for measuring leaf Rubisco activation status. (A) Appraising how MgCl\textsubscript{2} inclusion and quickness of soluble leaf protein extraction influences Rubisco activation quantification. NADH-linked assays were performed on N.\textsubscript{benthamiana} leaf discs (0.5 cm\textsuperscript{2}) taken from a young, nearly fully expanded upper canopy leaf (15 cm in diameter) and stored at –80 °C for up to 3 months without effect on recoverable activity. Circles indicate the total activities measured after 10 min activation relative to the 0.5 min centrifuged sample. (B) Representative NADH-linked spectrophotometric measures of initial (dashed lines) and total (solid lines) Rubisco activities made using low purity commercial RuBP (squares) or that purified according to Kane et al. (1998) (circles). Rates correspond to protein from 0.9 mm\textsuperscript{2} of leaf with a Rubisco active site concentration in each assay of ~34.4 nM (i.e. a k\textsubscript{cat} of 2.2 s\textsuperscript{-1}). (C) Incubation of leaf protein extract with purified tobacco Rubisco using the NADH-coupled assay. This is ~30% lower than those typically measured by \textsuperscript{14}CO\textsubscript{2} fixation assays (Sharwood et al., 2008; Whitney et al., 2011). Incubation of the leaf protein extracts with purified tobacco RCA or BSA (control) showed no difference in the measured total Rubisco activities (Fig. 3C). In corresponding control assays, the RCA treatment was able to reactivate ER inhibited Rubisco fully over 10 min (Supplementary Fig. S2). This indicates that the lower k\textsubscript{cat} was not due to residual ECMI complexes in the leaf protein extract. To confirm this, the same tobacco soluble leaf protein was used to quantify k\textsubscript{cat} by the \textsuperscript{14}CO\textsubscript{2} assay method of Sharwood et al. (2008). As indicated in Table 1, the expected k\textsubscript{cat} of 3.1 s\textsuperscript{-1} for tobacco Rubisco was obtained by the \textsuperscript{14}CO\textsubscript{2} assay. This finding questions the accuracy of the NADH-coupled assay for quantifying Rubisco carboxylase activity, a deficiency also evident in the comparative measurements made by Lilley and Walker (1974). Indeed, published k\textsubscript{cat} values determined by the NADH-coupled assay for cyanobacteria (Emlyn-Jones et al., 2006) and plant (Pearce and Andrews, 2003) Rubisco are also 20–25% lower than those measured by \textsuperscript{14}CO\textsubscript{2} fixation (Whitney et al., 1999; Mueller-Cajar and Whitney, 2008). To ensure that the differences were not due to components in the leaf extracts interfering with the coupling enzymes, comparative assays were undertaken in triplicate (technical repeats) using tobacco Rubisco purified by ion exchange chromatography (see the legend to Supplementary Fig. S2). Again, the k\textsubscript{cat} values determined by the NADH-coupled assay (1.9 ± 0.2 s\textsuperscript{-1}) were 30% lower than that quantified by \textsuperscript{14}CO\textsubscript{2} fixation assays with three separate leaf samples expressed as a percentage of the total activities measured after 10 min (C) and 25 min (D) activation. For (A), the significance level (\(P\)) for the [MgCl\textsubscript{2}] and centrifugation duration factors are shown. Letters indicate the ranking (lowest=a) of means within each centrifugation duration using a post-hoc Tukey test. Values followed by the same letter are not significantly different at the 5% level (\(P>0.05\)).
Table 1. Comparative values of Rubisco $k_{\text{cat}}$ at 25 °C quantified by the NADH-linked and $^{14}$CO$_2$ fixation assays

| Plant species | Photosynthetic biochemistry | $k_{\text{cat}}$ ($\pm$SE s$^{-1}$) | Significance ($P$) |
|---------------|-----------------------------|---------------------------------|------------------|
|               | NADH-linked assay           | $^{14}$CO$_2$ assay              |                  |
| Tobacco       | $C_3$                       | 2.15 ± 0.02 b n=24               | 3.07 ± 0.06 b n=23 <0.001 |
| P. bisulcatum | $C_4$                       | 1.79 ± 0.03 a n=22               | 2.72 ± 0.11 a n=7 <0.001 |
| M. maximus    | $C_4$,PCK                   | 3.85 ± 0.09 e n=9                | 5.17 ± 0.17 d n=10 <0.001 |
| T. aestivum   | $C_4$                       | 2.70 ± 0.06 c n=9                | 3.59 ± 0.04 c n=6  <0.001 |
| Z. mays       | $C_4$,NADP ME               | 3.70 ± 0.10 d n=9                | 5.46 ± 0.10 d n=6  <0.001 |

Values (means ±SE) obtained using NADH-linked and $^{14}$CO$_2$ assays were compared by one-way ANOVA, and the significance level ($P$) is shown. Species’ means obtained by each of the assay types were ranked separately using a post-hoc Tukey test. Values followed by the same letter are not significantly different at the 5% level ($P>0.05$). $n$=number of leaf protein samples (biological replicates) analyzed.

(2.7 ± 0.1 s$^{-1}$). This suggests that substrate limitations for one or more of the enzymes in the NADH-linked assay limit its potential for accurately quantifying $k_{\text{cat}}$, possibly the rate of 3-PGA reduction (Lilley and Walker, 1974).

The need for measuring Rubisco activation rate and stability over time

Given that the NADH-coupled assay allows for the continued ‘real-time’ monitoring of both initial and total Rubisco activity (Fig. 3B), it was used to examine the activation rate and stability of Rubisco activity at 25 °C in the soluble protein of leaves from the $C_3$ species P. bisulcatum and T. aestivum (wheat), and the $C_4$ species M. maximus and Z. mays. Linear rates of initial and total Rubisco activities were reproducibly found for each sample (Fig. 4A), with the rates of NADH oxidation significantly lower in the $C_4$ samples due to their low Rubisco contents. As shown in Fig. 4A, the $k_{\text{cat}}$ for both $C_4$ Rubiscos were seen to be higher than those of their $C_3$ counterparts when the activities were corrected for Rubisco content (quantified by $[^{14}$C]CABP binding).

Like tobacco, full activation of Rubisco in the soluble protein extracted from wheat and P. bisulcatum required 10min incubation at 25 °C (Fig. 4B, circles). In contrast, full activation of Rubisco in the M. maximus and Z. mays leaf protein required only 3–5min (Fig. 4B). Whether the faster rate of Rubisco activation in both $C_4$ species arises from a lower RuBP binding affinity remains a subject for future investigation. Nevertheless, variation in the time needed to activate Rubisco fully in leaf protein extracts questions whether shorter incubation times (e.g. 3min) are sufficient to evaluate Rubisco activation status accurately and extrapolate ECMI levels (Parry et al., 1997; Carmo-Silva et al., 2010; Galmes et al., 2011; Scales et al., 2014).

The $k_{\text{cat}}$ of $C_4$ plant Rubisco exceeds that of $C_3$ Rubisco

The $k_{\text{cat}}$ values of Rubisco from the $C_4$ species examined in this study were significantly faster relative to that of each $C_3$ plant Rubisco (Table 1). Notably the NADH-coupled assay measures of $k_{\text{cat}}$ were again 30–35% lower than corresponding $k_{\text{cat}}$ measurements made using $^{14}$CO$_2$ fixation assays (Table 1). Statistical ranking of the catalytic speed indicated
that the C₃ Rubisco from *P. bisulcatum* is slower than that of tobacco, with wheat outperforming both (*P*<0.001). In contrast to its ancestral *P. bisulcatum* Rubisco, the *k*ₐₘₐₓ for *M. maximus* Rubisco is ~2-fold higher but similar to the *k*ₐₘₐₓ of maize Rubisco despite originating from different biochemical subtypes and evolutionary origins (Table 1).

How do PEPC, Rubisco content, and activation status vary with leaf age?

Rubisco comprises a significant but variable N investment in plant leaves. In tobacco, wheat, and rice, Rubisco comprises 20–30% of the leaf N, which is equivalent to 30–60% of the leaf soluble protein (Evans, 1989; Makino et al., 2003; Whitney et al., 2011), while in C₄ plants it is 5–10% (Ghannoum et al., 2005). The lower Rubisco requirement of C₄ plants stems from their CCM that enables them to operate under near saturating CO₂ concentration, which has facilitated the evolution of increased Rubisco *k*ₐₘₐₓ (Ghannoum et al., 2005). Consistent with these findings, the variation in Rubisco content with leaf ontogeny and at different locations in the canopy of *M. maximus* and maize (Fig. 5A) was 3- to 25-fold lower than that measured in C₃ species (tobacco, *P. bisulcatum*, and wheat, Fig. 5B). Among the C₄ plants, significantly higher levels of Rubisco were measured in wheat relative to tobacco and *P. bisulcatum*, with the latter grass showing the greatest variation in Rubisco content (per leaf area) in the leaves from juvenile and mature plants.

The variation in Rubisco with leaf ontogeny evident in both C₄ species was somewhat mirrored by differences in their PEPC activities (Fig. 5B), resulting in similar PEPC:Rubisco activity ratios of ~3.9–5.7 in *M. maximus* that were more varied in maize (4.2–7.9 in juvenile plant leaves and 8.5–9.3 in the young leaves of exponentially growing plants; Fig. 5C). This ratio is typically used as an indication of the CO₂ supply to the CCM in C₄ plants and is normally in balance to minimize leakage of fixed CO₂ (von Caemmerer, 2000; von Caemmerer et al., 2014). In both *M. maximus* and *Z. mays*, the PEPC:Rubisco ratio tended to increase during ontogeny, in particular when the ratio is adjusted with regard to differences in Rubisco activation status (Fig. 5C). The higher Rubisco:PEPC ratio in mature *Z. mays* leaves relative to *M. maximus* may arise from their varying C₄ biochemical and/or evolutionary origin, a consideration beyond the objectives of this study.

Despite being produced in lower abundance, the activation status of C₄ Rubisco was similar or lower than that measured in the C₃ species. Similarly, low levels of Rubisco activation (~45–55%) have been measured in other C₄ species (von Caemmerer et al., 2005; Carmo-Silva et al., 2010) that correlate with those measured in mature *M. maximus* and *Z. mays* leaves (Fig. 5D). Higher Rubisco activation levels (~70–80%) were measured in the juvenile C₄ plant leaves. These levels matched those measured in tobacco and *P. bisulcatum*, where little variation in Rubisco activation status was found among the leaves sampled. In contrast, significant variation in Rubisco activation status was observed among the upper wheat panicle leaves, where Rubisco activation was significantly lower than those sampled from juvenile plants (Fig. 5D).

The level of variation in Rubisco content and activation status with leaf ontogeny identified within this explorative study using plants grown under non-stress conditions emphasizes the importance of determining these parameters to compare meaningfully values of *V*ₐₘₐₓ derived by extrapolation from leaf gas exchange (*A*-*C*₃ curves) for different biological samples. As demonstrated by Whitney and Sharwood (2014), quantifying the leaf Rubisco content is best achieved using the [¹³C]CABP binding methods as densitometry methods following PAGE separation of Rubisco are highly imprecise, unless appropriately calibrated. Accurate quantification of Rubisco site content in the *in vitro* assays of Rubisco activity are also critical for quantifying *k*ₐₘₐₓ. This parameter also provides a number of quality checks as reduced measures of *k*ₐₘₐₓ provide a useful indicator of reduced leaf sample viability (as found if ultra-cold temperatures are not maintained during transfer and storage at ~80 °C) and incomplete activation (e.g. insufficient activation time and/or presence of significant levels of ECMI complexes in the sample).

Variation in NADP-ME and PEPC activities in Z. mays and M. maximus.

Different aged leaves sampled from mature *M. maximus* and *Z. mays* plants were analysed for maximal NADP-ME and PEPC activities (Fig. 6). Higher PEPC activities were measured in the younger leaves from both species, with as much as 2-fold higher activity measured in the *Z. mays* samples. Conversely, NADP-ME levels were characteristically >10-fold higher in *Z. mays*, consistent with its C₄-NADP-ME photosynthetic biochemistry. While the low NADP-ME activity in *M. maximum* probably arose from an anaerobic reaction, the significance of the PEPC activity in *Z. mays* is not yet fully understood. Prior analysis of *Z. mays* exposed to salinity stress and shade treatments showed that there is plasticity in PEPC contents and activity (Sharwood et al., 2014). This suggests that the PEPC decarboxylation pathway may serve a role in responding to stressful environmental cues (Bellasio and Griffiths, 2014).

Conclusion

In this study, we demonstrate the need for carefully considering the experimental requirements needed to measure accurately, and reproducibly, the activity of key carboxylase and decarboxylase enzymes that are commonly used to evaluate physiological and biochemical parameters between plant samples. Of particular relevance to C₃ and C₄ photosynthetic modelling studies is how Rubisco content and activation can vary significantly with leaf ontogeny, in particular in C₄ plants where Rubisco activation appears characteristically low. Here we show that full Rubisco activity is recoverable *in vitro* without the need for RCA when extracted with no RuBP. We therefore propose that Rubisco inactivation in the chloroplasts of non-stressed, illuminated leaves is primarily attributable to ER complex formation. Removing Rubisco inhibitors using Na₂SO₄ and PEG treatments (Parry et al., 1997, 2002; Carmo-Silva et al., 2010; Galmes et al., 2011; Scales et al.,
that can potentially harm recoverable activity might therefore be unnecessary using the in vitro assay conditions described in this study.

As summarized in Supplementary Fig. S3, we identified the core requirements for measuring Rubisco activation status (fast extraction, include ~5 mM MgCl₂, use pure RuBP, activate for 10 min), PEPC (pH 8, 22 °C post-extraction), PEPCK (pH 7, >2 mM Mn²⁺ no Mg²⁺, 15 mM PEP), and NADP-ME activities using NADH-linked assays. We highlight how an unresolved limitation in the NADH linked assay underestimates Rubisco $k_{cat}$ by >20%. We also emphasize the advantage of quantifying Rubisco by $^{14}$C-CABP binding.
to normalize Rubisco activities per active site (i.e. \( k_{\text{cat}} \)) as it serves as a quality control indicator of sample integrity and full Rubisco activation. Understandably, the assay and extraction conditions used in this study probably need optimization for other plant samples where additives and conditions (pH, temperature) are required to sustain, or promote, enzyme activities (Supplementary Fig. S3). As shown here by the differing assay requirements of PEPC and PECK, this optimization should also assess the compatibility of additives on the activity of each enzyme measured.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Details for the preparation and storage of coupling enzymes used in the NADH-linked spectrophotometric assay of Rubisco activity.

Figure S1. Overview of the NADH-linked enzyme-coupled spectrophotometric assay for measuring Rubisco activity.

Figure S2. Time-dependent activation in vitro of inhibited tobacco Rubisco–RuBP (ER) complexes by RCA.

Figure S3. Core requirements for measuring Rubisco activation status, PEPC, PECK, and NADP-ME activities.

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