RESEARCH PAPER

Rubisco in planta $k_{\text{cat}}$ is regulated in balance with photosynthetic electron transport

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Abstract

Site turnover rate ($k_{\text{cat}}$) of Rubisco was measured in intact leaves of different plants. Potato (Solanum tuberosum L.) and birch (Betula pendula Roth.) leaves were taken from field-growing plants. Sunflower (Helianthus annuus L.), wild type (wt), Rubisco-deficient (–RBC), FNR-deficient (–FNR), and Cyt $b_{6}f$ deficient (–CBF) transgenic tobacco (Nicotiana tabacum L.) were grown in a growth chamber. Rubisco protein was measured with quantitative SDS-PAGE and FNR protein content with quantitative immunoblotting. The Cyt $b_{6}f$ level was measured in planta by maximum electron transport rate and the photosystem I (PSI) content was assessed by titration with far-red light. The CO2 response of Rubisco was measured in planta with a fast-response gas exchange system at maximum ribulose 1,5-bisphosphate concentration. Reaction site $k_{\text{cat}}$ was calculated from $V_{\text{m}}$ and Rubisco content. Biological variation of $k_{\text{cat}}$ was significant, ranging from 1.5 to 4 s$^{-1}$ in wt, but was >6 s$^{-1}$ at 23 °C in –RBC leaves. The lowest $k_{\text{cat}}$ of 0.5 s$^{-1}$ was measured in –FNR and –CBF plants containing sufficient Rubisco but having slow electron transport rates. Plotting $k_{\text{cat}}$ against PSI per Rubisco site resulted in a hyperbolic relationship where wt plants are on the initial slope. A model is suggested in which Rubisco Activase is converted into an active ATP-form on thylakoid membranes with the help of a factor related to electron transport. The activation of Rubisco is accompanied by the conversion of the ATP-form into an inactive ADP-form. The ATP and ADP forms of Activase shuttle between thylakoid membranes and stromally-located Rubisco. In normal wt plants the electron transport-related activation of Activase is rate-limiting, maintaining 50–70% Rubisco sites in the inactive state.

Key words: Photosystem I, Rubisco, Rubisco activase.

Introduction

Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) is a dominant rate-controlling enzyme of photosynthetic CO$_2$ assimilation, along with diffusion resistances in leaves. Rubisco is a regulated enzyme, its activity level being the result of a complicated balance between activation/deactivation.

An assembled Rubisco holoenzyme, consisting of eight small (14 kDa) and eight large (53 kDa) (Knight et al., 1990) subunits containing eight active sites, requires carbamylation of a Lys residue by a non-substrate CO$_2$ followed by binding of Mg$^{2+}$ for activation (Badger and Lorimer, 1976; Boyle and Keys, 1987). Binding of the substrate RuBP to the uncarbamylated enzyme blocks the activation process (Jordan et al., 1983). Protonation during the enediolization of RuBP bound to the carbamylated enzyme may generate different 5C phosphates that block the active site (Edmondson et al., 1990a, b). Several phosphorylated compounds bind to Rubisco sites and act as dead-end inhibitors (Parry et al., 2008). In many plants 2-carboxyarabinitol-1-phosphate (CA1P) is synthesized from 2-carboxyarabinitol which binds to the active site with high affinity precluding enzyme activation (Vu et al., 1984; von Caemmerer and Quick, 2000).

Rubisco activase (henceforth referred to as Activase) catalyses the carbamylation and releases inhibitory sugar
phosphates from uncarbamylated or carbamylated sites (Salvucci et al., 1987; Portis Jr, 1992; Portis, 2003). Activase is bound to Rubisco as an oligomer of up to 14 subunits, exhibiting ATPase activity (Robinson and Portis, 1989; Lilley and Portis, 1997). Activase is inhibited by ADP, hence the ATP/ADP ratio is a sensitive regulator of Rubisco activation state. In most plants there are two isoforms of Activase. Distinct from the smaller isoform (41–44 kDa), the larger isoform (45–46 kDa) is regulated by the ferredoxin-thioredoxin f system (Zhang and Portis, 1999; Zhang et al., 2002).

For some time it has been understood that, in plants growing under saturating light and optimal temperature, Rubisco is nearly fully activated. This notion emerged from the fact that the activation state (ratio) measured in vitro accurately matched the carbamylation ratio (Butz and Sharkey, 1989), and usually 80–90% of Rubisco sites are carbamylated under these optimal conditions (Cen and Sage, 2005; Yamori et al., 2006). At moderately high temperatures (30–40 °C) Activase is progressively unable to cope with the increasing rate of Rubisco inactivation, so the carbamylation (=activation) state decreases (Quick et al., 1991a; Cen and Sage, 2005; Sharkey, 2005; Yamori et al., 2006). These observations supported the notion that carbamylation by Activase is the major regulator of Rubisco activity—until variable activity of the presumably fully carbamylated enzyme was observed.

When extracted Rubisco protein was precipitated with sulphate ions and redissolved and carbamylated in a phosphate-free medium, the resulting ‘maximal activity’ was greater than the ‘total activity’ of the carbamylated enzyme before the precipitation. Precipitation evidently released an unknown inhibitor from carbamylated or uncarbamylated sites (Parry et al., 1997). When the Activase content was gradually decreased in anti-sense transgenic tobacco, the number (or in planta turnover rate) of carbamylated sites decreased finally about 10-fold (He et al., 1997; Parry et al., 2003). Similar impairment of the function of Rubisco was observed under the influence of moderately high temperatures in these anti-sense plants (Sharkey et al., 2001). Early experiments on lysed chloroplasts (Campbell and Ogren, 1990, 1992), as well as Cyt b6f-deficient and GAPDH-deficient tobacco (Rusuksa et al., 2000), revealed that activation of the Activase is not a simple function of the ATP/ADP ratio, but is somehow related to Cyt b6f content and PSI electron transport, demonstrating the importance of further in planta investigations.

A convenient metric of enzyme activity is kcat, the catalytic turnover rate of an active site. Extracted Rubisco from C4 plants exhibited the highest kcat of 4.86±0.89 at 28 °C, followed by 3.55±0.54 of Rubisco from C3 plants from cool habitats and 2.46±0.52 s⁻¹ of Rubisco from C3 plants from warm habitats (Sage, 2002). Such variation of kcat values could indicate an evolutionary adjustment (Tcherkez et al., 2006), but the active sites could be differently inhibited as well. The balance of Rubisco inhibitors and activators is best revealed directly in planta. An experimental difficulty is that Rubisco Vm cannot be measured in normal wild-type leaves, because photosynthesis becomes limited by RuBP regeneration far below the Vm of Rubisco. This problem was elegantly bypassed by using Rubisco-deficient transgenic plants, where Rubisco was rate-limiting over a wide range of CO2 concentrations (von Caemmerer et al., 1994). In these leaves kcat was 3.53 s⁻¹ at 25 °C, but was estimated to be 4.24 s⁻¹ after the carbamylation ratio of 0.8 at 1500 μmol quanta m⁻² s⁻¹ was taken into account. The latter kcat value is higher than most reported values in C3 plants.

The CO2 response curve of Rubisco was measured in planta at CO2 concentrations up to at least three times Kn(CO2) by accumulating RuBP at low CO2 and O2 concentrations and then measuring the initial rate of CO2 fixation after a rapid increase in CO2 concentration (Laisk and Oja, 1998; Laisk et al., 2002). The biological variation of kcat was wide. It decreased from 4 to 2.5 s⁻¹ with increasing Rubisco content in sunflower grown under different conditions (Eichelmann and Laisk, 1990). In mature birch (Betula pendula) leaves the average kcat was about 2 s⁻¹ (Eichelmann et al., 2004a). In developing birch leaves Rubisco Vm increased in proportion to the capacity of the developing photosynthetic machinery, kcat varied from 1.35 to 2.24 s⁻¹ (Eichelmann et al., 2004b). In Betula pendula and Tilia cordata leaves growing in a natural canopy the apparent kcat values were 2.3 and 1.6 s⁻¹, respectively, independent of sun/shade exposure in the vertical cross-section of the canopy (Laisk et al., 2005), all cited measurements at 22.5 °C. Significant correlation was detected between the amount of activated Rubisco (Vm) and PSI density, suggesting that the activity of Activase is related to the photosynthetic electron transport system.

This overview shows that in normally photosynthesizing leaves Rubisco is present in high quantities, but the variable kcat values reflect partial enzyme activation. It is likely that the stromal redox state and ATP/ADP ratio are not the only parameters controlling Rubisco activation via Activase; as, at least, unknown inhibitor controls the number of Rubisco sites that can be activated by carbamylation. In this work, the actual Rubisco activity in planta, as a function of the amount of holoenzyme and in relation to the photosynthetic light reactions, is investigated. It is shown that the activation state of Rubisco is generally low in wild-type plants containing high Rubisco levels. It is still low in N-starved leaves where Rubisco content is low, but PSI content is low as well. It is even lower in FNR-deficient and Cyt b6f-deficient tobacco leaves containing normal amounts of Rubisco, but impaired in electron transport capacity. Rubisco activation state approaches the maximum in transgenic leaves containing little Rubisco but sufficient PSI. In these leaves, the catalytic turnover rate of an active Rubisco site may exceed 6 s⁻¹ at 22.5 °C.

Materials and methods

Plant material

Birch (Betula pendula Roth.) leaves were taken either from young trees growing in open top chambers in Suonenjoki,
Finland (the control trees for CO₂ and ozone enrichment experiments, Eichelmann et al., 2004a, b) or from full-grown trees in a natural community in Järveselja, Estonia (Laisk et al., 2005). The petioles of excised leaves were immersed in water and leaves were fitted to the measuring chamber. Potato (Solanum tuberosum L.) was grown in pots in the field and in the laboratory (Laisk et al., 2007). Wild-type (wt), Rubisco-deficient (−RBC, Hudson et al., 1992) and Cyt b₆f deficient (−CBF, Price et al., 1995) transgenic lines of tobacco (Nicotiana tabacum L.) cv. W38 were kindly supplied by Professor D Price (Australian National University, Canberra). The FNR-deficient (−FNR) tobacco line (Hajirezaei et al., 2002) was kindly supplied by Professor U Sonnewald (Gatersleben, Germany). The transgenic and corresponding reference wt tobacco plants were grown in 4l pots and nutrient-rich soil. In the N gradient (−N series) wt tobacco plants were grown hydroponically at Ca(NO₃)₂ concentrations of 100, 50, 25, 20, 17, 12, and 5% of the standard Knop level. In all cases the laboratory growth conditions were PFD of 300–400 μmol quanta m⁻² s⁻¹ and a 12/12 h 25/20 °C day/night cycle. Three full-grown attached leaves (positions 5–7) were used in measurements for the −N series. The transgenic leaves were measured over a wider age range, resulting in highly variable Rubisco contents.

Gas exchange measurement system

The gas exchange measurement system (Laisk and Oja, 1998; Laisk et al., 2002) was designed to vary incident quantum flux densities and chamber CO₂ and O₂ concentrations and to measure fast transitions in transpiration, CO₂ uptake and O₂ evolution rates, Chl fluorescence, and 810 nm transmittance (oxidized plastocyanin and P700⁺). The leaf chamber of 32 mm diameter and 3 mm height was illuminated through a multi-arm light guide by two Schott KL 1500 tungsten halogen lamps (Schott GmbH, Mainz, Germany), providing actinic white light and saturation pulses of 11 000 μmol m⁻² s⁻¹. CO₂ exchange was measured with an infrared CO₂ analyser LI-6251 (Li-Cor, Inc. Lincoln, NE, USA) and transpiration was measured with a micropsychrometer incorporated in the gas stream. Intercellular air space and chloroplast CO₂ concentrations are represented as equivalent liquid phase molarities.

Incident quantum flux density was measured with a quantum sensor (LI-250, Li-Cor, Inc. Lincoln, NE). Leaf absorptance of white and far-red light (FRL) was measured using an integrating sphere and a spectroradiometer PC-2000 (Ocean Optics, Dunedin, FL).

Measurement of Rubisco protein

A disc of 1.86 cm² was excised from the leaf part that had been enclosed in the gas-exchange chamber. The disc was ground in liquid nitrogen and homogenized in 0.8 ml of 50 mM MES-NaOH buffer (pH 6.8) containing 20 mM MgCl₂, 50 mM 2-mercaptoethanol, and 1% (w/w) Tween-80 (all from Sigma-Aldrich, St Louis, MO, USA). Part of the homogenate was diluted with the SDS-PAGE loading buffer and incubated in a boiling water bath for 5 min. Such samples were kept at −20 °C until SDS-PAGE. The gels were stained with Coomassie Serva Blue G and scanned with UMAX Power Look III scanner in the light transmission mode and resolution of 500 dpi. The Rubisco large subunit band was denoted by a freehand line and the optical density of the selected area was integrated in the red colour channel using updated ImageQuant™ software considering the logarithmic relationship between the scanner signal and optical density. The SDS-PAGE sample volume was increased for extracts anticipated to contain less Rubisco, with the aim of adjusting the optical density of the band within the range of the calibration bands (three calibration bands were run in each gel along with samples). Performing the extraction procedure on the precipitate did not liberate any Rubisco.

The gels were calibrated using a known mass of Rubisco purified from young sunflower leaves grown with excess nutrients. Leaves (100 g) were ground in liquid nitrogen and extracted with 240 ml of extraction buffer. The homogenate was filtered, centrifuged, and proteins were sedimented from the supernatant at first with 37% and then with 50% (NH₄)₂SO₄. The sediment was dissolved and desalted with a Sephadex G-25 column. The fraction containing protein (48 ml) was subjected to chromatography with a DEAE-Toyopearl column and a 0.025–0.6 M NaCl gradient. Fractions containing Rubisco were collected and the protein was sedimented with 65% ammonium sulphate. The pellet was dissolved in 25 ml of buffer and desalted with Sephadex G-25. Equal volumes of the Rubisco fraction and buffer were dried at 90 °C in two repetitions. The mass of Rubisco was obtained by difference. Aliquots of the Rubisco preparation were stored in liquid N₂ and used later as standards during SDS-PAGE. The standard was about 95% pure Rubisco.

The Rubisco active site turnover rate kcat was calculated as

\[
k_{\text{cat}} = \frac{550 000 V_{\text{m}}}{8 m_{\text{RBC}}},
\]

where 550 000 is the molecular weight assumed to be invariant among plant species (www.brenda.uni-koeln.de,) and \( m_{\text{RBC}} \) is the Rubisco concentration (g m⁻²). Possible small differences in the molecular weight of Rubisco from different species were neglected in these experiments.

Measurement of photosystem I density

PSI density was measured by oxidative titration of the PSI donor side using a known rate of absorption of FRL (Oja et al., 2003). In order to avoid interference by reduced plastocyanin and cyclic electron transport (both donating additional electrons to P700, thus slowing down the rate of its oxidation), measurements were carried out near the steady-state FRL level. The speed of P700 re-oxidation under FRL after a brief dark exposure is an exponential
process. The product of the PAD of FRL (µmol absorbed quanta m\(^{-2}\) s\(^{-1}\)) and exponential time constant \(\tau(s)\) yields the PSI density \(N_1\) (µmol m\(^{-2}\)):

\[
N_1 = \tau \text{PAD}_{\text{FRL}}.
\]  

Equation (2) is based on a self-evident assumption that a larger pool of P700 requires a longer time for oxidation by a given FRL photon fluence rate, assuming the quantum efficiency is constant [close to 100% (Hiyama, 1985)]. The PSI densities were checked by reductive titration using O\(_2\) evolution measurement (Oja et al., 2004). The leaf was illuminated under FRL, a single-turnover flash of white light was applied and FRL was immediately turned off. The number of electrons generated by PSII was measured as four times the integral of O\(_2\) evolution after the flash. Electrons arriving at the PSI donor side via Cyt \(b\)\(_{\text{af}}\) caused partial reduction of the PSI donors P700\(^+\) and PC\(^+\). The extent of this fast reduction was relatively greater when the number of PSI donor side carriers was smaller and vice versa; hence, the number of PSI m\(^{-2}\) was deduced from this relationship (Oja et al., 2004). The S-states of the water-splitting complex were randomized due to the slow PSII excitation caused by FRL.

The in planta PSI density measurements were carried out with a PAM-101 fluorometer equipped with an emitter/detector unit ED P700DW (H. Walz GmbH, Effeltrich, Germany), redesigned for the wavelength difference of 810–950 nm. Far-red light (720 nm) was provided by a light-emitting diode source (Fast-Est Instruments, Tartu, Estonia) equipped with a longpass filter to minimize PSII excitation. Single turnover flashes were produced by a xenon arc flash lamp MVS 7060 (Perkin Elmer, Salem, MA, USA). O\(_2\) evolution produced by an individual flash was measured at very low ambient O\(_2\) concentration of 10–50 µmol mol\(^{-1}\) with a zirconium cell analyzer S-3A (Ametek, Pittsburgh, PA, USA). The incident FCD of FRL was measured with the spectroradiometer PC-2000 calibrated in absolute units against a standard lamp.

The in planta measurements of PSI density were also compared to in vitro redox titrations of PSI density in thylakoid preparations, based on the known differential optical extinction coefficient between oxidized and reduced P700 at 700 nm [64 m\(^{-1}\) cm\(^{-1}\) (Hiyama and Ke, 1972)]. Leaf discs (1.86 cm\(^2\)) were ground and washed in buffer (1 ml per 1 leaf disc) containing 50 mM MES-KOH (pH 7.0), 10 mM NaCl, 5 mM MgCl\(_2\), and 300 mM sorbitol. Triton-X100 was added after washing to a final concentration of 1% (all reagents from Sigma-Aldrich, St Louis, MO, USA). Finally, 20 µg polyvinyl pyrrolidone (PVPP) per leaf disc was added to avoid interference from phenolic compounds. Starch particles, PVPP and suspended plant material were sedimented by centrifugation at 3000 g for 5 min. Part of the thylakoid suspension (supernatant) was oxidized with 40 mM ferricyanide and the rest of the solution was reduced with 40 mM Na-ascorbate. The difference spectrum of these solutions was measured in a UV-2410PC spectrophotometer (Shimadzu Corporation, Kyoto Japan). The entire procedure, except optical measurements, was carried out on ice. Parallel determinations based on all three methods were proportional (data not shown), but the in vitro method tended to show somewhat more PSI than the two rather well-coinciding in planta optical titrations.

Quantitative immunoblotting of ferredoxin-NADP reductase

Leaf discs (3.72 cm\(^2\)) were ground in 300 µl of buffer containing 50 mM TRIS-HCl (pH 7.0), 5 mM MgCl\(_2\), 2 mM EDTA, and 0.5 mM phenylmethylsulphonylfluoride (PMSE). Protein extract of each sample corresponding to 5 mg leaf fresh weight was mixed with SDS-PAGE sample buffer and heated by boiling in a water bath for 3 min. Proteins were concentrated in a 5% stacking gel and separated by 12% SDS-PAGE. Immunoblotting was performed using standard protocols (Burnette, 1981). Prestained protein markers were run in neighboring lanes. FNR was detected immunologically by sheep anti-FNR (dilution 1:10 000), and visualized using the appropriate IgG secondary antibody conjugated with horseradish peroxidase (dilution 1:20 000, Sigma) and the chemiluminescence method (GE Healthcare, UK). Band intensities were quantified (Quantity One, Bio-Rad). Three separate blots were made of each sample and the band intensities were averaged and normalized to the most intense sample. On average, the ratio of FNR protein in the –FNR plants to wt was 0.41, with the lowest of 0.23 and highest of 0.77 of wt.

Measurement of leaf N content

The content of organic N in leaves was measured with the micro-Kjeldahl method (Kjeltech Auto 1030, Foss Tecator AB, Hoeganaes, Sweden).

Results

Rubisco kinetic curves in planta

Gas exchange and optical measurement routines were essentially the same as used before (Laisk et al., 2002). Maximal RuBP pool levels accumulated in leaves at an ambient CO\(_2\) concentration of 200 µmol mol\(^{-1}\) and O\(_2\) concentration of 20 mmol mol\(^{-1}\). Fast transitions were made from this steady-state to lower and higher CO\(_2\) concentrations. At lower CO\(_2\) concentrations the RuBP pool did not change and the initial slope of the CO\(_2\) response of Rubisco kinetics was measured in the steady-state. During the jumps to higher CO\(_2\) concentrations RuBP regeneration became rate-limiting and the RuBP pool began to decrease after the transition. The fast-response gas exchange measurement system correctly recorded the CO\(_2\) uptake rate beginning from 2 s after the transition. The CO\(_2\) uptake associated with CO\(_2\) solubilization and bicarbonate formation was recorded in a parallel measurement carried out in the dark. The time-course of the carboxylation rate was obtained by subtracting the trace recorded in the dark.
from the trace recorded in the light. The initial carboxylation rate was obtained by extrapolating the carboxylation rate to the moment of the CO₂ increase. The initial carboxylation rate was plotted against the chloroplast CO₂ concentration Cₒ calculated considering this initial rate and the diffusion resistances in the gas phase, rₙₒ, and in the liquid phase of mesophyll cells, rₙₙ. The latter was determined using the electron transport method of Harley et al. (1992), considering the reduction of alternative acceptors. These Rubisco kinetic curves were rectangular hyperbolas with Kₘ (CO₂) of 10 µM at a leaf temperature of 22.5 °C (Fig. 1), in close accordance with earlier in vitro (Yokota and Kitaoka, 1985) and in planta (von Caemmerer et al., 1994; Laisk et al., 2002) measurements. In wt plants the maximum reaction rate, Vₘ, of Rubisco (approaching 100 µmol CO₂ m⁻² s⁻¹, calculated by extrapolating the rectangular hyperbola to an infinitely high CO₂ concentration) was considerably faster than the maximum steady-state CO₂ and light-saturated rate of photosynthesis, Aₘ. In the Rubisco-deficient transgenic plants Vₘ was about equal to Aₘ, indicating that, in these plants, photosynthesis was Rubisco-limited at all CO₂ concentrations. In –FNR and –CBF plants, as in wt plants, the Vₘ of Rubisco, though low, was still higher than the steady-state photosynthetic rate.

Leaf N and Rubisco content

The growth of wt tobacco plants at different nutrient solution N contents and the selection of leaves of different age from control wt plants and from –RBC, –FNR, and –CBF plants resulted in different N contents in leaves. Rubisco content was generally higher at higher leaf N, however, the slope of the regression was different in different treatments (Fig. 2). The least variable dependence was obtained in the –N series, where Rubisco content increased linearly with N content, with a small offset on the N axis. At any leaf N content the Rubisco level of the –RBC plants was far below that of wt plants, but neither –FNR nor –CBF plants exhibited Rubisco deficiency.

Rubisco content and k₉ₐₜ

Among the leaves examined, Rubisco content ranged from very low values to a maximum of 80 µmol active sites m⁻². The interdependence between k₉ₐₜ and Rubisco content was reciprocal (Fig. 3). In the mutants with extremely low Rubisco content (and young sunflower leaves transferred from low to high growth light) k₉ₐₜ exceeded 6 s⁻¹. In wt tobacco plants the highest k₉ₐₜ values were 4 s⁻¹ detected in leaves with low Rubisco content. At high Rubisco levels k₉ₐₜ values were about 2 s⁻¹ over the range of active site concentrations of 50–70 µmol m⁻² in wt tobacco, potato, and birch leaves. The following empirical regression equation models the reciprocal dependence between Rubisco site content and the apparent (average) k₉ₐₜ assuming the maximum k₉ₐₜ of 4.3 s⁻¹ as recorded for wt plants.

\[
k₉ₐₜ = \frac{4.3}{1 + 0.03 RBC}
\]

where RBC is the active site concentration (µmol m⁻²).

Notably, the –FNR and –CBF tobacco leaves exhibited about the same Rubisco content as wt leaves, but their k₉ₐₜ values were only 0.5 s⁻¹, significantly lower than in wt plants with similar Rubisco content. A closer analysis of Fig. 4A shows a significant correlation (R²=0.9) between Rubisco k₉ₐₜ and the relative expression level of FNR in different plants. The transgenic down-regulation of Cyt b₅₆
was characterized by the exponential rate constant (reciprocal of the time constant) of post-illumination reduction of P700+. As with –FNR plants, a similarly high correlation was observed between Rubisco $k_{cat}$ and PSI electron transport rate in the –CBF tobacco (Fig. 4B).

PSI density and $k_{cat}$

Assuming that PSI density proportionally characterizes the capacity of other components of the electron transport chain (Graan and Ort, 1984) and that Activase is functionally related to ATP synthase, one may expect that more Rubisco is activated in leaves exhibiting a higher ratio of PSI per Rubisco active site. The content of PSI increased with leaf N (Fig. 5). A plot of Rubisco $k_{cat}$ versus the ratio of PSI per Rubisco active site exhibited a saturating relationship, where data points for wt plants formed the initial slope (low PSI/Rubisco) and data from the –RBC plants formed the saturation phase (high PSI/Rubisco, Fig. 6A). The hyperbolic relationship of equation (4) describes the dataset involving the maximal $k_{cat}$ of 8 s$^{-1}$ recorded for –RBC plants:

$$k_{cat} = \frac{8(\text{PSI/RBC})}{0.085 + (\text{PSI/RBC})^0.75}. \quad (4)$$

The initial part of the relationship is represented better in the reciprocal plot of Fig. 6B. In the latter, data from the –FNR and –CBF plants fall below the average of the wt plants, indicating that, in these plants, Rubisco is significantly less activated than expected, based on the average PSI/Rubisco ratio (in some –CBF plants Cyt $b_6f$ level was only slightly down-regulated and, thus, did not influence Rubisco $k_{cat}$). Equation (5) approximates the reciprocal relationship:

$$k_{cat} = \frac{8}{1 + 0.085(\text{RBC/PSI})}. \quad (5)$$

Discussion

Measurement of the in planta $k_{cat}$ Of Rubisco

The reported $k_{cat}$ values are based on the $V_m$ of Rubisco, measured in planta, divided by the content of Rubisco active sites in leaves. Therefore, the $k_{cat}$ values represent an average over all the Rubisco active and inactive sites, and therefore do not characterize a distinct biochemical species. The in planta method of measurement of the initial rate of CO$_2$ fixation by RuBP-saturated Rubisco was first used by Laisk and Oja (1974) and later by Ruuska et al. (1998). Reliable CO$_2$ fixation rates by RuBP-saturated Rubisco could be measured at CO$_2$ concentrations of at least 3 K$_m$(CO$_2$) (up to 75% of $V_m$) in wt plants (at higher CO$_2$ concentrations the pre-accumulated RuBP was carboxylated too fast), but full kinetic curves could be measured...
with high precision in –RBC plants, where the Rubisco content was low. Thus, reliability of the reported $k_{\text{cat}}$ values is mainly dependent on the measurement of Rubisco protein.

The amount of Rubisco was measured by quantitative SDS-PAGE calibrated gravimetrically, which is the most straightforward method to detect Rubisco protein. For example, the widely used method of binding radioactive carboxyarabinitol bisphosphate (CABP) requires a correction factor of unknown origin and constancy, considering that about 6.5 CABP molecules are bound per Rubisco instead of 8 (Butz and Sharkey, 1989; Sage and Seemann, 1993). A potential problem with the gel measurements used here was the occurrence of unidentified bands that co-migrate with the Rubisco large subunit. This could cause an overestimation at the lowest Rubisco levels. However, overestimation of Rubisco would lead to an underestimation of the highest $k_{\text{cat}}$ values. Underestimation of Rubisco levels (resulting in overestimated $k_{\text{cat}}$) could occur if some Rubisco was so tightly bound to the membrane system that it did not extract despite the stringent procedure employed (Makino and Osmond, 1991).

In our leaves Rubisco density varied from 2 to 70 µmol sites m\(^{-2}\), similar to the range reported for wt and –RBC tobacco measured by the CABP-binding method (von Caemmerer et al., 1994; Kubien and Sage, 2008). The maximum Rubisco density is also not substantially different from the value of 64 µmol sites m\(^{-2}\) measured in rice using a similar quantitative electrophoresis method (Makino et al., 2000). This maximum value is, however, significantly higher than detected by stoichiometric binding of \([^{14}\text{C}]\text{CABP/CRBP}\) in wild-type tobacco (Rususka et al., 2000). The difference is explainable by the adjustment of Rubisco content to growth conditions (for a review, see von Caemmerer and Quick, 2000). In the –RBC transgenic plants, Rubisco content remained generally below 10, and typically between 2 and 5 µmol sites m\(^{-2}\), in accordance with results reported by von Caemmerer et al. (1994) and Kubien and Sage (2008). Though Rubisco levels could be somewhat reduced in the –CBF and –FNR treatments (Palatnik et al., 2003) the remaining 30–50 µmol sites m\(^{-2}\) was well comparable with wt plants.

**Variation of in planta average $k_{\text{cat}}$**

In wt leaves the $k_{\text{cat}}$ values varied between 1.5 and 4 s\(^{-1}\) in tobacco (up to 6 s\(^{-1}\) in young sunflower) and increased with decreasing Rubisco content. This upper limit of $k_{\text{cat}}$ is somewhat higher than the in planta value obtained for tobacco by von Caemmerer et al. (1994) as well as the in vitro values for Rubisco from tobacco (Rususka et al., 1998) and from several C\(_3\) plants (at least those from cool habitats; Sage, 2002). The maximum value for $k_{\text{cat}}$ of 6–7 s\(^{-1}\) observed in some leaves could be overestimated due to problems of extraction of membrane-bound Rubisco as discussed above. But this is still unlikely since the value of 6 s\(^{-1}\) has been confirmed by the data of Kubien and Sage (2008) for Rubisco in –RBC tobacco. Similar high $k_{\text{cat}}$ values (up to 8 s\(^{-1}\)) were reported in young sunflower leaves adapting from low to high growth light (Eichelmann and Laisk, 1999).

**Fig. 5.** Dependence of PSI content on leaf N content. The meaning of the symbols is given in Fig. 2. Dashed line indicates the maximum slope of regression.

**Fig. 6.** Average in planta catalytic turnover rate, $k_{\text{cat}}$, of a Rubisco site as a function of PSI per Rubisco site (A) or as a function of Rubisco sites per PSI (B). The meaning of the symbols is given in Figs 2 and 3. The lines were calculated from equation (4), in (A) and equation (5) in (B).
The correlation between high $k_{\text{cat}}$ values and low Rubisco content has been observed before (Quick et al., 1991b; Cheng and Fuchigami, 2000) and recently documented by Kubien and Sage (2008) in –RBC tobacco, in complete agreement with our results. Similarly, the reciprocal dependence between Rubisco content and its activation state was confirmed by Suzuki et al. (2009), who over-expressed Rubisco in rice. But the increased Rubisco level was not accompanied by an increased photosynthetic rate, indicating lower $k_{\text{cat}}$ in leaves where the Rubisco content was higher.

The observed variability in average $k_{\text{cat}}$ could be (to some extent, but not all) ascribed to different carbamylation states of the enzyme. Although in planta and in vitro Rubisco activity were not compared, it is well known that the degree of carbamylation of extracted Rubisco is usually 80–90% in wt leaves under light saturating for photosynthesis at 23 °C (Butz and Sharkey, 1989; von Caemmerer et al., 1994; He et al., 1997; Ruuska et al., 2000; Cen and Sage, 2005; Yamori et al., 2006). Therefore, the wider variation of $k_{\text{cat}}$ in our leaves (from 1.5 to 4 s$^{-1}$ in wt tobacco leaves and to >6 s$^{-1}$ in –RBC tobacco) cannot readily be explained in terms of variable carbamylation.

A novel result of this work is the relationship between Rubisco activation state and the activity of the photosynthetic electron transport chain. Expression of $k_{\text{cat}}$ as a function of the ratio of PSI per Rubisco site revealed a hyperbolic dependence with a $K_{0.5}$ of about 0.1 PSI per Rubisco active site. In wt plants the PSI/Rubisco active site ratio varied from 0.02 to 0.1 (10 to 50 sites per PSI) with a median value of about 0.03 (33 sites per PSI). Therefore, typically the measured $k_{\text{cat}}$ was only 20–30% of the theoretical maximum. Important new information came from the experiments with –FNR and –CBF tobacco. In control leaves grown together with the –FNR series $k_{\text{cat}}$ was about 1.3 s$^{-1}$, typical for leaves with a relatively low PSI/Rubisco ratio in wt plants. In the –FNR plants Rubisco activity decreased, as was noticed by Palatnik et al. (2003).

Quantitatively, in the –FNR plants the in planta apparent $k_{\text{cat}}$ was suppressed to 0.5 s$^{-1}$ and correlated with the relative expression of FNR (Fig. 4A). Similarly, as soon as the expression of Cyt b$_6$f was low enough to limit electron transport, $k_{\text{cat}}$ values decreased in strong correlation with the Cyt b$_6$f-limited electron transport rate (Fig. 4B). It is unlikely that in the –FNR case the FNR control of Activase was observed, since FNR deficiency must have caused the increased reduction of ferredoxin and correspondingly more activation of Rubisco. Instead, the opposite was observed.

A shuttle model of Rubisco activase

Recent progress in understanding the mechanism of Rubisco activase has focused on interaction between Activase and Rubisco (Andrews et al., 1995; Mate et al., 1996; Portis, 2003; Portis et al., 2008), but little new knowledge has been obtained about the interactions between Activase and thylakoid membranes since the pioneer-
decreased about proportionally and the portion of the inhibitor-free sites available for further carboxylation remained about constant when Rubisco content decreased (Fig. 3). In the –RBC transgenic plants the activity of the electron transport chain remained relatively high, although Rubisco content decreased. In these plants the turnover of Activase became Rubisco-limited and nearly all of the Rubisco was maintained in the inhibitor-free state, ensuring \(k_{\text{cat}}\) was close to its intrinsic maximum. In accordance with such a shuttle model, immunogold labelling experiments indicated that 25% of Activase was bound to thylakoids in wt rice and this fraction decreased in the anti-Activase transgenic plants where Rubisco content increased (Jin et al., 2006).

Campbell and Ogren (1990, 1992) first observed that electron transport through PSI stimulated the activation of Rubisco by Activase, but it is generally believed now that, in these experiments, the underlying mechanism was based on the ATP/ADP sensitivity and redox activation of Activase (Portis, 2003). Experiments on Cyt b\(_{\text{6}}\) deficient and GAPDH-deficient tobacco revealed that the activation of Activase was not a simple function of the ATP/ADP ratio, but the ability of Activase to detect the light signal was somehow related to Cyt b\(_{\text{6}}\) deficiency, despite no change in the bulk ATP/ADP ratio (Ruuska et al., 2000). The authors concluded that the light regulation of Rubisco by Activase is not mediated by the stromal ATP/ADP ratio or the electron transport per se, but, rather, by some manifestation of the balance between the photosynthetic electron transport rate and the consumption of its products, in accordance with the essence of the above shuttle model.

**Physiological implications**

Although the amount of Rubisco protein varies dependent on growth conditions, for example, light intensity (Walters, 2005), normally Rubisco is still disproportionately abundant in relation to the capacity of the electron transport chain. The Activase system activates such a portion of Rubisco that the RuBP carboxylation/oxygenation capacity is balanced with the electron transport capacity of the light reactions of photosynthesis. Even in plants grown at the most severe N deficiency the capacity of the electron transport chain decreases in parallel with the amount of Rubisco, always leaving a part of the Rubisco protein in inactive reserve, functioning as a storage protein (Eichelmann et al., 2005). Such a strategy of N use may aim at the readiness of the plant to increase the turnover of the carbon reduction/oxidation cycle rapidly when the capacity of the electron transport system increases. The strong proportional correlation between the mesophyll conductance (initial slope of the \(A\) versus \(C_i\) curves) and maximum CO\(_2\)- and light-saturated (‘potential’) photosynthetic rate, observed under different environmental conditions (Eichelmann and Laisk, 1999, and references therein) and during the development of leaves (Eichelmann et al., 2004b) is an old mystery, because mesophyll conductance is determined mainly by the initial slope of the Rubisco kinetic curve (Rubisco activity), while the potential photosynthetic rate is determined by the RuBP regeneration capacity that is related to the potential of the electron transport chain. In the light of the present work we understand that Rubisco activity is under the control of the electron transport chain. Therefore, proportionality between the mesophyll conductance and the maximum CO\(_2\) and light-saturated photosynthesis is expected. The electron transport-related control of Rubisco may shed some light also on the mysterious constancy of the \(C_i/C_a\) ratio (intercellular versus ambient CO\(_2\) concentration) which stabilizes at about 0.7 under a wide variety of environmental conditions (Noormets et al., 2001).

To conclude, we suggest that the empirical equation (3) approximates the data of Fig. 3 for modelling purposes. Presently, the Farquhar–von Caemmerer–Berry model (Farquhar et al., 1980) is widely applied for canopy and global-scale modelling of photosynthesis. Usually Rubisco density is calculated from a correlation with N content, but \(k_{\text{cat}}\) is assumed to be constant and independent of Rubisco content. Incorporation of variable \(k_{\text{cat}}\) into the Farquhar–von Caemmerer model, dependent on leaf N (and Rubisco) content, would be a step toward a better prediction of canopy photosynthetic productivity.

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