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Prospects for increasing photosynthesis by overcoming the limitations of Rubisco

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SUMMARY

The low activity and the competing reactions catalysed by Rubisco are major limitations to photosynthetic carbon assimilation in C_3 plants; the present paper considers how these limitations can be overcome. The limitations could be most effectively addressed by introducing Rubisco with a higher catalytic rate and/or better able to discriminate between gaseous substrates. Although enzymes with desirable characteristics are available, technical advances are required before their potential can be realized in major crop plants. Significant improvements could be achieved also by increasing the concentrations of the productive substrates, CO_2 and RuBP, at the active site of Rubisco. Critically, it is essential that other environmental and genotype constraints are minimized, to realize the highest photosynthetic potential.

INTRODUCTION

Among the challenges posed by an increasing human population and a changing climate are finding ways to maximize carbon assimilation and improve crop yield. Carbon is essential to life since all molecular machines are built around a central scaffolding of organic carbon. It has been proven that increasing photosynthesis has the potential to increase crop yields, provided other constraints do not become limiting (Kruger & Volin 2006; Long *et al.* 2006). Of the possible strategies to improve crop yield, namely, increasing the efficiency of light capture, increasing the harvest index or optimizing photosynthesis (i.e. conversion of intercepted radiation into biomass), the last is the only one expected to substantially increase yield potential in the major C_3 crops (Long *et al.* 2006) since the other approaches have already been significantly optimized and already approach their theoretical maxima. In C_3 photosynthesis, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the assimilation of CO_2, marking the

conversion of inorganic carbon into organic compounds (Fig. 1*a*). A number of serious limitations to the efficiency of photosynthesis are caused by the catalytic properties and regulation of this enzyme. In addition to catalysing the CO_2 dependent carboxylation of ribulose 1,5-bisphosphate (RuBP), to form two molecules of 3-phosphoglyceric acid (PGA), Rubisco also catalyses the oxygenation of RuBP, to form one molecule each of PGA and 2-phosphoglycolate (PG). Conversion of the latter into PGA requires the process of photorespiration, which consumes energy and leads to the release of CO_2 (about one third of fixed carbon in C_3 plants; Monteith 1977) and ammonia. Furthermore, Rubisco is such a slow-working enzyme that large amounts of it must be present to achieve acceptable rates of photosynthesis (Mann 1999). Although Rubisco can account for half the soluble leaf protein, or a quarter of leaf nitrogen, it can still be limiting under some conditions (Kung 1976; Ellis 1979). Although the activity of Rubisco is regulated to match RuBP regeneration with RuBP utilization, including starch and sucrose synthesis, these processes are not always optimal for crop productivity. The present review examines the prospects for increasing photosynthesis by overcoming the numerous

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a

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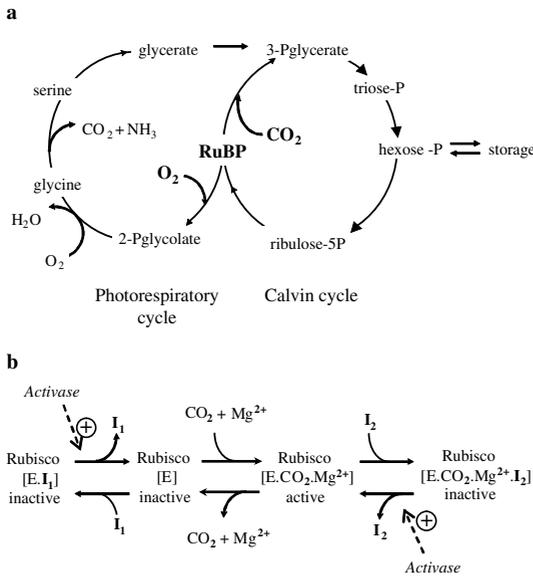
Fig. 1. (*a*) The fate of RuBP catalysed by the carboxylase and the oxygenase activities of Rubisco. (*b*) Regulation of Rubisco activity by reversible carbamylation and inhibitor (I_1 and I_2) binding to carbamylated or non-carbamylated Rubisco. I_1 = RuBP; I_2 includes CA1P and PDBP.

limitations presented by Rubisco, with consideration to Rubisco kinetic properties, the variation of Rubisco properties found in diverse species, Rubisco engineering, abundance, regulation and substrate availability, carbon concentration mechanisms and the modification of photorespiratory metabolism.

Both the carboxylase and oxygenase reactions of Rubisco occur at the same active site and have the same initial steps, i.e. the binding of the bisphosphate substrate (RuBP) and the generation of an enzyme-bound enediol intermediate (Gutteridge *et al.* 1984). CO_2 and O_2 compete to react with this enediol intermediate, since neither gaseous substrate has a formal binding site within the enzyme. Once the enediol has reacted with either CO_2 or O_2 the enzyme is committed to form products.

Clearly a major factor affecting the ratio between the carboxylase and oxygenase reactions is the relative concentration of O_2 and CO_2. The lower the CO_2 concentration the greater the losses through the oxygenase reaction and vice versa. Many environmental stresses decrease carboxylase activity relative to oxygenase, including:

(1) elevated temperature, since (a) the solubility of O_2 decreases less than the solubility of CO_2 as the temperature rises (Hall & Keys 1983); and (b) the oxygenase activity is differentially favoured at higher temperatures, since it has a higher activation energy (Nilsen & Orcutt 1996);

(2) drought, since under these conditions stomatal closure leads to a depletion of CO_2 within the leaf.

Another major factor determining the partitioning between carboxylase and oxygenase activities is the ability of Rubisco to discriminate between the two gaseous substrates, as expressed by the specificity factor (V_cK_o/V_oK_c) where V_c, V_o are the maximum velocities and K_c, K_o the Michaelis constants for CO_2 and O_2, respectively (Jordan & Ogren 1981). The specificity factor is high when the carboxylase activity is favoured and low when the oxygenase activity is favoured. The lowest known specificity factor is for Rubisco from the photosynthetic bacterium *Rhodospirillum rubrum*, moderate values are found in crop plants but the highest values are found in marine red algae (Parry *et al.* 1989; Read & Tabita 1994; Delgado *et al.* 1995; Uemura *et al.* 1996).

A recent study has even uncovered significant differences in specificity between closely related species and cultivars (Galmes *et al.* 2005) and differing carboxylase activities have been reported amongst wheat lines with differing gene ploidy (Evans & Austin 1986) but it has not yet clearly been established whether or not significant differences in Rubisco kinetics can be found between cultivars for the major C_3 crops (Parry *et al.* 1987). Furthermore, attempts to increase the genetic variability (methods for the rapid introgression of traits either from diverse germplasms or from wild relatives) should be considered in addition to other methods for the generation of novel diversity, such as chemical or radiation mutagenesis. Where such variability exists it should be exploited in breeding programmes (Parry *et al.* 2005).

EXPLOITING NATURAL VARIATION

One strategy to increase photosynthetic carbon fixation would be to generate C_3 crop plants expressing Rubisco, with high specificity factor and catalytic rate (Spreitzer & Salvucci 2002; Parry *et al.* 2003*a*; Long *et al.* 2006). Attempts to introduce *rbc*L & *rbc*S operons for the high specificity factor Rubiscos of *Galdieria sulphuraria* and *Phaeodactylum tricornutum* into the plastid genome of tobacco (Whitney *et al.* 2001) have only partially succeeded. Whilst the transgenes directed the synthesis of transcripts in abundance, the subunits of these foreign Rubisco proteins were insoluble, indicating problems with folding or assembly. More encouragingly, substitution of tobacco *rbc*L with sunflower *rbc*L produced a catalytically active enzyme composed of sunflower large subunits (LSU) and tobacco small subunits (Kanevski *et al.* 1999). Exploiting the existing variability in Rubisco specificity factor among C_3 plants appears a promising way to increase photosynthetic carbon assimilation (Parry *et al.* 2003*a*; Raines 2006).

It has been argued that there is an inverse correlation between specificity factor and catalytic rates and uncertainty exists as to the extent to which these two components can be uncoupled (Bainbridge *et al.* 1995; Tcherkez *et al.* 2006; Gutteridge & Pierce 2006). However, some natural variants (e.g. Rubisco from *Griffithsia monilis* which would increase the photosynthetic performance of a C₃ plant by 27%; Zhu *et al.* 2004) indicate that potential candidates for crop improvement remain (Gutteridge & Pierce 2006) and highlight the need to determine the kinetic parameters for more Rubiscos from diverse sources. Furthermore, whilst the adverse effects on photosynthetic rate of the relationship between specificity and catalytic rate will hold under conditions where plants can express their maximum catalytic rates, it may not apply under conditions of severely restricted availability of the substrate CO_2, such as under drought stress. Thus, increased specificity may result in improved photosynthesis even if maximum catalytic rates are lowered (Parry *et al.* 2005).

Recently, Galmes *et al.* (2005) tested the hypothesis that C₃ plants growing in hot arid conditions may have evolved forms of Rubisco with higher specificity factors. Consistent with this hypothesis, they identified two species of the genus *Limonium* whose constituent Rubisco had high specificity factors. Both species are found in coastal regions of the Mediterranean basin, where they are exposed to both severe drought and high temperatures (Galmes *et al.* 2005). The corresponding V_c, V_o and K_c, K_o for the higher of these (*Limonium gibertii*) were determined (Table 1) following rapid enzyme isolation. According to the optimized, inverse relationship between specificity and catalytic rate (Zhu *et al.* 2004) represented by the solid line in the inset to Table 1, it is apparent that Rubisco from *L. gibertii* (and others, e.g. *Galdieria partita* and *Griffithsia monolis*, Table 1, graph) is superior, in terms of both V_c and specificity, to those of many other plant species. Such large deviations from the 'average' imply that Rubisco from different species constitutes an important resource that could assist in optimizing crop productivity under a range of environmental conditions.

Models evaluating the effect on net photosynthesis of replacing wheat and tobacco Rubisco with that from *L. gibertii* predict that this would give increases in net assimilation of 12% and 30% respectively (Galmes *et al.* 2005) assuming stromal concentrations of 7·4 μM CO_2 and 252 μM O_2 and that the difference in specificity factor is due to differences in V_c, which is affirmed in Table 1. Thus, replacement with Rubisco from *L. gibertii* would be expected to have a significant effect on crop productivity.

Whilst these approaches are appropriate there are additional technological obstacles (such as chloroplast transformation of major crops, stability of expression, assembly and regulatory control) to

overcome before they can be exploited (Pastori *et al.* 2001; Parry *et al.* 2003*a*, 2005; Mitchell *et al.* 2004; Shewry & Jones 2005).

ENGINEERING RUBISCO

Another approach to crop improvement is to identify and manipulate amino acid residues that confer key catalytic properties. A comprehensive understanding of structure/function relationships is an essential prerequisite to this approach. Research in this area is thus facilitated by the availability of Rubisco structures at atomic resolution, e.g. *Rhodospirillum rubrum* (Schneider *et al.* 1986), *Chlamydomonas rheinhardtii* (Taylor *et al.* 2001), *Galderia patita* (Sugawara *et al.* 1999), the blue green alga *Anacystis nidulans* (Newman & Gutteridge 1993), spinach (*Spinacea oleracea*; Andersson *et al.* 1989; Andersson 1996) and tobacco (*Nicotiana tabacum*; Chapman *et al.* 1988). The enzymes from most species have a very similar structure; a hexadecamer made up from eight large and eight small subunits. The small subunits are nuclear encoded. The large subunits are chloroplast encoded and are arranged as 4 dimers. Each LSU has two major domains, an N-terminal domain and a larger C-terminal domain, which is an alpha/beta barrel. Most of the active site residues that interact with the substrates (or substrate analogues) occur in peptide loops at the mouth of the alpha/beta barrel structure, with the remaining residues supplied by two peptide loops in the N-terminal domain of the second large subunit within the dimer. When the 3D structures for enzymes with different kinetic properties are compared, the structural differences are very small, especially around the residues that interact directly with the substrate analogue CABP (and by inference, the substrates themselves). It appears that differences in kinetic properties between species are due to very small differences in the position of these residues, brought about by differences in more distant residues.

Whilst extensive directed mutagenesis of *rbc*L and *rbc*S of *R. rubrum*, *C. rheinhardtii* and *A. nidulans* has provided much additional and valuable information on structure/function relationships it has thus far failed to produce an enzyme 'as good' as those already found in crop plants (Parry *et al.* 2003*a*; Zhu *et al.* 2004; Raines 2006). The possibility of improving the kinetic properties of Rubisco by intelligent design appears to be beyond our current knowledge (Andersson & Taylor 2003).

Attempts to improve plant productivity by selecting Rubisco from plants able to survive at low CO_2 concentrations proved unsuccessful, although other characteristics conferring improved dry matter accumulation were identified (Medrano *et al.* 1995). More recently, a directed evolutionary approach has been attempted. Directed evolution of Rubisco, as outlined by Morell *et al.* (1992), takes advantage of

Table 1. *Rubisco kinetic constants for the carboxylase and oxygenase activities of* Limonium gibertii

Kinetic constant	Carboxylase activity		Oxygenase activity
	in N$_2$	*in Air*	*(derived)*
k$_{cat}$ (s^{-1})	4·6 ± 0·2	4·6 ± 0·2	1·9 ± 0·1
K$_m$ (μM)	8·2 ± 0·2	14·0 ± 0·4	370 ± 34·8
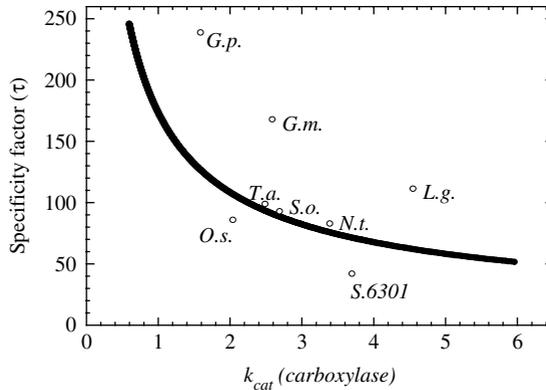
Immediately before assays were initiated, leaves (0·5 gFW) from young, pot-grown *L. gibertii* sampled in full sunlight and frozen in N$_2$(l), were rapidly homogenized in 2 mL {100 mM Bicine-NaOH, pH 8·0; 50 mM β-mercaptoethanol; 20 mM di-thiothreitol; 10 mM NaHCO$_3$; 2 mM MgCl$_2$; 6 % (w/v) PEG 4000; 1 mM benzamidine; 1 mM ε-aminocaproic acid; 1 mM PMSF; 2 μM pepstatin A; 10 μM E64; 7 μM chymostatin and 2·5 % (w/v) PVPP} at 0 °C. The homogenate was clarified by centrifugation (12 842 × g; 4 min; 4 °C) and 1 mL of the supernatant applied immediately to a PD-10 column (GE Healthcare, UK) pre-equilibrated with {100 mM Bicine-NaOH, pH 8·0; 20 mM MgCl$_2$; 10 mM NaHCO$_3$; 10 mM dithio-threitol; 1 mM benzamidine; 1 mM ε-aminocaproic acid; 1 mM potassium phosphate; and 0·5 mM EDTA} at 0 °C. The protein peak (in 1 mL) was supplemented with pepstatin A, E64, and chymostatin (as above) and 250 μL (containing 2·542 μmol NaHCO$_3$) of this was supplemented with 2·542 μCi NaH^{14}CO$_3$ (GE Healthcare, UK) and the remainder frozen immediately in N$_2$(l), for assay of Rubisco active sites. Measurement of V$_{max}$ and K$_m$ for the carboxylase activity in nitrogen (N$_2$) or air were as described elsewhere (Bird *et al.* 1982). It was assumed that this preparation was fully active and 1 min assays were conducted in glass scintillation vials, fitted with caps and silicone rubber septa, in a final volume of 1 mL at 25 °C. Each determination utilized 10 different concentrations of bicarbonate, chosen to provide CO$_2$ (aq) between 0·80 and 82 μM, each with a specific radioactivity of 3·7 × 10^{10} Bq mol^{-1} and containing 375 nmols RuBP and 10 μL of the protein extract. The K$_m$ for the oxygenase activity was calculated from the relationship, K$_m$ (*carboxylase, Air*) = K$_m$ (*carboxylase, N$_2$*)·{1 + ([O$_2$]/K$_m$ (*oxygenase*)}, assuming an O$_2$ concentration of 265 μM. The V$_{max}$ for the oxygenase activity was derived from the equation, τ = [V$_{max}$ (*carboxylase*)/K$_m$ (*carboxylase*)]/[V$_{max}$ (*oxygenase*)/K$_m$ (*oxygenase*)], assuming a specificity factor (τ) of 110·5, previously measured in our laboratory (Galmes *et al.* 2005). The concentration of Rubisco active sites was determined using [^{14}C]CABP (Yokota & Canvin 1985). The means and standard deviations for four separate experiments are shown.

Graph: Solid line shows the specificity versus catalytic rate representing the least square, best-fit, inverse relationship for a variety of wild-type forms of Rubisco, according to the exponential equation defined by Zhu *et al.* 2004. Also shown are the corresponding values (○) for *Galdieria partita* (Uemura *et al.* 1997), *Griffithsia monolis, Nicotiana tabacum* (Whitney *et al.* 2001), *Triticum aestivum, Spinacea oleracea* (Zhu *et al.* 1998), *Oryza sativa* (Kane *et al.* 1994), *Synechococcus* PCC6301 (Read & Tabita 1994) and *L. gibertii* (above).

the rapid pace of bacterial reproduction and combines random mutagenesis with genetic selection. This permits access to 'sequence space' that has been ignored by the site-directed approaches and the potential evaluation of millions of mutations (Griffiths 2006). Parikh *et al.* (2006) engineered the Calvin cycle in *E. coli* so that active Rubisco was required for growth and then screened mutants with randomly mutated *rbc*L. Initial results are encouraging as these authors have identified a

number of mutants with apparently increased activity (Parikh *et al.* 2006). With this approach it will be essential to discriminate between strains with increased expression and increased activity. If successful, the most efficient Rubisco genes will become candidates for introduction into crop plants.

Recent studies have called into question the feasibility of attempts to improve Rubisco as a means of increasing plant productivity. As already mentioned, comparisons between specificity factor and the

corresponding catalytic rate of Rubisco from C_3 and C_4 plants and many algae, has led to the observation that Rubisco specificity and rate may be inversely related, as illustrated graphically by the inset to Table 1 (Zhu *et al.* 2004) and elsewhere (e.g. Bainbridge *et al.* 1995). Such a relationship has been explained as being due to tighter binding of the carboxylase reaction intermediate by higher specificity forms of Rubisco, leading to slower rates of turnover (Tcherkez *et al.* 2006). Whilst such constraints may limit the type of optimization achievable by protein engineering, there are many types of Rubisco with very different combinations of rate and specificity (many more of which remain to be discovered) and so the starting point for such approaches is broad (for a more extensive appraisal of this work, see Gutteridge & Pierce 2006; Griffiths 2006). A specific, inverse relationship between specificity and rate has been derived and used to explore the relationship between specificity factor and canopy photosynthesis, by means of computational analysis (Zhu *et al.* 2004). Surprisingly, it was shown that a hypothetical C_3 plant with a lower specificity but faster Rubisco could have a higher rate of carbon uptake at both current and elevated atmospheric CO_2. Even so, when experimentally determined combinations of Rubisco specificity and rate (chosen because they deviated from the idealized inverse relationship shown in the inset to Table 1) were modelled, significantly higher rates of carbon assimilation were also predicted (Zhu *et al.* 2004). In conclusion, this study highlighted the importance of considering (1) both specificity and rate in predicting the benefit of substituting Rubisco in crop species with those from other species, and (2) the value and limitations of predictive analyses in deciding the correct way forward.

RUBISCO AMOUNT

In plants grown in the field or with high or variable irradiance and ambient CO_2, the maximum photosynthetic rate is highly correlated with Rubisco content (Hudson *et al.* 1992; Lauerer *et al.* 1993). In contrast, under light-limiting conditions (Hudson *et al.* 1992; Quick *et al.* 1991) or at elevated CO_2 (Stitt *et al.* 1991), there is little or no correlation between Rubisco content and photosynthetic rate as predicted by the model of Farquhar *et al.* (1980). This suggests that under variable field conditions it may still be advantageous to increase Rubisco content or activity. Attempts to increase Rubisco content and thereby photosynthetic productivity by over-expressing the *rbc*S gene have failed and even resulted in decreased Rubisco content by co-suppression. However, Rubisco content on a leaf area basis has been increased in plants transformed with other transgenes (e.g. Pellny *et al.* 2004) and in semi-dwarf hexaploid wheat cultivars (Pyke & Leech 1985). Interpretation

of such results is equivocal, since other components may also have been altered.

Since Rubisco already accounts for up to half of the soluble leaf protein, and factors regulating its abundance may be complex (possibly being determined by cell or chloroplast volume; Pyke & Leech 1987) the value of attempts to further increase its concentration are questionable. On the contrary, in the face of increasing atmospheric CO_2, the case has been made for decreasing Rubisco content to enable N to be invested in other photosynthetic components (Nakano *et al.* 1997; Theobald *et al.* 1998). However, the potential benefits of this would only be realized under N-limited conditions and would be small (Parry *et al.* 2003*a*).

RUBISCO REGULATION

The activity of Rubisco is regulated to match the capacity for RuBP regeneration with the capacity for RuBP utilization (through carboxylation and oxygenation). Rubisco activity is modulated *in vivo* in two ways, either by the carbamylation of an essential lysine on the large subunit and its subsequent stabilization by a Mg^{2+}-ion to form an active ternary complex or by the binding of inhibitors before and/or after carbamylation to block the active site of the enzyme (Fig. 1*b*). Both of these processes are affected by the activity of another enzyme, Rubisco activase, which facilitates the activation of all forms of Rubisco. For this reason, Rubisco activase is an important target in attempts to understand and manipulate Rubisco activity. At moderately high temperatures, photosynthesis declines. This loss of activity is paralleled by a decline in the activation state of Rubisco, caused by the thermal inactivation of Rubisco activase. Attempts to sustain productivity at elevated temperatures by sustaining Rubisco activity are currently being pursued, either by over-expression of Rubisco activase, or by introduction of more stable forms of Rubisco activase (Feller *et al.* 1998; Crafts-Brandner & Salvucci 2000; Salvucci *et al.* 2001; Rokka *et al.* 2001). A combination of directed evolution and high-throughput screening has already identified thermotolerant forms of Rubisco activase (Kurek *et al.* 2003).

In many species in darkness and low light, carboxyarabinitol 1-phosphate (CA1P) is responsible for the low activity of Rubisco (Gutteridge *et al.* 1986). CA1P is released by Rubisco activase (Robinson & Portis 1988) after which it may be rendered noninhibitory by a specific, redox-modulated phosphatase (Holbrook *et al.* 1989; Heo & Holbrook 1999). Under photorespiratory conditions, accumulation of the potent inhibitor, pentadiulose bisphosphate (PDBP) can also decrease Rubisco activity (Kane *et al.* 1998; Kim & Portis 2004). Elucidation of the biosynthetic and degradative pathways of these

inhibitors is an important area for research (Andralojc et al. 1996, 2002; Martindale et al. 1997; Parry et al. 1999). Manipulating the abundance of inhibitors like CA1P and PDBP, by targeting their synthesis or degradation, offer further opportunities to modulate Rubisco activity and even to control the stability of Rubisco under stress, on account of the protective effect conferred by tight binding inhibitors (Mehta et al. 1992; Khan et al. 1999).

RuBP REGENERATION

Sustained photosynthetic CO_2 assimilation and photorespiration requires the continuous regeneration of RuBP. At high irradiances, or when the CO_2 partial pressure is relatively low (e.g. during water stress), RuBP is present in excess and the observed rate of CO_2 assimilation is limited by the rate of CO_2 incorporation catalysed by Rubisco. However, as the irradiance decreases (e.g. by shading or less favourable solar angles) or as the CO_2 partial pressure increases (atmospheric CO_2 has almost doubled since the Industrial Revolution and continues to rise) the rate of assimilation is increasingly limited by the amount of RuBP (Farquhar et al. 1980; von Caemmerer 2000). Although dependent upon leaf position, time of day, etc., both situations are significant and often occur simultaneously within a crop stand. Free-air CO_2 enrichment studies clearly demonstrate increased rates of CO_2 assimilation by existing crop species at levels of CO_2 likely to prevail by the middle of this century and, in the context of RuBP regeneration, highlight the need for increasing the capacity for RuBP regeneration relative to Rubisco carboxylation, if the potential benefits of increasing CO_2 are to be maximized (Long et al. 2004, 2006).

The regeneration of RuBP depends upon the concerted action of the other enzymes of the Calvin cycle (discussed at greater length by Raines 2003), the supply of ATP and NADPH from the light reactions of photosynthesis and the supply of PGA from the carboxylase and oxygenase activities of Rubisco (the latter in C_3 plants, via the photorespiratory cycle).

The rate of RuBP regeneration is limited by the slowest step in the process (in terms of total flux). Antisense expression in tobacco (Harrison et al. 1998) and computer-based modelling studies of the Calvin cycle (Poolman et al. 2000) have both highlighted sedoheptulose-1,7-bisphosphatase (SBPase) as being a likely rate limiting enzyme in this process. This was dramatically confirmed in transgenic tobacco either by expression of a cyanobacterial SBPase (Miyagawa et al. 2001) or by expression of Arabidopsis SBPase (Raines 2003; Lefebvre et al. 2005), both of which resulted in higher photosynthetic rates, increased leaf area and biomass (up to 1·3-fold with the Arabidopsis enzyme). In addition, antisense reduction in the expression of the Rieske FeS protein of the cytochrome

b_6/f complex indicates that this component may also limit RuBP regeneration by limiting the capacity of photosynthetic electron transport (Price et al. 1998). Thus, both SBPase and the cytochrome b_6/f complex show promise for providing the necessary enhancement of RuBP regeneration required by increasing atmospheric CO_2. Even greater enhancement of RuBP regeneration capacity may be required to complement the introduction of more active forms of Rubisco into crop species, in the face of rising CO_2.

CO₂ PUMP

There is also the potential to improve photosynthetic carbon fixation by increasing the (internal) concentration of CO_2 at the catalytic site of Rubisco. Atmospheric (or external) CO_2 does not have direct access to Rubisco but must pass through open stomata in the leaf epidermis and then diffuse into the mesophyll cells to the site of photosynthesis within the chloroplasts. There is a strong positive correlation between photosynthesis and internal CO_2 concentration, which is determined by the stomatal and mesophyll conductances (Bernacchi et al. 2002; Hetherington & Woodward 2003; Flexas et al. 2004). Substantial improvements to photosynthesis would follow from the introduction of mechanisms to increase the internal CO_2 concentration, independent of stomatal conductance. Concomitant selection for characteristics that maintain large stomatal conductances under stress conditions is also highly desirable (Lu et al. 1994).

Increasing the mesophyll conductance – and therefore CO_2 at the catalytic site – through biotechnology will not be achievable until the process is better defined, after which suitable approaches can be pursued. However, recent reports suggest the involvement of aquaporins (Uehlein et al. 2003; Hanba et al. 2004) and carbonic anhydrase (Moroney et al. 2001) in mesophyll CO_2 conductance. Thus, it is likely that mesophyll conductance can be improved in the near future.

C_4 and CAM plants as well as many photosynthetic micro-organisms have developed carbon concentrating mechanisms to increase the CO_2 concentration in the vicinity of Rubisco to such an extent that the oxygenase reaction is almost negligible (Cushman & Bohnert 2000). This has been achieved through a variety of mechanisms. For example, Kranz anatomy and segregation of the biochemistry in maize, enables CO_2 to be initially incorporated as C_4 acids (oxaloacetate and malate) in surrounding mesophyll cells, which are then transported into deeper layers containing the bundle sheath cells whose suberized cell walls are gas-impermeable. Thus when the C_4 acids are subsequently decarboxylated, virtually all the CO_2 released is recaptured by Rubisco. Attempts by hybridization to introduce sufficient C_4 characteristics
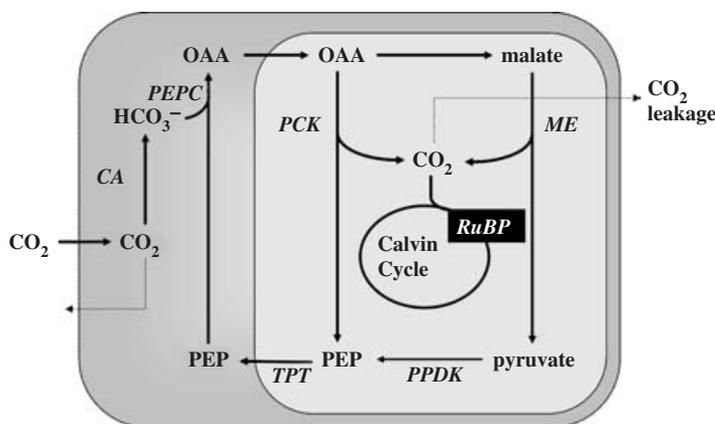
Fig. 2. Idealized scheme indicating the enzymes necessary to operate a single cell CO_2 concentrating mechanism. *CA*, carbonic anhydrase; *PEPC*, phophoenolpyruvate carboxylase; *PCK*, phospho*enol*pyruvatecarboxykinase, *ME*, malic enzyme; PPDK, pyruvate orthophosphate dikinase; *TPT*, triose phosphate translocator; OAA, oxalacetate; PEP, phopho*enol*pyruvate; RuBP, ribulose bisphosphate.

into C_3 plants to accomplish this ambitious goal have not yet succeeded (Brown & Bouton 1993). The International Rice Research Institute (IRRI) is currently screening 6000 wild rice varieties for characteristics of C_4 plants (http://www.checkbiotech.org/root/index.cfm?fuseaction = search&search = IRRI&doc_id=13263&start=1&fullsearch=0; verified 23/10/2006).

Other species (e.g. *Hydrilla verticillata*) operate a CO_2 pump within a single cell (Fig. 2). Elegant biotechnological approaches are seeking to exploit the introduction of this simpler system into a number of C_3 plants including rice, potato, tobacco and wheat (Leegood 2002). To be successful, this approach must achieve coordinated expression, targeting the gene products to the appropriate subcellular compartment, and appropriate regulation of all necessary enzymes and transporters involved in the pathway. This has yet to be achieved. A theoretical analysis has shown that, whilst the introduction of such a system could beneficially increase the CO_2 concentration at the catalytic site when energy was not limiting, it is an energy requiring process and so the benefits when photosynthesis is light limited are less certain (von Caemmerer 2003; Raines 2006). Nevertheless, such systems would be particularly advantageous in certain conditions, such as drought, where stomata are closed and low intercellular CO_2 leads to increased photorespiration (von Caemmerer 2003). However, the lack of success when key enzymes associated with this mechanism have been expressed singly or together in transformed plants (e.g. Hudspeth *et al.* 1992; Kogami *et al.* 1994; Gallardo *et al.* 1995; Gehlen *et al.* 1996; Ishimaru *et al.* 1997; Beaujean *et al.* 2001; Fukayama *et al.* 2001; Ku *et al.* 2000; Matsuoka *et al.* 2001; Hausler *et al.* 1999, 2001, 2002; Miyao 2003) indicates the additional need to provide

structural changes (Kranz anatomy) as well as the cell-specific expression of pivotal enzymes (Parry *et al.* 2005).

An even simpler approach involves the cyanobacterium *ict*B gene product. Whilst the exact role of this component, which is highly conserved amongst cyanobacteria, is not known, it is thought to be involved in concentrating CO_2 for utilization by Rubisco (Bonfil *et al.* 1998). Transgenic plants expressing *ict*B from *Synechococcus* and *Anabaena*, had faster photosynthetic rates than the wild-types under CO_2 limiting – but not at saturating – CO_2 concentrations (Lieman-Hurwitz *et al.* 2003). These changes in photosynthetic rates were reflected in increased biomass accumulation in the transgenic plants. Expression of the *ict*B gene product in crop plants appears to have the potential to increase photosynthetic carbon assimilation, particularly under drought when stomata are closed and low internal CO_2 results in increased photorespiration.

ENGINEERING PHOTORESPIRATORY METABOLISM

Recently, attention has been given to alternative strategies to overcome the limitations of Rubisco and its consequences. For example, the photorespiratory pathway (Figs 1*a* and 3) is a potentially important target – it is required to recover assimilated carbon in the form of 2-phosphoglycolate – but also causes the release of fixed carbon and nitrogen (as CO_2 and NH_3, respectively). Although the CO_2 can be, and the NH_3 invariably is, re-assimilated, energy is required for both processes (2 ATP and 1 NADPH equivalent per CO_2 and 1 ATP and 1 NADPH equivalent per NH_3). Indeed, the re-assimilation of NH_3 lost through photorespiration occurs at 5–10
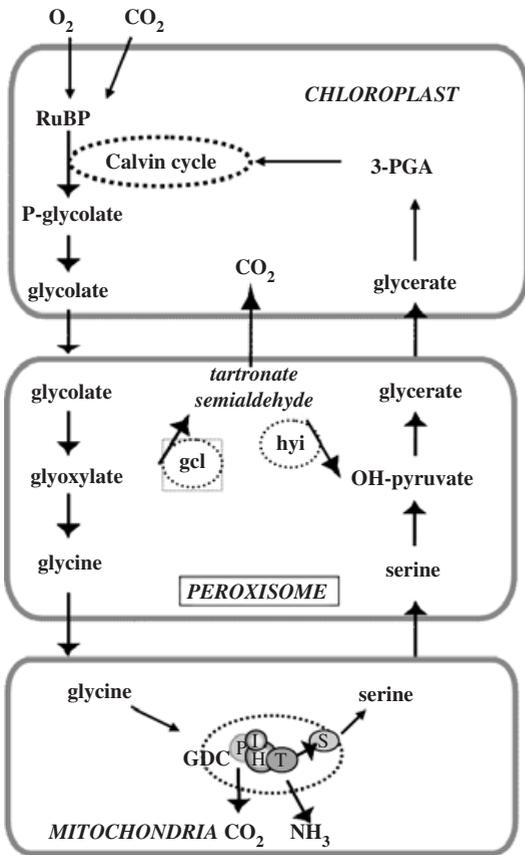
Fig. 3. A pathway for short circuiting photorespiratory metabolism, by-passing the conversion of glycine to serine (e.g. Parry *et al.* 2003*b*) by introducing glyoxylate carboligase (gcl) and hydroxypyruvate isomerase (hyi).

times the rate of NH_3 production through nitrate assimilation.

Strategies have been developed to short-circuit photorespiration, eliminating the need for photorespiratory ammonia assimilation, by removing the need for the conversion of glycine to serine (e.g. Carvalho *et al.* 2003; Parry *et al.* 2003*b*; Fig. 3). This approach uses two bacterial genes, *gcl* and *hyi*, which respectively encode glyoxylate carboligase (gcl), which converts glyoxylate to tartronate semialdehyde + CO_2 and hydroxypyruvate isomerase (hyi), which converts tartronate semialdehyde to hydroxypyruvate. Transgenic tobacco transformed with these two genes developed lesions on their leaves when grown under bright light and ambient concentrations of CO_2. A similar approach involving expression of glyoxylate carboligase and tartronate semialdehyde reductase in the chloroplast of Arabidopsis also resulted in leaf chlorosis (Hain *et al.* 2003; Bari 2004). The extent to which these phenotypes were the result of retaining

the existing photorespiratory pathway is unclear and warrants further investigation.

INTEGRATED APPROACHES

Whilst it may be tempting to focus exclusively on those components directly related to photosynthetic rate, this approach would be naïve. Considerable gains in carbon assimilation could be achieved simply by ensuring that the existing photosynthetic apparatus is used optimally. Often the available photosynthetic capacity is not fully utilized and can be in apparent excess; this may be caused by genetic or environmental factors. For example, there is compelling evidence that in some cultivars of wheat, post anthesis photosynthetic carbon assimilation is decreased by sink limitation (Reynolds *et al.* 2005). Significant increases in photosynthetic rate and yield could be achieved by removing such constraints, e.g. in the above case, by increasing grain number. There are huge potential benefits by breeding for (or manipulating other characteristics that allow) the existing photosynthetic capacity to be fully utilized. It is therefore essential that an integrated systems approach is adopted, combining the latest genomics resources and the new technologies in quantitative genetics, genomics and biomathematics with a better understanding of the interactions between crop plant genotypes and the growing-environment (Araus 2004).

CONCLUSIONS

Rubisco is a major limitation to photosynthetic CO_2 assimilation in C_3 plants. In principle, these limitations could be alleviated or overcome through genetic modifications and by selection. Improvements can be achieved by identifying and introducing a Rubisco with either higher catalytic rate or that was better able to discriminate between gaseous substrates, or both. Although there appears to be a negative correlation between these two factors, significant natural variation remains that could be exploited either directly or after protein engineering. However, a number of major technical obstacles must still be overcome before the benefits of this approach can be realized. An alternative, or complementary, approach is to increase the concentrations of CO_2 and/or RuBP, at the active site of Rubisco; in each case, this will require the coordinated expression, targeting and regulation of a 'suite' of suitable enzymes. Whilst such complex approaches are being developed, a number of single gene insertions (*ict*B, *SBPase* and *Reiske FeS subunit*) have all had encouraging results.

Significant increase in photosynthetic carbon assimilation could be achieved simply by ensuring that the existing photosynthetic capacity is more fully

exploited. This lost potential could be realized both by conventional breeding and by genetic manipulation.

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