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REVIEW ARTICLE

Manipulation of Rubisco: the amount, activity, function and regulation

M. A. J. Parry[1], P. J. Andralojc, R. A. C. Mitchell, P. J. Madgwick and A. J. Keys

Crop Performance and Improvement, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK

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Abstract

Genetic modification to increase the specificity of Rubisco for CO_2 relative to O_2 and to increase the catalytic rate of Rubisco in crop plants would have great agronomic importance. The availability of three-dimensional structures of Rubisco at atomic resolution and the characterization of site-directed mutants have greatly enhanced the understanding of the catalytic mechanism of Rubisco. Considerable progress has been made in identifying natural variation in the catalytic properties of Rubisco from different species and in developing the tools for introducing both novel and foreign Rubisco genes into plants. The additional complexities of assembling copies of the two distinct polypeptide subunits of Rubisco into a functional holoenzyme *in vivo* (requiring sufficient expression, post-translational modification, interaction with chaperonins, and interaction with Rubisco activase) remain a major challenge. The consequences of changing the amount of Rubisco present in leaves have been investigated by the use of antisense constructs. The manipulation of genes encoding Rubisco activase has provided a means to investigate the regulation of Rubisco activity.

Key words: Photosynthesis, *rbc*L *rbc*S, Rubisco, specificity factor, transgenic.

Introduction

Rubisco is the key enzyme responsible for photosynthetic carbon assimilation in catalysing the reaction of CO_2 with ribulose 1,5-bisphosphate (RuBP) to form two molecules of D-phosphoglyceric acid (PGA). It also initiates photorespiration by catalysing the reaction of oxygen, also with RuBP, to form one molecule each of phosphoglycolate and PGA. It is a complex enzyme and catalyses these reactions at rather slow rates. It constitutes some 30% of the total protein in many leaves for which reason it is of considerable interest in relation to the nitrogen nutrition of plants. Biochemists have shown much interest because of the catalytic mechanism, lack of specificity, regulation, and turnover aspects. Physiologists have been concerned because of the consequences of the properties of Rubisco for the gas exchange characteristics of photosynthetic tissues and because of the consequences of the amount of nitrogen tied up in the enzyme and its recycling upon senescence of leaves.

The significance of the inhibition of photosynthesis in many organisms by oxygen (Warburg, 1920; Ogren, 1984) became evident with the discovery of the oxygenation of RuBP and consequent stimulation of photorespiration (Bowes *et al.*, 1971; Ogren and Bowes, 1971; Lorimer, 1981). Increased CO_2 concentration diminished the inhibitory effect of oxygen on photosynthesis and this also finds explanation in the properties of Rubisco as a catalyst; the carboxylation and oxygenation reactions are catalysed at the same active site on the enzyme and CO_2 and O_2 are competitive substrates (Andrews and Lorimer, 1978). Evolution in various environments, usually hot or deficient in available inorganic carbon (CO_2, HCO_3^-, CO_3^{2-}), has resulted in photosynthetic organisms that can concentrate CO_2 in cells or organelles containing their Rubisco. Terrestrial plants with a CO_2 concentrating mechanism, C_4 plants, have much higher rates of photosynthesis in warm conditions at high light intensities than C_3 plants that have no CO_2 concentrating mechanism (Hatch, 1976; Edwards and Walker, 1983) Also, C_3 plants in atmospheres with low O_2, or elevated CO_2, assimilate CO_2 and grow more quickly than in ambient conditions, provided that nutrients and temperature are not limiting. Mechanistic models (Farquhar *et al.*, 1980; Collatz *et al.*,

[1] To whom correspondence should be addressed. Fax: +44 (0)1582 763010. E-mail: martin.parry@bbsrc.ac.uk

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1990) of photosynthetic gas exchange based upon Rubisco kinetics have proved very successful in representing the effects of light, temperature and atmospheric composition on assimilation of carbon by plants.

Genetic manipulation of Rubisco to double its specificity for CO_2 would theoretically increase $A_{(max)}$ by perhaps 20%, and photosynthesis at sub-saturating light intensities would also be improved (Reynolds *et al.*, 2000). Consequently, it has been accepted by many that manipulating Rubisco to decrease the inhibitory effect of oxygen and its competitive involvement in reaction with RuBP, as opposed to reaction with CO_2, is a worthwhile target to increase the productivity of plants.

Essential to the activity of Rubisco is the carbamylation of an active site lysine residue (Lorimer and Miziorko, 1980). The extent of this carbamylation depends on the concentrations of CO_2 and Mg^{2+}, the absence from the non-carbamylated sites of certain phosphorylated compounds and particularly RuBP, and the activity of an enzyme called Rubisco activase (Portis, 1992). The activity of this latter enzyme is controlled by the ratio of ATP/ADP (Streusand and Portis, 1987) and redox potential, in effect by light intensity (Zhang *et al.*, 2002). Rubisco activase also facilitates the removal of 2-carboxyarabinitol 1-phosphate (CA1P) from carbamylated sites of Rubisco (Robinson and Portis, 1988). CA1P is a tight binding naturally occurring inhibitor of Rubisco which is present bound to the enzyme in many species at night. The significance of the presence of CA1P is subject to some debate. It could be a regulator of activity at low light intensities, but may be more important in protecting Rubisco from degradation by proteases (Khan *et al.*, 1999) when the natural substrate, RuBP, is present at low concentrations. Manipulation of the activity of Rubisco activase or of the synthesis and breakdown of CA1P may be of value. These aspects are explored. The large amounts of Rubisco in leaves has had consequences for the development of research on this enzyme (Kung, 1976; Ellis, 1979). There is so much present that not only can it sometimes be seen as crystals in the chloroplast stroma (Steer *et al.*, 1966) but it also crystallizes very readily from relatively crude extracts (Chan *et al.*, 1972). It has been estimated to be normally present at a concentration of 240 mg ml^{-1} in the stroma of chloroplasts (Wildner, 1981) and constitutes some 30–50% (Kung, 1976; Ellis, 1979) of the soluble protein in the leaves of C_3 plants and a very high percentage of the total protein in leaves. Nevertheless, particularly in bright light it may exert considerable limitation over the rate of CO_2 fixation (Hudson *et al.*, 1992). The fate of Rubisco during leaf senescence has been intensively studied and the nitrogen from this source has been shown to be extensively reutilized in the synthesis of proteins in seeds and perennating organs (Dalling *et al.*, 1976; Peoples *et al.*, 1983; Millard and Catt, 1988). Thus the function of Rubisco as a store of nitrogen has resulted in much speculation and research.

The genes for the Rubisco polypeptide subunits from many species have been cloned and sequenced, as have genes for Rubisco activase polypeptides. Furthermore, the crystal structure of Rubisco from several species and the extensive homology of amino acid sequences has allowed the advance of genetic manipulation, protein engineering and transformation experiments (Spreitzer and Salvucci, 2002). One problem with the manipulation of Rubisco in higher plants is that it is composed of eight large and eight small polypeptide subunits and that the genes for the small subunit are in the nuclear genome (Kawashima and Wildman, 1972), but those for the large subunit are encoded in the chloroplast genome (Chan and Wildman, 1972; Ellis, 1981). Problems have also been encountered in assembling large and small subunits into the hexadecameric holoenzyme following manipulation (Gutteridge and Gatenby, 1995). Many protein engineering projects have, therefore, been conducted using cyanobacterial, algal and bacterial Rubiscos for which assembly into the holoenzyme is less problematic. Mutagenesis *in vitro* has been used to make changes to DNA encoding both large and small subunits. The effects of such changes on the expressed protein have been used to increase understanding of the catalytic properties of Rubisco and the extent to which the specificity and activity can be altered. The use of antisense constructs to alter the amount of expression of Rubisco has been used both to determine whether the amount of Rubisco in plants can be decreased to save nutrient nitrogen and to determine the extent to which Rubisco controls the rate of photosynthesis. Transgenic plants expressing altered amounts of Rubisco activase or Rubisco activase polypeptides with mutations or from different species have also increased the understanding of the details of Rubisco activation. This review aims to examine the information obtained by all these forms of manipulation as well as the prospects, and appropriate objectives, for future experiments.

Manipulating specificity factor

Directed mutagenesis

One approach to crop improvement is the identification of amino acid residues that confer key catalytic properties, such as the specificity factor (τ, expressed as the ratio of V_cK_o/V_oK_c) that is an important diagnostic parameter widely used as an indication of overall efficiency. Such studies have been facilitated by the availability of high resolution 3-D structures for Rubisco from both microorganisms (e.g. *Rhodspirillum rubrum*, Schneider *et al.*, 1986; chlamydomonas (*Chlamydomonas rheinhardtii*), Taylor *et al.*, 2001; *Galderia patita*, Sugawara *et al.*, 1999; cyanobacteria, Newman and Gutteridge, 1993) and

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higher plants (spinach (*Spinacea oleracea*), Andersson *et al*., 1989; Andersson, 1996; tobacco (*Nicotiana tabacum*) Chapman *et al*., 1988). The initial studies of Rubisco structure/function used *R. rubrum* Rubisco genes since they could be expressed in *E. coli* to generate active Rubisco (Somerville and Somerville, 1984; Gutteridge *et al*., 1984). This enabled potential substitutions to be evaluated *in vitro*, but it is difficult to relate engineered changes in structure of the homodimeric *R. rubrum* enzyme (together with the resulting subtle changes in kinetic characteristics) to Rubiscos with the more complex hexadecameric structure found in crop plants. Unfortunately, the expression in *E. coli* of genes for the hexadecameric Rubisco from crop plants (e.g. wheat (*Triticum aestivum*) and maize (*Zea mays*) (Gatenby *et al*., 1981; Bradley *et al*., 1986)) did not yield active enzyme. This is probably attributable to mismatches between the higher plant Rubisco and the host chaperone system involved in the assembly of holoenzyme (Gutteridge and Gatenby, 1995). Despite these limitations, considerable insight into the structure/function relationships of hexadecameric forms of Rubisco have been gleaned, either from prokaryotes such as cyanobacteria that can be expressed and assembled into an active holoenzyme in *E. coli* (Gatenby *et al*., 1985; Gatenby and Ellis, 1990) or following chloroplast transformation and classical genetics with the green alga chalmydomonas (Spreitzer, 1993, 1999). The Rubisco from these sources has over 80% amino acid and nucleotide homology with Rubiscos in crop plants.

One major objective for Rubisco manipulation has been to alter the discrimination between CO_2 and O_2 (i.e. to alter the specificity factor, τ). Altering specificity is a difficult goal since neither gas binds directly to the active site: the formation of the enediol of RuBP, with which CO_2 or O_2 react directly, is common to both the caboxylation and oxygenation reactions. Several residues have been shown to influence specificity, but, in many cases, the effect must be indirect since many of these are not close enough to interact directly with the substrate or reaction intermediates.

Many attempts to manipulate the specificity factor have focused on the C-terminal loop 6 region of the large subunit. Chen and Spreitzer (1989) working with chlamydomonas provided the first clue that this region was important to specificity for the gaseous substrates. Mutation of valine 331 of the large subunit to alanine decreased the specificity factor by almost 40%. Loop 6, at the mouth of the alpha/beta barrel, is an integral part of the catalytic site, the analysis of various 3-D structures suggests that the loop is flexible in the initial stages of carboxylation but then, early in catalysis, folds or slides to occlude the active site. This closed conformation of loop 6 is maintained by the residues of the C-terminal tail (Knight *et al*., 1990) and the N-terminal loops (Newman and Gutteridge, 1993) of the large subunit. Valine 331 at the N terminal end of loop 6 is the 'spring' responsible for the movement of the loop (Newman and Gutteridge, 1993). Characterization of a loop 6 deletion mutant has demonstrated that loop 6 is vital to normal processing of the enediolate intermediate (Larson *et al*., 1995). Many of the loop 6 residues are conserved, but only lysine 334 at the apex of the loop has ionic interactions with the transition state analogue 2-carboxyarabinitol 1,5-*bis*phosphate (CABP). During carboxylation, lysine 334 polarizes both oxygens of CO_2, thereby enhancing the electrophilic status of the carbon atom of CO_2, promoting electrophilic attack by CO_2 on C2 of the enediol to form the 2-carboxy, 3-keto intermediate (Cleland *et al*., 1998). Although site-directed mutants of lysine 334 catalysed enediolate formation they were unable to catalyse the reaction of the enediolate with CO_2 or form a stable complex with CABP (Soper *et al*., 1988; Hartman and Lee, 1989; Gutteridge *et al*., 1993). Thus lysine 334 is thought to play a specific role in stabilizing the transition state intermediates of both the carboxylation and oxygenation reactions, thereby facilitating the reaction between the gaseous substrate and the enediolate.

Within loop 6 there are some differences between the residues found in model systems and crop plants. These residues have been mutated either individually or together in several studies and analysis of the resultant enzymes has confirmed that loop 6 plays a role in determining the specificity factor of Rubisco (Table 1). Changing the chlamydomonas large subunit residue leucine 326 together with methionine 349 for the corresponding higher plant residues, isoleucine 326 and leucine 349, by *in vitro* mutagenesis and chloroplast transformation caused a 21% decrease in specificity factor (Zhu and Spreitzer, 1996). In tobacco, changing leucine 335 for valine decreased both the specificity and carboxylation rates to 25% of the wild-type values (Whitney *et al*., 1999). Similarly in cyanobacteria, substitution of leucine 332 with methionine, isoleucine, valine, threonine, or alanine decreased the specificity factor by as much as 67% (Lee *et al*., 1993). Although replacement of alanine 340 by histidine increased the specificity factor by 13%, this was accompanied by a 33% fall in V_c, replacement of alanine 340 by asparagine increased the specificity factor by 9% and V_c by 19% (Madgwick *et al*., 1998). The specificity factor of Rubisco from higher plants is greater than that from cyanobacteria. When four consecutive residues from cyanobacterial Rubisco were substituted for the analogous cassette of residues found in higher plants, the specificity was significantly increased (Parry *et al*., 1992; Kane *et al*., 1994). However, since these increases were relatively small (5–10%) they could not fully account for the difference in kinetic properties between cyanobacterial and higher plant enzymes. None of the residues in loop 6 interact directly with CABP and so any effect of loop 6

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Table 1. *Per cent increase or decrease in relative specificity factor of cyanobacterial, chlamydomonas and tobacco Rubisco in which residues within loop 6 and helix 6 have been substituted by site-directed mutagenesis and yielded assembled active Rubisco*

The sequence number preceded by the single-letter representation for the wild-type residue at that position is followed by the single letter representation for the replacement.

Mutation	% Change	Species	Authors
V 331A	–42	chlamydomonas	Chen *et al*., 1991
V331G	–64	cyanobacteria	Gutteridge *et al*., 1993
V331A	–46	cyanobacteria	Gutteridge *et al*., 1993
V331L	–13	cyanobacteria	Gutteridge *et al*., 1993
V331M	–71	cyanobacteria	Gutteridge *et al*., 1993
K334R	–99	cyanobacteria	Gutteridge *et al*., 1993
L335M	–54	cyanobacteria	Lee *et al*., 1993
L335I	–67	cyanobacteria	Lee *et al*., 1993
L335V	–60	cyanobacteria	Lee *et al*., 1993
L335T	–35	cyanobacteria	Lee *et al*., 1993
L335A	–55	cyanobacteria	Lee *et al*., 1993
L335V	–75	*N. tabacum*	Whitney *et al*., 1999
D338E	+4	cyanobacteria	Parry *et al*., 1992
K339R	0	cyanobacteria	Parry *et al*., 1992
K339P	–3	cyanobacteria	Read and Tabita, 1994
A340L	–8	cyanobacteria	Read and Tabita, 1994
A340E	–18	cyanobacteria	Parry *et al*., 1992
A340H	+13	cyanobacteria	Madgwick *et al*., 1998
A340N	+9	cyanobacteria	Madgwick *et al*., 1998
A340D	+5	cyanobacteria	Madgwick *et al*., 1998
A340G	–6	cyanobacteria	Madgwick *et al*., 1998
A340R	+3	cyanobacteria	Madgwick *et al*., 1998
A340Y	+12	cyanobacteria	Madgwick *et al*., 1998
S341I	–1	cyanobacteria	Parry *et al*., 1992
S341M	+8	cyanobacteria	Read and Tabita 1994
T342I	–36	chlamydomonas	Chen *et al*., 1991
T342I	–18	cyanobacteria	Read and Tabita, 1994
	–7		Gutteridge *et al*., 1993
T342L	–7	cyanobacteria	Gutteridge *et al*., 1993
T342M	–11	cyanobacteria	Gutteridge *et al*., 1993
T342V	–23	cyanobacteria	Read and Tabita, 1994
DKAS338–341 EREI	+7	cyanobacteria	Parry *et al*., 1992
DKAS338–341 ERDI	+3	cyanobacteria	Gutteridge *et al*., 1993
	+5		Kane *et al*., 1994
L326I and M349L	–21	chlamydomonas	Zhu and Spreitzer, 1996

mutations on the reactivity of the enediolate intermediate mediated by lysine 334 must have been indirect. Interactions between residues distant from the catalytic site must also be important. In cyanobacteria the ε-amino group of lysine 334 may not be optimally positioned within the active site, since mutation of other residues at the C-terminal end of loop 6 resulted in a 3–13% increase in the specificity factor (Parry *et al*., 1992; Gutteridge *et al*., 1993; Madgwick *et al*., 1998). In these instances, the improved specificity was attributed to repositioning the ε-amino group of lysine 334. Subsequent studies focusing on the adjacent alpha helix (helix 6) confirmed the importance of this region to the specificity factor (Ramage *et al*., 1998).

Structural studies have revealed that the conformation of loop 6 is maintained by the residues of the C-terminal tail (Knight *et al*., 1990) and of the N-terminal loop (Newman and Gutteridge, 1993). Portis (1990) demonstrated the importance of the C-terminus of the large subunit to catalytic function. Removal of the C-terminal residues of the spinach or chlamydomonas Rubisco large subunit by carboxypeptidase-A reduced carboxylase activity by 60–70%. Similarly, mutants in which the C-terminus of the large subunit was truncated lost catalytic activity and were no longer able to bind CABP (Gutteridge *et al*., 1993). Variations in length and charge of the C-terminus were found to have little effect on specificity factor at 35 °C. However, the specificity factor at 10 °C for a mutant with a two amino acid (aspartate–lysine) extension at the C-terminus was at least 10% higher than the wild type Rubisco (Zhu *et al*., 1998). It was suggested that the extended C-terminus established additional interactions with the protein surface, which altered specificity (Zhu *et al*., 1998). The eight large subunits of hexadecameric Rubisco occur as four homodimers in the holoenzyme. The side chain of lysine 128 is sandwiched between loop 6 and the C-terminal tail of the other large subunit of the dimeric pair. Examination of the 3-D structure of the large subunits (Bainbridge *et al*., 1998) reveals that the ε-amino group of lysine 128 is capable of forming hydrogen bonds with the backbone carbonyls of loop 6 (valine 331 and glycine 333) and with C-terminal phenylalanine 467, close to the apex of loop 6. Substitution of lysine 128 loosened the binding of CABP to activated Rubisco (Bainbridge *et al*., 1998). All substitutions for lysine 128 were detrimental, decreasing both specificity factor and catalytic activity. Disruption of hydrogen bonds between the ε-amino group and backbone carbonyls of loop 6 may cause the ε-amino group of lysine 334 to assume a different position within the catalytic site, impairing its ability to polarize the two oxygens of CO_2, thereby decreasing its potency as an electrophile. In addition, asparagine 123 on the same loop interacts directly with CABP; mutation of the equivalent residue in *R. rubrum* to glycine decreased the specificity factor more than 10-fold and drastically decreased $K_{\text{cat carboxylation}}$ to 1% of wild type (Chène *et al*., 1992; Soper *et al*., 1992). The region around lysine 128 is highly conserved. Changing lysine 128 to arginine, glycine, asparagine, histidine or glutamine did not cause a major disruption to the tertiary structure of the large subunit and so alterations in kinetic parameters are likely to result from disruption in the local environment of residue 128 rather than from gross structural changes.

Although providing valuable information on the relationship between structure and function, engineering cyanobacterial and algal Rubiscos has so far failed to produce an enzyme even 'as good' as those already found in crop plants. Considerable increases in the specificity factor of Rubisco of crop plants could be achieved by

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Table 2. *Manipulation of Rubisco in tobacco*

The symbol in the protein and activity columns indicate whether (+) or not (–) protein or Rubisco activity were found in those studies.

Introduced *rbc*L or *rbc*S	Transformation approach	Protein	Activity	Authors
Tobacco *rbc*L	Nuclear	+	+	Kanevski and Maliga, 1994
Tobacco *rbc*S	Chloroplast	+	+	Whitney *et al*., 2001
*Chromatium vinosum rbc*L	Nuclear	–	–	Madgwick *et al*., 2002
Cyanobacteria *rbc*L	Nuclear	–	–	Madgwick *et al*., 2002
Cyanobacteria *rbc*L	Cloroplast	–	–	Kanevski *et al*., 1999
*Galdieria sulphuraria rbc*L and *rbc*S	Chloroplast	+	–	Whitney *et al*., 2001
*Phaeodactylum tricornutum rbc*L and *rbc*S	Chloroplast	+	–	Whitney *et al*., 2001
*Helianthus annuus rbc*L	Chloroplast	+	+	Kanevski *et al*., 1999
*R. rubrum rbc*M	Chloroplast	+	+	Whitney *et al*., 2001

exploiting the natural variation in the catalytic properties of Rubisco isolated from different species. The highest reported value for Rubisco specificity factor is 238, found in the red alga *Galdieria partita* (Uemura *et al*., 1996) which is almost 3-times greater than that reported for Rubisco from most crop plants (Parry *et al*., 1989; Read and Tabita, 1994). Introduction of both subunits of a foreign Rubisco may not be necessary as, in some cases, it is possible to assemble subunits from different species to obtain functional holoenzyme. Expression in *E. coli* of the small subunit gene, *rbc*S, from two eukaryotic marine organisms, *Cylindrotheca* sp. N1 and *Olisthodiscus luteus*, together with the gene encoding the large subunit, *rbc*L, from cyanobacteria has generated functional holoenzyme (Read and Tabita, 1992). The specificity factor for both hybrid enzymes was increased nearly 60% relative to the cyanobacterial Rubisco. By contrast, although expression in *E. coli* of *rbc*S from rice, tobacco or wheat, together with the *rbc*L from cyanobacteria generated functional holoenzyme (Wang *et al*., 2001), the specificity factor of some of the hybrid enzymes was much lower than that of cyanobacterial Rubisco (Wang *et al*., 2001). Evidently, although the small subunits are remote from the active site, they can nevertheless still affect key kinetic characteristics like the specificity factor. Whilst these results confirm that hybrid Rubiscos may be catalytically competent they also highlight the difficulty of accurately predicting the outcome of such manipulations.

Transforming higher plant Rubiscos

Recent advances in chloroplast transformation have circumvented many of the previous obstacles to alter higher plant Rubisco. Moreover this approach allows the consequences for leaf photosynthesis and productivity to be determined. For example, in tobacco changing the loop 6 residue leucine 335 for valine decreased both specificity factor and carboxylation rates to 25% of the wild-type values; consequently, the plants were unable to survive without elevated CO_2 (Whitney *et al*., 1999). Further mutations of this type not only have the potential to improve the specificity factor, but, more importantly, allow analysis of consequential changes on the physiological properties of the whole plant.

Nuclear transformation has been used to relocate the plastid *rbc*L gene to the nucleus (Table 2). *Agrobacterium*-mediated transformation of tobacco lacking the chloroplast *rbc*L with the *rbc*L coding region preceded by a plastid targeting sequence was able to supply the defective plastids with fully functional Rubisco (Kanevski and Maliga, 1994). Conversely, although *rbc*S relocated to the tobacco plastid genome folded correctly and assembled into active holoenzyme, it contributed less than 1% of the total small subunits in the holoenzyme. The scarcity of the transplastomic small subunits may result from inefficient translation or assembly, although the assembled small subunits were as stable as the native counterparts (Whitney and Andrews, 2001*a*). Of possible significance is the demonstration that sequences downstream of the translation initiation codon are important determinants of translation efficiency in chloroplasts (Kuroda and Maliga, 2001).

Models for photosynthesis suggest that at elevated CO_2 and temperatures below 25 °C Rubisco from the photosynthetic bacterium *Chromatium vinosum* should outperform higher plant Rubiscos (Bainbridge *et al*., 1995). However, attempts to use nuclear transformation to introduce the *C. vinosum rbc*L into Rubisco-deficient tobacco were not successful. Although the *C. vinosum rbc*L was transcribed into mRNA, no *C. vinosum* large subunits were detectable (Madgwick *et al*., 2002). Similarly, lines in which the native *rbc*L of tobacco was replaced with the cyanobacterial *rbc*L by chloroplast transformation had no large subunit protein or enzyme activity although mRNA was produced (Kanevski *et al*., 1999). The failure to recover a fully active enzyme could be caused by incompatibility between the large subunits and the small subunits or by inability of the foreign Rubisco subunits to fold or assemble efficiently in the plastid. Further attempts that avoided the problem of assembling hybrid enzymes involved introduction of both *rbc*L and *rbc*S operons of *Galdieria sulphuraria* and *Phaeodactylum tricornutum* into the inverted repeats of the

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plastid genome of tobacco (Whitney *et al*., 2001). Whilst the transgenes directed the synthesis of transcripts in abundance, the subunits of these foreign Rubiscos were insoluble, indicating problems with folding or assembly. In addition, the accumulation of large amounts of insoluble protein decreased the amount of tobacco Rubisco, CO_2 assimilation, and growth.

Greater promise has been shown by tobacco lines in which the native *rbc*L was replaced with *rbc*L from another higher plant, sunflower (*Helianthus annuus*), by means of chloroplast transformation, which produced a catalytically active enzyme composed of sunflower large subunits and tobacco small subunits (Kanevski *et al*., 1999). Whilst the specificity factor of this hybrid enzyme was similar to that of wild-type tobacco it had decreased affinities for both CO_2 and ribulose *bis*phosphate and greatly decreased activity. The performance of a further line that expressed a chimeric (sunflower–tobacco) large subunit was similar to that of the other hybrid enzyme (Kanevski *et al*., 1999). Furthermore, Whitney and Andrews (2001*b*) were able successfully to replace the native *rbc*L of tobacco with *rbc*M from *R. rubrum* by chloroplast transformation and recover catalytically active Rubisco. The ability to recover active Rubisco protein probably reflects the simple dimeric structure of the holoenzyme and consequential simplicity of subunit assembly. Consistent with the kinetic properties of the *R. rubrum* Rubisco (whose specificity factor is very small) survival of these lines required elevated CO_2.

Clearly, the introduction of a high specificity factor Rubisco into crop plants remains a realistic goal. Modest changes in key catalytic properties achieved through small changes in *rbc*L sequences may have considerable significance to the whole plant (Sage, 2002). Such differences occur naturally amongst higher plants with high overall sequence homology. Further technological advances will expedite progress. These include developing chloroplast transformation techniques for the major crop species and overcoming the additional complexities of sufficient expression, post-translational modification, interaction with chaperonins and assembly (Gatenby and Ellis, 1990; Spreitzer and Salvucci, 2002). This remains a major challenge.

Selection

Even prior to the discovery of the oxygenase activity of Rubisco (Bowes *et al*., 1971) there had been attempts to manipulate the specificity factor of Rubisco by selection. Such selection relied on maintaining plants at, or slightly above the compensation point; any plant with relatively low rates of photorespiration should thrive whereas plants with relatively high rates of photorespiration should die (Menz *et al*., 1969; Cannell *et al*., 1969). The method is very simple and offers the possibility of screening very large populations. The discovery of the oxygenase activity stimulated further attempts to screen both different genotypes (Smith *et al*., 1976; Nasyrov, 1978) and induced mutants (Medrano and Primo-Millo, 1985; Somerville and Somerville, 1986). However, in higher plants the method was not effective at selecting genotypes with increased specificity factor, but did identify some genotypes with improved capacity for dry matter accumulation (Medrano *et al*., 1995) because of other characteristics.

Investigations with photosynthetic bacteria exploit the potential to screen very large populations (e.g. *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (Paoli and Tabita, 1998); cyanobacteria (Ogawa *et al*., 1994)). However, the potential of the unicellular green alga chlamydomonas in selection systems has been studied most. Changes in photosynthesis have been directly linked to alterations in the Rubisco large subunit gene (Spreitzer, 1993). Mutants were selected by requirement for acetate as a carbon source and then revertants that were no longer acetate-dependent were selected and characterized (Chen and Spreitzer, 1989; Chen *et al*., 1991; Thow *et al*., 1994; Spreitzer *et al*., 1995). Some of the revertants restored the gene sequence to the wild-type sequence while some were pseudo-revertants, with a second mutation that partly restored the ability to photosynthesize. The interpretation of these results was based on the crystal structures for tobacco and spinach Rubisco available at the time; now that the *C. reinhardtii* crystal structure is available (Taylor *et al*., 2001), this will aid both interpretation and future work in this area. These results, like those from site-directed mutants, demonstrate how alterations in amino acids remote from the active site of an enzyme can play a significant role in the stability and function of Rubisco. However, in none of the revertants was the specificity factor greater than that of the wild type. Nevertheless, such non-directed approaches have greatly increased understanding about certain regions involved in catalysis.

In many cases, photosynthetic mutants have been isolated that are found to have mutations in genes that encode proteins involved in the control of Rubisco expression, rather than within Rubisco itself. The ability of such approaches to mutate and screen very large populations fully justifies their inclusion in future research projects.

Amount

The first successful genetic manipulation of the amount of Rubisco in a higher plant was by transformation of tobacco with a construct containing an antisense *rbc*S sequence (Rodermel *et al*., 1988). This decreased the amount of Rubisco via a decrease in the level of endogenous *rbc*S transcript (Jiang *et al*., 1994). Since then, the same antisense approach has been used to decrease the amount of Rubisco in tobacco (Hudson *et al*., 1992), the C_4 plant *Flaveria bidentis* (Furbank *et al*., 1996), rice (Makino

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et al., 1997) and wheat (Mitchell *et al.*, unpublished results). There have been several attempts to increase Rubisco content by overexpressing the *rbc*S gene, but these have failed, often resulting in decreased Rubisco content by cosuppression. Increases in Rubisco content on a leaf area basis have been observed in plants transformed with transgenes aimed at other targets (Pellny *et al.*, 2002). In such cases, interpretation is equivocal, since other components may also have been altered (such as chlorophyll content in the example cited).

Effects on physiology of decreasing Rubisco content

Much of the physiological work on antisense-*rbc*S tobacco lines has been previously reviewed (Stitt and Krapp, 1999; Stitt and Schulze, 1994). Nearly all the results serve to confirm the existing hypotheses concerning the degree to which C_3 photosynthesis is limited by Rubisco content as represented in the model of Farquhar *et al.* (1980). Thus, decreasing Rubisco content decreased photosynthesis nearly proportionately at ambient CO_2 and high light (Hudson *et al.*, 1992; Lauerer *et al.*, 1993). By contrast, there was little or no effect of small reductions under light-limiting conditions (Hudson *et al.*, 1992; Quick *et al.*, 1991) or at elevated CO_2 (Stitt *et al.*, 1991). Growth at low N supply lowers photosynthetic capacity and extremely low N supply results in photosynthesis being saturated by quite low growth light intensities. Thus decreased Rubisco content increasingly limits photosynthesis at lower N supplies (Quick *et al.*, 1992). Decreased Rubisco content decreases the sink for electrons and thus photoprotective mechanisms are induced at lower light intensities (Schoefs *et al.*, 2001). Transformants of the C_4 plant *Flaveria bidentis* with lower Rubisco content had lower photosynthetic rates, despite the high CO_2 concentration in the bundle sheath (Furbank *et al.*, 1996). The amount of Rubisco in wild-type *Flaveria* plants must therefore be close to the point at which it would limit photosynthetic capacity. An unexpected result of lowering Rubisco content by antisense *rbc*S was that stomatal conductance was greater for a given photosynthetic rate, thus giving a higher internal CO_2 concentration in all species (Hudson *et al.*, 1992; Makino *et al.*, 1997; Stitt *et al.*, 1991; von Caemmerer *et al.*, 1997). There were differences in findings on whether a constant ratio to external CO_2 remained (Hudson *et al.*, 1992; Stitt *et al.*, 1991), but in any case the effect would be expected to lower the water-use efficiency of photosynthesis, and this is consistent with the greater C isotope discrimination seen in the transgenic plants (Hudson *et al.*, 1992; von Caemmerer *et al.*, 1997).

Possible benefits of decreasing Rubisco content under elevated CO_2

It has been suggested that the increasing atmospheric CO_2 concentration makes a reduction in Rubisco amount desirable. This is because it becomes increasingly in excess for a given light environment as compared to other photosynthetic components which increasingly limit light-saturated photosynthesis at high CO_2 (Makino *et al.*, 1997; Mitchell *et al.*, 2000; Theobald *et al.*, 1998). This depends on the extent to which acclimation to elevated CO_2 occurs to redress this balance. Excess Rubisco is only a problem when the resources invested in it could be usefully deployed elsewhere, for example, when growth is N-limited. It now seems clear that the reduction in Rubisco content at elevated CO_2 occurs only when there is demand for N elsewhere in the plant (Stitt and Krapp, 1999), which fits with this view. When N content of cereal leaves is decreased by low N supply or senescence, Rubisco decreases more than other photosynthetic components (Nakano *et al.*, 1997; Theobald *et al.*, 1998). In some cases acclimation to elevated CO_2 simply involves earlier leaf senescence that also has the net effect of a relatively larger decrease in Rubisco content. However, this acclimation is usually slow and incomplete (Medlyn, 1996; Sage, 1994). Nevertheless, decreasing the Rubisco content would increase the N-use efficiency both at elevated CO_2 and even at current CO_2 concentrations in moderate light environments (Mitchell *et al.*, 2000).

No evidence of greater efficiency was found for tobacco antisense lines at elevated CO_2 (Masle *et al.*, 1993; Quick *et al.*, 1992). However, rice plants transformed with a construct with an antisense *rbc*S gene driven by the endogenous *rbc*S promoter (in contrast to the constitutive promoters used in other studies) did show increased photosynthetic rate at high CO_2 concentration for a given leaf N content (Makino *et al.*, 1997). However, the benefit became less at lower leaf N content, so there may be less effect under N-limiting conditions. The growth of the transgenic plants was not greater than the wild type under conditions of saturating CO_2 concentration (Makino *et al.*, 2000), but it is not clear whether N supply was limiting growth in this experiment. However, interesting traits associated with decreased Rubisco were identified, including greater allocation of N to leaves and delayed leaf senescence.

Future manipulation of Rubisco amount

There remains a case for continued attempts to decrease the Rubisco content of crops for nutrient-limited conditions as atmospheric CO_2 concentration increases. However, an important agronomic goal is to increase N uptake by crops during periods of high N supply following fertilizer application before it is lost into the environment. Since high plant N status suppresses N uptake (King *et al.*, 1993), uptake may be stimulated by increasing N demand. In many crop species during vegetative growth, N is stored primarily as increased photosynthetic capacity in leaves, with a proportionally greater increase in Rubisco content.

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There may therefore be a case for increasing photosynthetic capacity in crops under conditions of high N supply to maximize storage and decreasing it at low N supply to maximize N-use efficiency. These changes could be brought about using existing natural genetic variation or by direct genetic manipulation of the signalling processes that determine the amounts of Rubisco, given a better understanding of these than currently exists. Such approaches might overcome the undesirable pleiotropic effects of direct manipulation of *rbc*S expression, such as altering the link between photosynthetic rate and stomatal conductance, discussed above.

Regulation

The reversible formation of a carbamate by reaction of CO_2 with the amino group of a lysine residue in the catalytic site and its stabilization by Mg^{2+} is a basic mechanism underlying the control of Rubisco activity. However, since the carbamate group is directly involved in catalysis of both carboxylation and oxygenation, changing residues in Rubisco to change the reactivity of this lysyl residue are reflected in changes in activity. The identification and characterization (Somerville *et al*., 1982; Salvucci *et al*., 1985) of a mutant (*rca*) of arabidopsis (*Arabidopsis thaliana*), in which the carbamylation of the catalytic site lysine was impaired, because of the absence of another protein, Rubisco activase, has provided an alternative and adaptable target for changing the regulation of Rubisco activity in leaves. Rubisco activase has been likened to a molecular chaperone (Jimenéz *et al*., 1995) and there is clear evidence that it requires a binding site on Rubisco in order to facilitate carbamylation of the lysine residue. Thus Wang *et al*. (1992) showed that Rubisco activase from petunia or tobacco was not effective in the activation of Rubisco from spinach, barley, wheat, soybean, arabidopsis, pea, maize or chlamydomonas, *in vitro*. Conversely, Rubisco activase from barley or spinach was ineffective in the activation of Rubisco from petunia, tobacco or tomato. Larson *et al*. (1997) showed that changing proline 89 to arginine in the large subunit polypeptide of Rubisco from chlamydomonas rendered the enzyme susceptible to activation by tobacco Rubisco activase. Although the need for the formation of a complex between Rubisco and Rubisco activase has been established, no such complex has yet been crystallized to allow full structural characterization. Activation of Rubisco by Rubisco activase requires ATP (Streusand and Portis, 1987; Wang and Portis, 1992). Activation of Rubisco is decreased by a high ADP/ATP ratio (Robinson and Portis, 1989), and is increased by light through the operation of a ferredoxin/thioredoxin-linked mechanism involving redox-sensitive cysteine residues in Rubisco activase itself (Zhang and Portis, 1999).

Effects of decreasing Rubisco activase on Rubisco activity

The arabidopsis mutant (*rca*) completely lacking Rubisco activase is unable to survive in ambient air because the active site lysine does not become fully carbamylated. The mutant survives in air enriched with CO_2 where carbamylation is favoured (Somerville *et al*., 1982; Salvucci *et al*., 1985). Tobacco plants in which activase expression was decreased by transformation with antisense DNA showed no distinct phenotype until the activase was very low (Jiang *et al*., 1994; Mate *et al*., 1996; Hammond *et al*., 1998) although Mate *et al*. (1993) observed effects with even minor decreases in the activase activity. Slow growth of these transgenics was associated with decreased rates of photosynthesis although Rubisco amount in the tobacco leaves was increased, especially as the leaves aged. Plants with very little Rubisco activase needed CO_2-enriched atmospheres for survival. Such transgenic plants eventually reached a size similar to the wild type because senescence was delayed (He *et al*., 1997). Transgenic arabidopsis plants with 40% of normal activase concentration showed decreased growth and photosynthesis compared to wild-type plants especially as light intensity was increased.

Recent interesting observations have been reported with the *rca* mutant of arabidopsis that has been transformed with DNA coding for either or both isoforms of the Rubisco activase normally found in this plant. The 46 kDa isoform contains unique C-terminal cysteine residues. In the absence of this isoform, but in the presence of the smaller 43 kDa isoform, the associated Rubisco was not down-regulated at night. This response involves a redox-sensitive disulphide formed between two cysteine residues in the C-terminus (Zhang and Portis, 1999) unique to the larger isoform. Substitution of either C-terminal cysteine for alanine diminished the ATP/ADP sensitivity of activase (Zhang and Portis, 1999) and the light responsiveness of Rubisco activity *in vivo* (Zhang *et al*., 2002). Further manipulations using arabidopsis *rca* plants have been described, into which either the 43 kDa or the 46 kDa isoform of activase were introduced. Rubisco activity in plants expressing the shorter isoform was not down-regulated following a light–dark transition, while that in plants expressing the larger isoform was strongly down-regulated (Zhang *et al*., 2002).

Future prospects for manipulating Rubisco regulation

Evidence is accumulating showing that Rubisco activase may be more susceptible to heat denaturation *in vivo* than Rubisco (Feller *et al*., 1998; Crafts-Brandner *et al*., 1997; Crafts-Brandner and Salvucci, 2000; Rokka *et al*., 2001). Thus there may be scope for over-expression of Rubisco activase, or changing it to a more stable form, to make plants more fitted to stressful or extreme environments.

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CA1P is responsible for low activities of Rubisco in many species in darkness and low light. Rubisco activase releases CA1P from the carbamylated sites of Rubisco (Robinson and Portis, 1988) after which CA1P may be rendered non-inhibitory by the action of a specific, light-modulated phosphatase. The phosphatase has been purified and the gene coding for it should soon be available. The effect of manipulating the abundance of the CA1P phosphatase on CA1P abundance and Rubisco activity will be of considerable interest. It may be possible to change the activity of Rubisco activase by light, redox regulators, or exposure to elevated temperature, so that CA1P is not released from the catalytic sites of Rubisco. Alternatively, the amount of CA1P may be increased by decreasing the expression of CA1P phosphatase. In either case, earlier investigations (Mehta *et al*., 1992; Khan *et al*., 1999) suggest that Rubisco would then be protected from proteolysis. It should soon be possible to test this hypothesis.

Alternative means of manipulating Rubisco activity may arise once genes for the enzymes involved in the synthesis of CA1P have been identified. Very strong evidence is available for the pathway of synthesis of CA1P from fructose 1,6-*bis*phosphate in the chloroplast involving the sugar hamamelose (Andralojc *et al*., 2002), but none of the enzymes involved have been characterized and none of the genes coding for these enzymes have been identified.

At least three other tight-binding inhibitors of Rubisco occur naturally, but these may be merely misfire products of Rubisco catalysis. Little is known about the factors controlling the amounts of these inhibitors in steady-state photosynthesis and manipulation of these factors to control Rubisco activity cannot yet be envisaged. Several phosphorylated metabolites in the chloroplast are competitive inhibitors of Rubisco activity or of its carbamylation (Portis, 1992; Parry *et al*., 1999). Mutations and genetic manipulation affecting chloroplast metabolism may be expected to change steady-state concentrations of such metabolites in the chloroplast and, consequently, affect Rubisco activity. Glyoxylate is thought to be increased in certain photorespiratory mutants (Wingler *et al*., 1999) and to decrease the activation of Rubisco (Cook *et al*., 1985).

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