Engineering Photosynthetic Enzymes Involved in CO₂–Assimilation by Gene Shuffling

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Summary

Enhancing photosynthesis is a promising approach for increasing plant productivity. Advances in plant transformation technology make it possible to manipulate photosynthesis by overexpressing particular genes for alleviating bottleneck steps, diverting the flux of Calvin cycle intermediates and photoassimilates, or introducing new enzymes and pathways that can positively influence photosynthesis. Furthermore, directed molecular evolution makes it possible to target selected key enzymes in photosynthetic pathways for modifying their specific catalytic or protein properties and tailoring them to best function under specified growth conditions. In this chapter, advances in directed molecular evolution technology and the use of gene shuffling methodology to modify Rubisco and Rubisco activase to enhance plant photosynthesis and growth are described. By shuffling the *Chlamydomonas reinhardtii* Rubisco large subunit and utilizing competitive growth selection, several mutated Rubisco enzymes with increased carboxylase activity or CO_2/O_2 specificity were identified. The mutations identified in the modified *Chlamydomonas* Rubisco variants were then introduced into the tobacco enzyme by site-directed mutagenesis. Enzyme kinetic assays indicated that the modified tobacco Rubisco enzymes displayed increased CO_2/O_2 specificity, carboxylase activity and reduced K_m for CO_2. Similarly, gene shuffling technology was used to generate several *Arabidopsis thaliana* Rubisco activase variants exhibiting improved thermostability in order to alleviate the

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inhibition of plant photosynthesis by elevated temperatures. The thermostable activase variants were then expressed in an *Arabidopsis* Rubisco activase deletion line created by fast-neutron mutagenesis. The positive effects of the shuffled thermostable Rubisco activase variants on Rubisco activation state, rates of photosynthesis, and growth under moderate heat stress were demonstrated.

I Introduction

Increasing plant productivity is necessary to meet future worldwide food demands and may also be needed to alleviate the dependence on fossil fuels by providing more plant- based alternative energy sources. Photosynthesis, the process through which plants accumulate biomass by using light energy to convert inorganic carbon to carbohydrates, is a major target for improving plant productivity via conventional breeding practices and crop biotechnology (Richards, 2000; Sinclair et al., 2004). In the past century conventional breeding increased many crop yields by more than double. These achievements were accomplished mainly through: (1) genetic selection and agronomic management improvements, including increased photosynthesis per unit land area by maximizing leaf area index (LAI) via optimizing leaf orientation within a canopy; (2) extended duration of leaf photosynthesis by increasing disease resistance combined with utilizing inorganic fertilizer and improving agronomic practice; and (3) increased partitioning of crop biomass to the harvested product (harvest index – the ratio of yield biomass to the total cumulative biomass at harvest). Since the selection for

increased yield by plant breeders has not resulted in a genetic increase in photosynthetic rate per leaf area (Richards, 2000), increasing genetic yield potential through such an approach is perhaps approaching its ceiling. The leaf area index is already high in many crop plants and the harvest index for many major crops, such as corn and rice has reached or exceeded 0.5 (Sinclair, 1998; Peng et al., 2000). Improving the net photosynthetic rate per leaf area to increase the inherent crop yield potential is a logical target for the next stage of agricultural research (Horton, 2000).

Realizing yield potential in an agricultural setting is often limited by environmental stress. Tollenaar and Lee (2002) believe that most of the improvement in corn yield has resulted from increased stress resistance. Yield loss from many stress conditions is directly or indirectly caused by effects on plant photosynthesis. In the field, drought is a very common stress which affects plant photosynthesis almost instantly by limiting CO_2 diffusion from the atmosphere into the chloroplasts by reducing stomatal opening. A tight positive relationship between the grain yield of wheat and maize and stomatal conductance has been observed (Evans and Fischer, 1999). Other factors such as light intensity, low or high temperatures, and high salinity can affect plant photosynthetic performance and hence, crop yield. To avoid a plateau in crop yield potential and to realize a higher percentage of yield potential under farming conditions, increasing plant net photosynthetic rate at the leaf level under normal conditions and improving the stability of photosynthesis under stress conditions are becoming two major challenges.

II Potential Targets for Improving Plant Photosynthesis

In the biochemical model of photosynthesis (Farquhar et al., 1980), Rubisco (ribulose-1,5-bisphophote carboxylase/oxygenase) plays a central role in the determination of leaf photosynthetic rate and is often a rate-limiting enzyme under many physiological conditions. Much of the limitation can be attributed to the catalytic properties of the Rubisco enzyme. Rubisco is notorious for its low turnover number (k_{cat}^c) and catalyzes a wasteful oxygenation reaction which competes with its CO_2 fixing activity, the carboxylation reaction

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Abbreviations: *aadA* – aminoglycoside 3" adenyltransferase gene; *ble* – bleomycin resistant gene; GFP – green fluorescent protein; HTP – high-throughput; *ictB* – A gene involved in HCO_3^- accumulation within the cyanobacterium *Synechococcus* sp. PCC 7942; k_{cat}^c – Rubisco k_{cat} for carboxylation; K_m – the substrate concentration at which an enzyme yields one half maximum velocity; k_{cat}^o – Rubisco k_{cat} for oxygenation; LAI – the ratio of total leaf surface area of a crop to the surface area of the land on which the crop grows; LSU – Rubisco large subunit; *otsA* – trehalose synthase gene; PGA – 3-phosphoglyceric acid; *rbc*L – Rubisco large subunit gene; *Rbc*S – Rubisco small subunit gene; RCA – Rubisco activase; RuBP – ribulose-1, 5-bisphosphate; SBPase – sedoheptulose-1,7-bisphosphatase; SSU – Rubisco small subunit; V_c – maximum velocity of Rubisco carboxylation reaction; Ω – Rubisco CO_2/O_2 specificity

(Laing et al., 1974). The oxygenation reaction product enters into the photorespiratory pathway through which 25% of the fixed carbon is released (Ogren, 1984). The ratio of Rubisco's carboxylation catalytic efficiency (k_{cat}^c of carboxylation over K_m for CO_2) to its oxygenation catalytic efficiency (k_{cat}^o of oxygenation over K_m for O_2) is defined as Rubisco CO_2/O_2 specificity (Ω). As predicted by the model, improvement in k_{cat}^c without altering its Ω value and K_m for CO_2 or vice versa will benefit photosynthetic CO_2 fixation under ambient growth condition. Significant work to genetically modify Rubisco proteins has been performed in the past decades with the aim of improving Rubisco k_{cat}^c and Ω (reviewed by Spreitzer, 1993, 1999; Hartman and Harpel, 1994; Tabita, 1999; Spretzer and Salvucci, 2002). Due to constraints in expressing a functional higher plant Rubisco in microbial hosts (Cloney et al., 1993; Gutteridge and Gatenby, 1995), Rubisco engineering has mainly focused on enzymes from only a few photosynthetic microorganisms, such as *Rhodospirillum rubrum*, cyanobacteria or the eukaryotic green algae, *Chlamydomonas reinhardtii*. The kinetic information generated from analyzing various genetically modified Rubisco mutants assisted in establishing the catalytic mechanisms and helped to identify some structural regions that may determine a specific catalytic parameter or structural stability, as well as Rubisco-activase specificity (Larson et al., 1997). It is hoped that the accumulation of the knowledge on Rubisco structure-function relationships will finally enable the engineering of a better plant Rubisco for improving crop photosynthesis.

Investigation of the natural variation in Rubisco catalytic properties from different species revealed that the Rubisco from red alga exhibited a surprisingly high Ω value, approximately two to three times that of crop plant Rubiscos (Read and Tabita, 1994; Uemura et al., 1997; Whitney et al., 2001). A general inverse relationship between Ω and K_{cat}^c among Rubiscos existing in nature has also been observed, although the data are considerably scattered along the trend line (Bainbridge et al., 1995; Zhu et al., 2004; Tcherkez et al., 2006). Recently, Tcherkez et al. (2006) hypothesized that a conflict may exist between the structural requirements for a higher catalytic turnover rate and increased discrimination between CO_2 and O_2. As such, a compromise has to be made

between Ω and k_{cat}^c. Through long periods of natural selection, such a compromise between Ω and k_{cat}^c for a particular Rubisco may be nearly perfectly optimized for adaptation to its ecosystem, especially to the gaseous and thermal environments where the organism lives (Tcherkez et al., 2006). Tcherkez et al. (2006) further suggested that the potential for improving Rubisco catalytic efficiency may only be modest (within the range of the scatter). If this hypothesis is true, possible improvements from engineering Rubisco alone in order to enhance crop photosynthesis at leaf level might not be dramatic. A 15–20% increase in photosynthesis on a leaf area basis, however, could still have significant impacts on plant growth and yield.

Besides directly targeting Rubisco, there are some alternative approaches to increase plant photosynthesis. One possibility is to increase the Rubisco activation state under certain conditions. Rubisco must be "activated" in order to fix CO_2 (Lorimer and Miziorko, 1980). The Rubisco activation state is the ratio of catalytically competent sites to total Rubisco sites. The net photosynthetic rate is proportional to Rubisco activation state, but not necessarily to total Rubisco sites (Perchorowicz et al., 1981; Crafts-Brandner and Salvucci, 2000a). Rubisco activation in vivo is controlled by Rubisco activase (Portis, 1992), which is a thermolabile protein (Feller et al., 1998). Inhibition of plant photosynthesis by moderately elevated temperatures appears primarily due to temperature damage to Rubisco activase and perhaps also the specific activity of activase via influencing ATP/ADP ratio, which results in the loss of Rubisco activation state (Crafts-Brandner and Salvucci, 2000a). Rubisco from crop plants is considerably more thermostable and its catalytic activity increases with increases in temperature beyond 40–45°C. At these temperatures, Rubisco activase activity for most crop plants is significantly reduced or diminished (Feller et al., 1998; Crafts-Brandner and Salvucci, 2000a). Engineering a thermostable Rubisco activase that will stabilize or even increase plant photosynthesis at moderately elevated temperatures (Crafts-Brandner and Salvucci, 2000a, b) will be discussed separately in the section V.

Recent advances in plant transformation technology make it possible to manipulate photosynthesis by overexpressing particular genes or introducing new enzymes or pathways that can positively

influence photosynthesis (reviewed by Parry et al., 2003; Raines, 2006). It has been reported that overexpression of the Calvin cycle enzymes, fructose-1,6-bisphosphtase or sedoheptulose-1,7-bisphosphatase (SBPase) in tobacco plants, not only increased RuBP concentration, but also Rubisco activation state (Miyagawa et al., 2001; Tamoi et al., 2006). A 1.2-fold higher activation state than that of the untransformed wild-type resulted in both photosynthetic rate per leaf area basis and growth of the transgenic plants being significantly increased (Miyagawa et al., 2001). Although it is not clear whether the increased SBPase activity in the chloroplast enhances Rubisco activation state due to elevated RuBP concentrations, the research demonstrates an alternative approach to manipulate plant photosynthesis by other key Calvin cycle enzymes besides Rubisco. Other research showing positive effects on plant photosynthesis by introducing a single enzyme include: (1) the overexpression of sucrose-phosphate synthase, which influences partitioning of photoassimilates and has resulted in the extended duration of older leaf photosynthesis and increased the biomass of transgenic tobacco and tomato plants (Baxter et al., 2003; Lunn et al., 2003); and (2) the overexpression of a *Escherichia coli* gene, *otsA*, for trehalose synthesis in tobacco that enhanced Rubisco activity and photosynthesis as well as biomass (Pellny et al., 2004). In attempts to increase the CO_2 concentration at the Rubisco site, overexpression of a C_4 cycle enzyme, phosphoenolpyruvare carboxylase, in rice, a C3 plant, as well as a cyanobacterial gene, *ictB*, involved in HCO_3^- accumulation in *Arabidopsis* and tobacco showed positive effects on photosynthesis in the transformed plants (Ku et al., 1999; Lieman-Hurwitz et al., 2003). A successful example for introducing an *E. coli* glycolate catabolic pathway into *Arabidopsis* chloroplasts aimed to alleviate photorespiratory losses has been recently published by Kebeish et al. (2007). In their approach, three *E. coli* enzymes, i.e. glucolate dehydrogenase, glyoxylate carboligase and tartronic semialdehyde reductase, expressed with chloroplast targeting peptides in transgenic *Arabidopsis*, convert glycolate directly into glycerate within the chloroplast. This short-circuited photorespiratory pathway releases CO_2 around Rubisco site which facilitates CO_2 refixation without extra energy input, and reduces NH_3 release that saves energy for NH_3 refixation, resulting in better plant growth and biomass accumulation.

III Directed Molecular Evolution Provides a Useful Tool to Engineer Selected Enzymes

An alternative approach to overexpression of naturally existing genes to manipulate photosynthetic pathways is by modifying selected endogenous or exogenous photosynthetic enzymes for best function under preferred growth conditions. Directed evolution, a powerful method to evolve proteins by generating libraries of mutants (variants) and selecting/screening for desirable properties not found in nature enables this approach. It mimics the natural evolution process in which protein variants are generated and tested for their improved properties in vitro or in vivo every cycle/generation (Fig. 1).
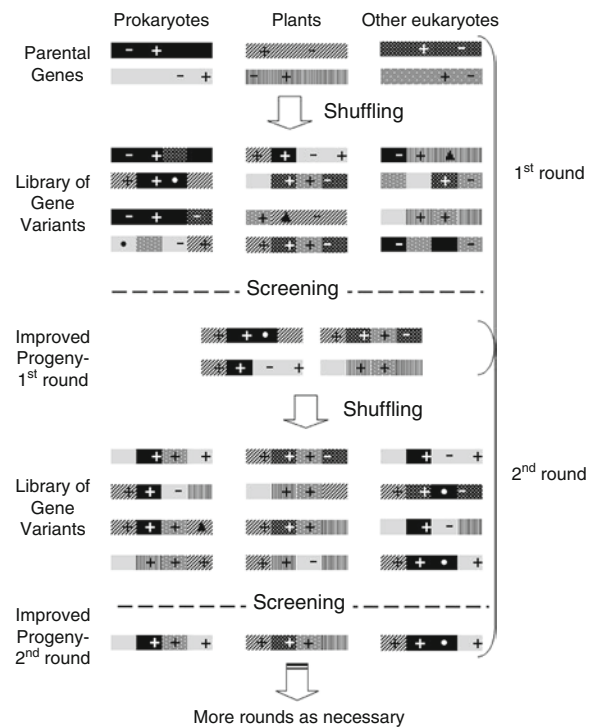

Fig. 1. Directed evolution through gene shuffling. Parental genes related by sequence from species of different kingdoms that possess beneficial mutations (+) and deleterious mutations (−) are fragmented and reassembled using the PCR reaction. During the gene shuffling process, random mutations with positive (*circle*) and negative (*triangle*) mutations are introduced into the library. The resultant library of shuffled variants is screened for desired properties and the selected variants with improved fitness are used as parents for the next round of directed evolution. Each round increases the amount of beneficial and positive random mutations and eliminates deleterious and negative random mutations. The process can be repeated until shuffled variants with the desired properties are identified.

Libraries of variants are generated through a variety of mutagenesis techniques or by gene shuffling in which multiple parental genes generate chimeric sequences. The libraries are then screened for desired properties such as improved catalytic activity, substrate specificity, pH or temperature stability etc. During the screening process, useful mutations and random beneficial mutations are accumulated and deleterious ones are discarded. The resultant progenies that exhibit higher fitness for the desired properties can be used as parental genes for additional rounds of directed evolution by gene shuffling. By consolidating the beneficial mutations in poolwise recombination and discarding the deleterious mutations in each round, directed evolution dramatically accelerates the rate of improvement compared to sexual evolution.

The concept of directed evolution was first demonstrated by evolving the *E. coli* enzyme, EbgA (evolved β-galactosidase activity), to hydrolyze o-nitrophenyl-β-D-galactoside (Campbell et al., 1973). An *E. coli* LacZ⁻ deletion strain that expresses all the other genes involved in lactose uptake and metabolism was adapted to grow on lactose as the carbon source by evolving a novel lactose hydrolyzing enzyme. Campbell and colleagues demonstrated the major advantage of directed evolution, namely, the ability to generate a new protein with improved activity regardless of structure-function information. Moreover, directed evolution can overcome the lack of three-dimensional structural information and can enhance the activity of proteins that are linked to poorly folded polypeptides by improving their folding properties. This was recently demonstrated by generating an active green fluorescent protein (GFP) fused to a poorly folded polypeptide that interfered with the correct folding of the GFP (Pedelacq et al., 2006). Four rounds of shuffling and screening for bright fluorescent variants that can still fold in the presence of the misfolded fusion peptide resulted in an active super-folder GFP that is unaffected by the poorly folded polypeptide.

Over the last 3 decades direct protein evolution has become the key technology in protein engineering and is widely used in academic laboratories and industry (Minshull and Stemmer, 1999; Yuan et al., 2005; Matsuura and Yomo, 2006). Enhanced enzymatic performance such as protein specific activity, stability in extreme conditions such as temperature and pH, and new enzymatic functions by altering substrate specificity for nucleic acid modifying enzymes, reporter genes,

biochemical catalysts and cellulolytic enzymes have been reported (Arnold and Moore, 1997; Minshull and Stemmer, 1999; Powell et al., 2001; Yuan et al., 2005; Kaur and Sharma, 2006). While changing substrate specificity by site-directed mutagenesis often negatively affects the specific activity on the natural substrate, enhancing catalytic activity and expanding the substrate selectivity of enzymes by directed evolution are frequently linked and provide a rapid and powerful method to optimize enzymes. For example, evolved β-fucosidase from the *E. coli lacZ* β-galactosidase exhibited over tenfold improvement of catalytic efficiency (k_{cat}/K_m) and dramatic improvement for fucose substrates compared to the parent activity (Zhang et al., 1977). While the native β-galactosidase acts only weakly on β-D-fucosyl moieties, the evolved β-fucosidase exhibits high specificity for *o*-nitrophenyl substrates and *p*-nitrophenyl substrates. Similarly, two rounds of shuffling of the glycosynthase β-glucosidase (Abg) increased the catalytic efficiency 27-fold and significantly expanded the repertoire of acceptable substrates (Kim et al., 2004).

The three major advantages of directed evolution in comparison to natural evolution are: larger diversity pool, a rapid screening process and increased selective pressure. While natural evolution is limited to two parental genomes per generation, directed evolution can incorporate a large number of genes from different species through gene shuffling methodology (Fig. 1). Random fragmentation of multiple genes and then reassembly into full-length chimeric sequences through PCR was first demonstrated by Stemmer (1994a, b) as an efficient gene shuffling method that generates direct recombination of beneficial mutations. Additional diversity can be introduced into the library during the reassembly process by controlling the fidelity of the DNA polymerase. Recognizing the significance of the size of the diversity pool, scientists have developed methods that allow the introduction of non-homologous and very small crossover fragments (reviewed by Yuan et al., 2005).

Each round of screening during the directed evolution process is equivalent to a single generation of an organism in natural evolution. Therefore, rapid HTP screens enhance the process and provides faster results compared with natural evolution in which the screen is determined by the life cycle of the organism. However, HTP assays for protein

function are the major bottleneck in directed evolution. They are labor-intensive with limited screening capacity of about 10^4 variants per library (Boersma et al., 2007). Methods for recombinant protein production such as *E. coli* expression and purification systems, phage display and cell surface display are widely used for the screening and selection processes (Lin and Cornish, 2002). Therefore, increasing screening capacity depends on the optimization of automatic HTP liquid handling for processing and assay monitoring.

Positive genetic selection enables the organism to survive only in the presence of improved target protein variants under certain desired conditions. It is the most demanding approach that significantly increases the selection capacity to 10^{10}–10^{13} variants per cycle. This is in contrast to a screening approach in which one has to analyze each individual variant in the library. The power of a selection system for identifying improved prokaryotic Rubisco variants was successfully demonstrated by Smith and Tabita (2003) using a Rubisco deletion mutant host of the photosynthetic bacterium *Rhodobacter capsulatus* (SBI-II⁻). *Rhodobacter capsulatus* SBI-II⁻ was unable to grow photoautotrophically in the presence of either 1.5 or 5% CO_2. Complementation of this deletion host with the *Synechococus* PCC6301 *rbc*LS allowed photoautotrophic growth in the presence of 5% CO_2 but not 1.5% CO_2. Mutant variants of *rbc*LS with improved kinetics properties that enable the host to grow photoautotrophically in the presence of 1.5% CO_2 were identified using the deletion host. This system was also used for negative selection of many *rbc*LS mutants that could not complement photoautotrophically the deletion host growth in the presence of 5% CO_2. Further biochemical analysis indicated that different kinetics properties were affected for positive and negative clones. Similarly, positive selection was used to screen for increased resistance against β-lactam antibiotics (Stemmer, 1994a, b) and moxalactam degradation by recursive shuffling of the cephalosporinase enzyme (Crameri et al., 1998).

More recently, a modified *E. coli* host in which only the active Rubisco enzyme can restore the growth of the mutant cell line was used as a selection host to identify improved prokaryotic Rubisco (Parikh et al., 2006). Expression of the phosphoribulokinase in *E. coli* converts irreversibly ᴅ-ribulose-5-phosphate into RuBP. Since *E. coli* can not use RuBP, the carbon flux diverts from the pentose phosphate shunt into a metabolic dead end that causes growth arrest. Co-expression of functional Rubisco in this genetically engineered *E. coli* strain will rescue the bacteria by converting RuBP into PGA that serves as a metabolic intermediate in glycolysis. Selection of three rounds of randomly mutagenized libraries of the *Synechococcus* PCC6301 LSU and co-expression in the host system with its wild type SSU resulted in identification of improved LSU variants. The mutant variants exhibited four- to fivefold improvement in specific activity and produced significant amounts of Rubisco proteins relative to the wild-type enzyme.

Recombinant DNA techniques were successfully utilized to improve herbicide and fungicide control in agricultural biotechnology during the twentieth century (Miflin, 2000). Thus, directed evolution technology offers great opportunity in the transgenic plant approach to study structure–function relationships and to produce commercially viable genetically modified (GM) products (Lassner and Bedbrook, 2001; Lassner and McElroy, 2002). In this respect, Castle et al. (2004) demonstrated a novel catalytic activity of glyphosate *N*-acetyltransferase (GAT) that provides herbicide tolerance by gene shuffling technology. Detoxification of the herbicide glyphosate (*N*-phosphonomethylglycine) can be achieved by *N*-acetylation. Screening of a microbial diversity collection consisting of predominantly *Bacillus licheniformis* identified three genes encoding glyphosate *N*-acetyltransferase (GAT) enzymes with poor glyphosate acetylation activity. Eleven iterations of gene shuffling improved the enzymatic efficiency by 9,000-fold. Transgenic maize lines expressing the improved GAT variants tolerate six times the concentration that causes severe symptoms to untransformed plants. This is the first agricultural product developed by gene shuffling technology that will be commercialized in the nearest future.

IV Improving Rubisco Catalytic Efficiency by Gene Shuffling

A Attempts to Express Arabidopsis thaliana Rubisco in Chlamydomonas reinhardtii

Our ultimate goal for Rubisco engineering is to improve crop plant productivity. It is therefore

desired to directly evolve higher plant Rubisco through gene shuffling and the selection/screen process. Unfortunately this approach is greatly limited by the lack of a host system suitable for library screening. *Chlamydomonas* is the eukaryotic green alga that is often viewed as a plant cell model system (Weeks, 1992). The amino acid sequence of the *Chlamydomonas* Rubisco large subunit (LSU) shares nearly 90% identity to that of higher plant enzymes. To test if a higher plant Rubisco LSU can be expressed in *Chlamydomonas*, we generated a Rubisco LSU deficient mutant strain named MX3312 from wild type strain 2137 provided by Dr. Spreitzer, University of Nebraska. MX3312 has its entire *rbc*L coding sequence replaced by a bacterial *aadA* gene through homologous recombination and antibiotic selection on spectinomycin. This strain can heterotrophically grow on an acetate containing medium, but dies after withdrawal of acetate from the medium. The photoautotrophic growth of MX3312 can be easily restored by transforming the construct of *Chlamydomonas* wild type *rbc*L (*Cr-rbc*L) with both 5' and 3' flanking sequences and the transformation efficiency is high. Namely, one bombardment with approximate 1 μg plasmid carrying wild type *Cr-rbc*L and 6×10^7 MX3312 cells could typically generate 200–300 photosynthesis-competent colonies. If a plant Rubisco LSU expressed in *Chlamydomonas* can form a functional holoenzyme with the *Chlamydomonas* small subunit (SSU), the mutant phenotype of MX3312 can then be complemented. To test such possibility, the construct of *Arabidopsis rbc*L (*At-rbc*L) coding region linked to 5' (2.3 kb) and 3' (1 kb) flanking sequences of *Cr-rbc*L was delivered to MX3312 chloroplasts by particle bombardment (PDS 1000-He Biolistic Delivery System-BioRad). The transformed cells were plated on minimal medium to select for photosynthesis-competent colonies. After extensive transformation and selection, we were unable to recover any photoautotrophic colonies. There are at least three possible reasons to explain this outcome: first, *At-rbc*L was not expressed in *Chlamydomonas* at either the transcriptional or translational level; second, *At-rbc*L was expressed at the protein level, but could not fold correctly into a functional form; third, *Arabidopsis* LSU was not compatible with *Chlamydomonas* SSU to form a functional Rubisco. This approach, however, cannot

explore any of these possibilities because of the life-or-death selection. To address this shortcoming, we used a cell wall-less strain cc349/CW15 (from *Chlamydomonas* genetic center, Duke University), which is suitable for both chloroplast (particle bombardment, Boynton et al., 1988) and nuclear (electroporation, Shimogawara et al., 1998) transformations subsequently, to further test the possibility of expressing *Arabidopsis* Rubisco in *Chlamydomonas*. A construct containing *At-rbc*L flanked with *Cr-rbc*L 5' and 3' sequences followed by an *aadA* cassette was transformed into the cc349/CW15 strain. Hundreds of spectinomycin resistant colonies were recovered and the replacement of *Cr-rbc*L by *At-rbc*L was confirmed by DNA analysis. The transformants could not grow photoautotrophically, which is to be expected based on the outcome of MX3312 transformed with *At-rbc*L. RT-PCR analysis with *At-rbc*L specific primers, indicated that the mRNA levels in the *At-rbc*L transformants were normal (Fig. 2). Western analysis, however, could not detect either LSU or SSU, indicating no Rubisco holoenzyme was formed in the transformants. By analyzing a Rubisco SSU deficient mutant strain, T60-3, Khrebtukova and Spreitzer (1996) have observed in a pulse labeling experiment that the Rubisco LSU could not be produced in T60-3 cells in the absence of SSU even though the *rbc*L mRNA level was normal, an indication that *rbc*L expression is suppressed at the translational level. To test if the lack of both LSU and SSU in cc349/CW15-*At-rbc*L transformants is due to incompatibility between *Arabidopsis* LSU and *Chlamydomonas* SSU, a construct containing a *At-Rbc*S cDNA with a *Cr-Rbc*S promoter and 5'-transit peptide followed after by a 3' *ble* cassette (from Dr. Saul Purton, University College London) conferring zeocin resistance, was constructed. Since *Cr-Rbc*S genomic DNA contains 3 introns, a separate experiment was performed complementing T60-3 (From Dr. Spreitzer, University of Nebraska) with two *Cr-Rbc*S constructs containing either all three introns or only intron 1. We found that the T60-3 strain could not be complemented by *Cr-Rbc*S cDNA, but was complemented by both *Cr-Rbc*S intron-containing constructs, indicating intron 1 is essential. But it was also observed that the construct containing 3-intron recovered at least five-fold more photosynthesis-competent transformants than the construct carrying 1-intron by using the
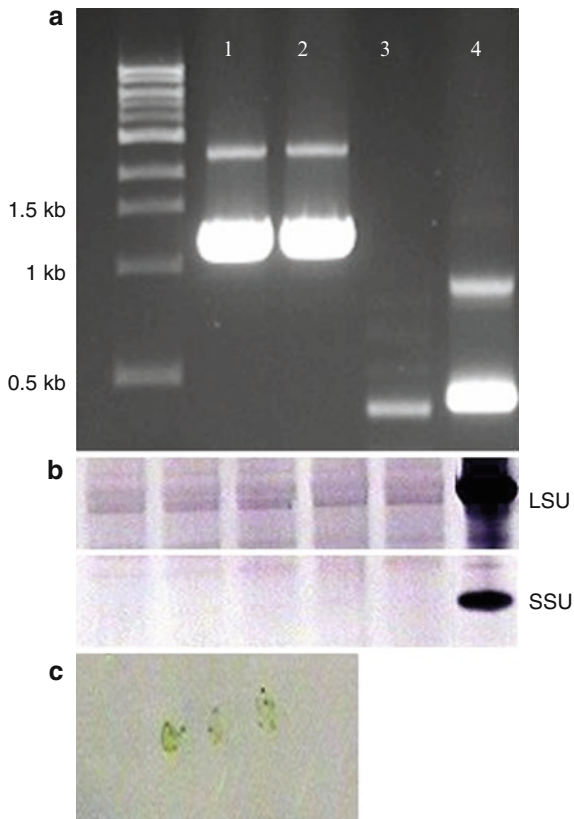
Fig. 2. Expression of *At-rbc*L and *At-Rbc*S in *Chlamydomonas* strain cc349/CW15. Panel A. RT-PCR results. Lane 1: RT-PCR product using *At-rbc*L specific primers; lane 2: RT-PCR product using *Cr-rbc*L specific primers; lane 3: RT-PCR product using *At-Rbc*S specific primers; lane 4: RT-PCR product using *Cr-Rbc*S specific primers. Panel B. Western analysis of five independent transformants containing *At-rbc*L and *At-Rbc*S. The cells were grown in acetate medium under continuous light (150 μE m^2 s^2) at 23°C. Right lane: LSU, SSU standards. Panel C. *At-rbc*L and *At-Rbc*S transformants growing on minimal medium under light for 8 weeks after transferring from antibiotic selection plates.

same amount of DNA for transformation. Based on this information, we also inserted the *Cr-Rbc*S intron 1 into *At-Rbc*S cDNA in the corresponding position. After transforming the *At-Rbc*S construct into cc349/CW15-*At-rbc*L cells, many zeocin resistant colonies were recovered. When the transformed cells containing both *At-rbc*L and *At-Rbc*S were transferred from acetate containing antibiotic selection media to the minimal media, the cells survived under light for months but hardly grew (Fig. 2). RT-PCR with *At-Rbc*S specific primers indicated the existence of mRNA, but at a highly reduced level compared to

*Cr-Rbc*S mRNA (Fig. 2). Western analysis detected a very faint band at the expected LSU position after prolonged color development (Fig. 2), suggesting only a trace amount of Rubisco formation, but certainly not enough to support photoautotrophic growth even under elevated CO$_2$. In the transformants of *At-rbc*L and *At-Rbc*S, the endogenous *Cr-rbc*L was completely replaced by *At-rbc*L, but the native *Cr-Rbc*S genes in the nucleus were retained. Although it is not clear whether or not the extremely low expression of the *At-Rbc*S transgene in the transformants is due to the presence of the native *Cr-Rbc*S product or the lack of introns 2 and 3 in the *At-Rbc*S construct, it seems that further optimization of *At-Rbc*S expression in *Chlamydomonas* cells is needed in order to succeed. However, we also cannot rule out the possibility that the lack of a compatible chaperone for correct folding of *At*-Rubisco subunits in *Chlamydomonas* is responsible for the minimal holoenzyme accumulation. In this case, co-expression of a *At*-chaperone and/or *At*-RCA may be necessary.

B Shuffling the Chlamydomonas reinhardtii Rubisco Large Subunit

Because of the technical difficulties discussed above in developing a plant Rubisco expression and selection system, we have shuffled *Chlamydomonas* Rubisco in order to test: (1) if the catalytic properties of a eukaryotic Rubisco can be improved by gene shuffling; and (2) if changes in the catalytic properties resulting from the substitutions introduced into *Chlamydomonas* Rubisco variants can be achieved with the plant enzyme.

To shuffle *Chlamydomonas* LSU, the *Cr-rbc*L coding region with 2.3 kb 5' and 1 kb 3' flanking sequences was cloned into the pBluescript plasmid. The libraries were constructed according to Stemmer (1994a) and Crameri et al (1998). Single gene shuffling and semi-synthetic shuffling (Ness et al., 2002), in which oligos containing some of the natural-occurring diversity of the Rubisco gene family were spiked into the *Cr-rbc*L fragments during assembly, were performed in the 1st round shuffling. The parental genes for the 2nd and 3rd shuffling rounds were selected from the previous round's hits. The library variants were transformed into the *rbc*L deletion mutant strain, MX3312, by particle bombardment (PDS 1000-He Biolistic Delivery

System-BioRad). Approximately 150 bombardments for the 1st round and 120 bombardments each for 2nd and 3rd rounds were performed for subsequent selection and screening.

In order to identify Rubisco variants with improved catalytic properties from the shuffled libraries, we developed a three-tier selection/screen procedure (Fig. 3). The 1st tier is based on functional complementation. As discussed above, MX3312 can grow on acetate containing medium but not on minimal medium. The photoautotrophic growth of MX3312 can only be restored by introducing a functional Rubisco LSU. After transforming the shuffled Cr-rbcL variants into MX3312, only Rubisco LSU variants which are functional can be recovered as photosynthesis-competent colonies obtained from selection on minimal medium. With this single selection step, all non-functional variants in the library and those with inadequate catalytic activity to support photoautotrophic
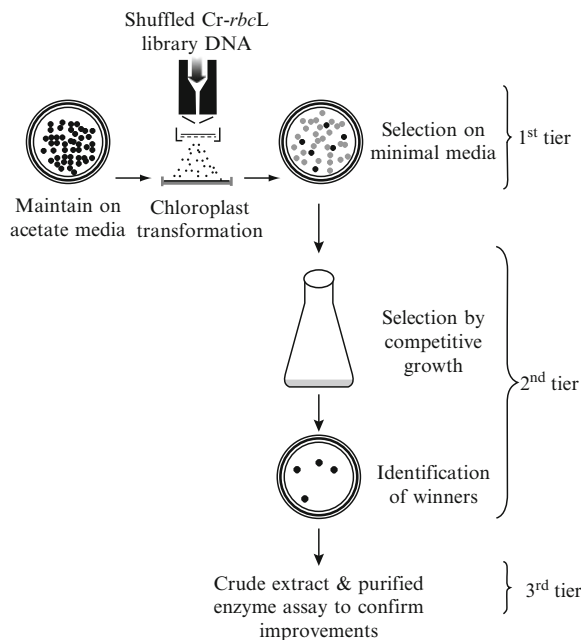

growth are eliminated. The 2nd tier screen relies on competitive growth. The photosynthesis-competent clones recovered from the 1st tier selection were pooled (usually 30 clones per group) with similar amounts of cells and grown together in a liquid culture for >30 generations as monitored by OD_{600} changes. As a single cell organism, the growth response of *Chlamydomonas* to photosynthesis is more sensitive than that of plants. It can be expected that the clones containing Rubisco variants with improved catalytic properties will grow better (faster) than those with less capable Rubisco variants. The consequence of the competitive growth is that the fast growing clones will become the dominant population in the resulting culture after a sufficient number of growth cycles (It is necessary to increase the number of growth cycles to enrich clones with only slightly improved growth rates). To increase the selection pressure, we also included 25–50 μM of a carbonic anhydrase inhibitor (6-ethoxy-2-benzothiazole-sulfonamide) in the competitive growth medium in the later rounds of shuffling to disrupt the CO_2 concentrating mechanism existent in *Chlamydomonas* cells. The resulting culture was plated on solid minimal agar medium to obtain single cell clones. The enriched variants after competitive growth were identified by *rbc*L sequence analysis and photosynthesis measurements for O_2 evolution using an O_2 electrode. The 3rd tier screen is an enzyme kinetic property assay. The cell crude extracts and ion exchange column purified Rubisco enzymes of the clones from competitive growth were used to measure Rubisco V_c and Ω to identify variants with improved catalytic properties.

After three rounds of shuffling, approximately 80,000 library variants were selected and screened. We were able to identify multiple clones showing increased in vitro carboxylase activity up to 56% greater and Ω values up to 18% greater as compared to the wild-type (Zhu et al., 2005). Sequence analysis of these clones displayed on average around three residue substitutions per variant. Most clones contained a substitution in the hydrophobic core of the N-terminal domain and another in the C-terminal tail region. A few substitutions at the surface of the C-terminal domain were also found. Examination of the crystallographic structure indicates that the substituted residue in C-terminal tail interacts with the loop between the αB and βC helices in the N-terminal domain.

Fig. 3. Three-tier selection/screening procedure designed for identifying improved enzyme variants from shuffled *Chlamydomonas rbc*L library. For 3rd tier assay, Rubisco from *Chlamydomonas* crude extracts was purified by ammonium sulfate fractionation (35–55% of saturation) and polyethylene glycol precipitation (20%) followed by an anion exchange chromatographic separation (Poros HQ/20 column). Rubisco carboxylase activity (V_c) was determined by $^{14}CO_2$ incorporation. The Ω value was determined by quantifying 3-phosphoglyceric acid and 2-phosphoglycolic acid directly from Rubisco reaction mixtures with a LC/MS method.

Three active site residues (E60, T65 and W66) are located in this loop region. The substituted residues at the surface of the C-terminal domain are usually involved in interactions between the subunits, either within a L2 dimer or between L2 dimers, but not with SSU. Most of the substitutions resulting in improved catalytic properties are located at non-conserved regions.

To test if the mutations that positively impacted *Chlamydomonas* Rubisco catalytic properties can produce the same effect with a plant Rubisco enzyme, several mutation sets identified from the *Chlamydomonas* Rubisco variants were introduced into tobacco (Petite Havana) *rbc*L by site-directed mutagenesis and chloroplast transformation. One mutation set contains three mutations which are all novel to both *Chlamydomonas* and plant Rubiscos. Kinetic analysis indicated that the modified tobacco Rubisco with the triple mutations exhibited an increase in both V_c (1.5 ± 0.08 μmol·mg^{-1}·min^{-1}) and Ω (89 ± 2.98) by 15% and 14%, respectively by comparison to wild type (1.3 ± 0.03 μmol·mg^{-1}·min^{-1} and 78 ± 0.98 respectively). The triple mutant also displayed lower K_m for CO_2 (7.55 μM) than the wild type enzyme (12.63 μM). Another mutation set contains four mutations, but two already exist in tobacco. The V_c of the mutant tobacco Rubisco with this mutation set increased by 20%, but its Ω value remained unchanged or slightly reduced. This preliminary data suggested that it was possible to engineer higher plant Rubisco by using substitutions identified from shuffling *Chlamydomonas* Rubisco *rbc*L.

V Improving Rubisco Activase Thermostability by Gene Shuffling

Identifying ways to maintain high photosynthetic CO_2 fixation rates in plants exposed to moderately elevated temperatures remains a challenging task for academic labs, breeders and agriculture biotechnology companies. At elevated temperatures that are slightly higher than optimum and with sufficient water availability, plants maintain the stomatas open in order to cool their leaves by evapotranspiration. Under these conditions, the inhibition of photosynthesis is reversible for short periods (hours) of stress, while permanent inhibition occurs under more severe heat stress

due to irreversible damage of the photosynthetic apparatus (Salvucci and Crafts-Brandner, 2004a). The inhibition of photosynthesis under moderate heat stress conditions in both C3 and C4 plants is hypothesized to be due to the extreme heat sensitivity of the Rubisco activase (RCA) enzyme, which constantly maintains Rubisco at a high activation state. The *Arabidopsis rca* mutant reported by Somerville et al. (1982) was the key to the discovery of RCA and its role in photosynthesis. This mutant, which requires high CO_2 concentrations to survive, lacks two polypeptides (Salvucci et al., 1985) that were later purified and shown to promote the activation of Rubisco at physiological concentrations of CO_2, Mg, and RuBP (Portis et al., 1987). RCA was subsequently characterized as a member of the AAA$^+$ family of <u>A</u>TPases <u>a</u>ssociated with diverse cellular <u>a</u>ctivities that interacts with inactive Rubisco and removes sugar-phosphate inhibitors from Rubisco's catalytic sites (Portis, 2003)**.**

The hypothesis that photosynthesis is limited by inactivation of Rubisco due to heat sensitivity of RCA to moderately elevated temperature was first suggested by Feller et al. (1998). The extreme sensitivity of RCA to elevated temperatures is now well characterized for both C3 (Crafts-Brandner and Salvucci, 2000a; Salvucci and Crafts-Brandner, 2004a, b) and C4 plants (Crafts-Brandner and Salvucci, 2000b). Therefore, generating a thermostable RCA that can effectively activate Rubisco under moderately elevated temperatures is a potential target for agriculture biotechnology. Maintaining Rubisco at a high activation state under moderately elevated temperatures can be achieved by recombinant DNA technologies that stabilize RCA through: (1) overexpression of thermostable RCA from plants grown in warm regions such as creosote bush, or cotton; (2) increasing the concentrations of osmoprotectants such as glycine betaine in the chloroplasts; or (3) directed evolution of a thermostable RCA. Overexpression of a thermostable activase is a possibly limited solution due to the species dependence of Rubisco and RCA, namely, the incompatibility between Solanaceae RCA to fully activate non-solanaceae Rubisco and vice versa (Wang et al., 1992). In addition, a foreign gene typically possesses different GC content and thus requires codon optimization in order to ensure high expression levels.

Increasing glycine betaine concentration in chloroplasts is an indirect strategy to stabilize RCA and maintain Rubisco at a high activation state during heat stress (Yang et al., 2005). The osmoprotectant nature of glycine betaine was shown to prevent inactivation of RCA and positively affected Rubisco activation state and photosynthetic rate at elevated temperatures. However, glycine betaine levels are limited by the levels of the precursor, choline, in the chloroplast and high levels can affect other metabolic pathways. Therefore, directed evolution through gene shuffling of the endogenous *rca* gene was deemed a more favorable method to modulate the thermal properties of RCA. This approach will allow us to identify shuffled variants with enhanced thermostability and/or improved specific activity of the endogenous enzyme. The expression of the mutagenized genes coding for improved RCA variants could be controlled using the endogenous *rca* promoter and untranslated regulatory sequences without the needs for codon optimization and worries of incompatibility of the expressed RCA variants with the host Rubisco.

Arabidopsis contains two RCA polypeptides: the 43 kDa short (β) form and the redox-regulated 46 kDa long (α) form (Zhang et al., 2002). The two forms are generated by alternative splicing of a single pre-mRNA (Werneke et al., 1989). In order to enhance photosynthesis under moderately elevated temperatures, we have increased the thermostability of *Arabidopsis* RCA short form through directed evolution (Kurek et al., 2007). Variants of *Arabidopsis* RCAβ that effectively maintain Rubisco at high activation state under normal and elevated temperature were generated using diversity provided by relatively thermostable RCAs such as wheat, barley, maize and cotton. To increase the diversity pool, additional random mutations were incorporated. The libraries containing the shuffled variants were screened using a Rubisco activation assay that monitored the incorporation of ^{14}C into the Rubisco product, PGA, through the activation of inactive Rubisco (decarbamylated) in the presence of the shuffled RCAβ. This was achieved with an ATP-regenerating system, RuBP and [^{14}C] $NaHCO_3$ (Fig. 4a). Assaying the activation of Rubisco by the purified RCAβ or by the soluble fraction of *E. coli* cell lysates expressing the shuffled variants in a HTP format was achieved in three tiers (Fig. 4b). The first tier is a HTP screen using *E. coli* cell lysates expressing RCAs exposed to
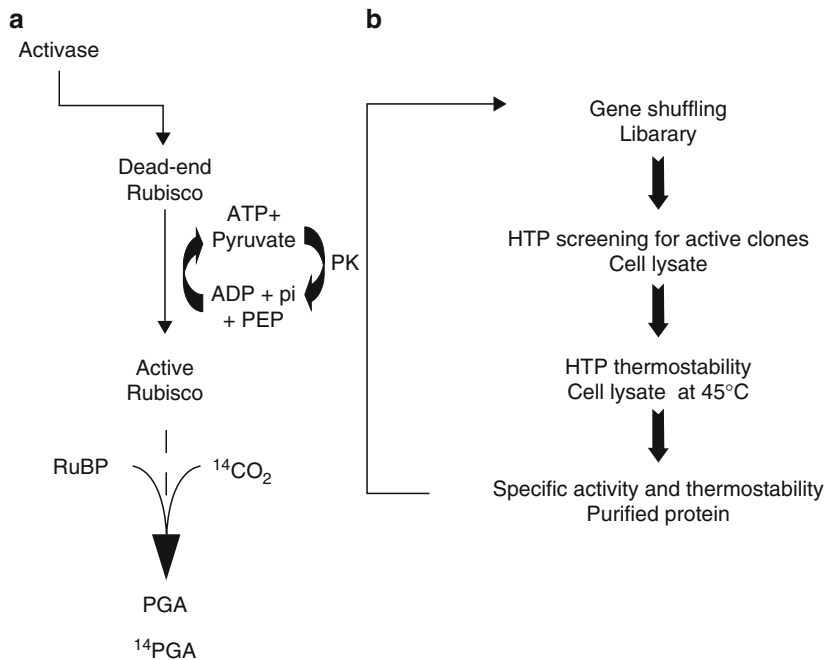

Fig. 4. Overview of the directed evolution of thermostable RCA. Schematic presentation of (**a**) Rubisco activation assay and (**b**) three tier shuffling cascade. PK – pyruvate kinase; PEP – phosphoenolpyruvate; PGA – 3-phosphoglyceric acid.

normal temperature prior to the Rubisco activation assay. The second tier screens for thermostability of the *E. coli* cell lysates of the selected variants from the first tier, exposed to higher temperatures prior to the Rubisco activation assay. The third tier is the characterization of selected RCAβ variants from the second tier for activation of Rubisco at normal and moderately elevated temperature by purified shuffled RCA. That in turn confirmed the superiority of the shuffled variants and determined the specific activity and thermostability. Two rounds of shuffling enhanced the thermostability of the shuffled RCA variants by 80% at 45°C compared to the activity the wild type RCA at the same temperature. The activity at 45°C was only 10% less than the activity of wild type RCA at 25°C. When monitoring the ability of wild type and shuffled variants to maintain Rubisco in an active state during heat treatment (Rubisco activation under catalytic conditions; Crafts-Brandner and Salvucci, 2000a), wild type RCA maintained a Rubisco activation state of 0.5 at 40°C, while the three selected shuffled leads were able to maintain activation states of 0.62–0.72 under the same condition. Relative to reactions at 25°C, the activation state of Rubisco maintained by the thermostable variants at 40°C was in the range of 78–98%, versus 70% for the wild type enzyme (Kurek et al., 2007).

Sequence analysis revealed that one amino acid substitution (T274R) was sufficient to improve activity and thermostability. Three amino acid substitutions (F168L, V257I, K310N and M131V, V257I, K310N) resulted in a 10°C increase in stability of *Arabidopsis* RCA1. The variant containing the mutations F168L, V257I, T274R, and K310N exhibited relatively low activity at 25°C (82% of the T274R activity), but maintained high activity at 40°C (102% of F168L, V257I, K310N activity). The substitutions V257I and K310N shared by two selected leads are also present as natural variation in plant species: the former is present in the cucumber enzyme and the later is conserved in wheat, rice, spinach and maize.

To test the effect of the improved RCA *in planta*, the shuffled RCA variants (sRCA) were expressed in an *Arabidopsis* mutant that lacks the endogenous *rca* gene (ΔRCA). This mutant line was selected in order to demonstrate that the phenotype, photosynthesis and growth rates under normal and moderately elevated temperatures were directly and solely affected by the properties of the shuffled variants and not by the presence of

the wild-type endogenous RCAβ. In addition, the presence of endogenous RCA α and β (in wild-type plants) that potentially forms heterocomplexes with the shuffled variants (the active complex RCA-Rubisco consists of multiple RCA subunits) could affect the shuffled variants' properties. Finally the absence of endogenous RCAs mimics the screening for improved recombinant shuffled variants that was performed in the absence of the wild type RCA forms. Transgenic ΔRCA lines expressing wild-type RCAβ (wRCAβ) and sRCA exhibited normal photosynthetic rates and phenotypes under ambient growth conditions. Daily exposure of 2-week-old transgenic lines to moderately elevated temperature (30°C for 4 h day^{-1} in the middle of the light cycle), which mimics moderate heat stress during the day, demonstrated the positive effect of thermostable sRCA on growth and photosynthetic performance (Fig. 5). sRCA plants exhibited higher leaf area (about 15–20%) (Fig. 5a) than wRCAβ plants (Fig. 5b) and higher photosynthetic rates (about 10%) during the heat stress period (Fig. 5c–e). The leaves of wRCAβ grown under moderately elevated temperature were severely damaged and displayed discoloration. When wRCAβ and sRCA plants were grown continuously at 26°C under higher light intensity and humidity, the sRCA plants possessed 50–100 more siliques per plant than wRCAβ plants. In addition, the siliques of sRCA plants were larger and produced more seeds, higher seed weight and better seed viability (higher germination rate) than the wRCAβ plants (Kurek et al., 2007). The phenotype and photosynthetic performance of wRCAβ and sRCA strongly support the hypothesis that RCA limits Rubisco activity and therefore photosynthesis under elevated temperatures. The improved phenotype of the transgenic lines expressing the shuffled RCA under moderately elevated temperatures is most likely due to the improved thermostability of RCA, minimizing the negative effect on photosynthetic performance and the inhibition of biomass accumulation.

VI   Future Prospects

Genetic adaptation of a crop plant to its growth environment is a key determining factor for yield potential. Current crop elites have experienced extensive genetic selection under their growth conditions to achieve higher yield potential. It is
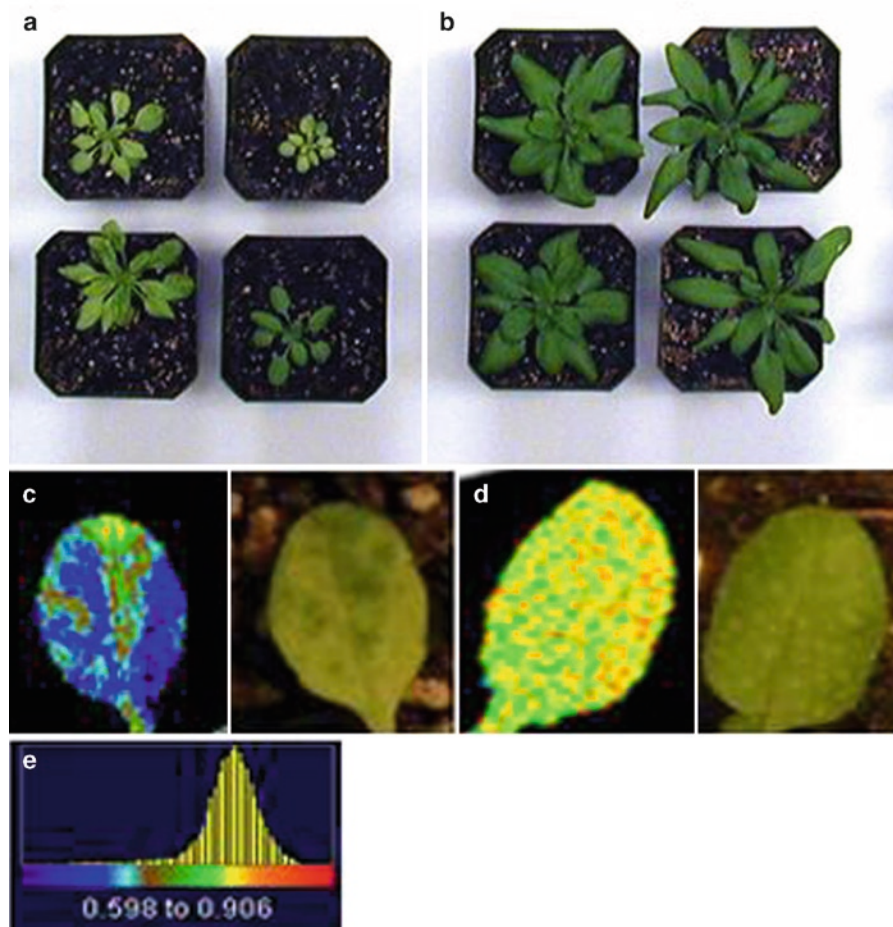
Fig. 5. Expression of thermostable RCA in ΔRCA mutant. Phenotype of 4-week-old transgenic plants overexpressing RCAβ. (**a**) and sRCA (**b**) grown under 16 h light (225 µmol photons m^{-2} s^{-1}) 8 h dark regime exposed daily to moderately elevated temperatures (6 h 22°C; 4 h 30°C; 6 h 22°C during the day cycle) for 2 weeks. Photosynthetic performance (Fq'/Fm') of a single leaf from RCAβ (**c**) and sRCA (**d**) was measured using fluorescence image analysis. The Photosynthetic performance scale from low (blue) to high (red) Fq'/Fm' values is indicated (**e**).

most likely that the potential enhancement of photosynthetic rate at the leaf level by modifying any single enzyme will not be dramatic under normal growth conditions because of other genetic constraints. The improvements from modifying a single enzyme or enhancing a single catalytic step for alleviating limitations under certain growth conditions, however, might generate significant positive impacts on photosynthesis. Environmental conditions change during the growth season. For instance, temperature and light, which are two key environmental factors for photosynthesis, fluctuate significantly within a growth season and even within a day. Enzymes have their own optimal temperature range within which they perform best. The optimal temperature ranges for

some photosynthesis enzymes are quite narrow compared to the magnitude of temperature fluctuations during the growth season. This can often become limiting for the overall photosynthetic light use efficiency at suboptimal growth temperature. Adjusting the expression level of relevant enzymes is one of the common strategies used by plants to acclimate to a changed environment. Selection with genetic markers and gene overexpression could be an option to address some specific limitations, but both are limited to the use of naturally existing enzyme properties. With the aid of directed molecular evolution technology, it is possible to generate novel enzymes with catalytic properties that cannot be found in naturally existing enzymes. For example it is possible to

maximize enzyme performance over the whole growth season, by broadening enzyme optimum temperature ranges. In this way, overall light use efficiency might be improved significantly and the sum of photosynthesis can be increased, as can biomass and yield.

Acknowledgements

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