A critical review on the improvement of photosynthetic carbon assimilation in C$_3$ plants using genetic engineering

Cheng-Jiang Ruan, Hong-Bo Shao & Jaime A. Teixeira da Silva

To cite this article: Cheng-Jiang Ruan, Hong-Bo Shao & Jaime A. Teixeira da Silva (2012) A critical review on the improvement of photosynthetic carbon assimilation in C$_3$ plants using genetic engineering, Critical Reviews in Biotechnology, 32:1, 1-21, DOI: 10.3109/07388551.2010.533119

To link to this article: https://doi.org/10.3109/07388551.2010.533119

Published online: 24 Jun 2011.
A critical review on the improvement of photosynthetic carbon assimilation in C₃ plants using genetic engineering

Cheng-Jiang Ruan¹, Hong-Bo Shao², and Jaime A. Teixeira da Silva³

¹Key Laboratory of Biotechnology & Bio-Resources Utilization, Dalian Nationalities University, Dalian City, Liaoning, China, ²Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, China, and ³Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Kagawa, Japan

Abstract
Global warming is one of the most serious challenges facing us today. It may be linked to the increase in atmospheric CO₂ and other greenhouse gases (GHGs), leading to a rise in sea level, notable shifts in ecosystems, and in the frequency and intensity of wild fires. There is a strong interest in stabilizing the atmospheric concentration of CO₂ and other GHGs by decreasing carbon emission and/or increasing carbon sequestration. Biotic sequestration is an important and effective strategy to mitigate the effects of rising atmospheric CO₂ concentrations by increasing carbon sequestration and storage capacity of ecosystems using plant photosynthesis and by decreasing carbon emission using biofuel rather than fossil fuel. Improvement of photosynthetic carbon assimilation, using transgenic engineering, potentially provides a set of available and effective tools for enhancing plant carbon sequestration. In this review, firstly different biological methods of CO₂ assimilation in C₃, C₄, and CAM plants are introduced and three types of C₄ pathways which have high photosynthetic performance and have evolved as CO₂ pumps are briefly summarized. Then (i) the improvement of photosynthetic carbon assimilation of C₃ plants by transgenic engineering using non-C₄ genes, and (ii) the overexpression of individual or multiple C₄ cycle photosynthetic genes (PEPC, PPDK, PCK, NADP-ME and NADP-MDH) in transgenic C₃ plants (e.g. tobacco, potato, rice and Arabidopsis) are highlighted. Some transgenic C₃ plants (e.g. tobacco, rice and Arabidopsis) overexpressing the FBP/SBPase, ictB and cytochrome c₆ genes showed positive effects on photosynthetic efficiency and growth characteristics. However, over the last 28 years, efforts to overexpress individual, double or multiple C₄ enzymes in C₃ plants like tobacco, potato, rice, and Arabidopsis have produced mixed results that do not confirm or eliminate the possibility of improving photosynthesis of C₃ plants by this approach. Finally, a prospect is provided on the challenges of enhancing carbon assimilation of C₃ plants using transgenic engineering in the face of global warming, and the trends of the most promising approaches to improving the photosynthetic performance of C₃ plants.

Keywords: Global warming, increase in CO₂ concentration, biotic carbon sequestration, C₃ plants, C₄ plants, CAM plants, overexpression of C₄ enzymes, transgenic C₃ plants, improvement of photosynthesis

Introduction
The concentration of carbon dioxide (CO₂) in the Earth’s atmosphere has increased by 31% from 280 parts per million (ppm) in 1850 to 380 ppm in 2005 (IPCC, 2007). It is presently increasing at 1.7 ppm yr⁻¹ or 0.46% yr⁻¹, which will lead to 50% higher levels in 2050 than today (Sun et al., 2009). Global surface temperature has increased by 0.88°C since the late 19th century, and 11 out of the 12 warmest years on record have occurred during 1995–2006 (IPCC, 2007). The Earth’s mean temperature is projected to increase by 1.5–5.8°C during the 21st century (IPCC, 2001). In addition to a rise in sea level by 15–23 cm during the 20th century (IPCC, 2007), there have been notable shifts in ecosystems (Greene and Pershing, 2007) and in the frequency and intensity of occurrence of wild fires (Running, 2006; Westerling et al., 2006). The increase in atmospheric CO₂ and other greenhouse gases (GHGs) e.g. methane (CH₄) and nitrous oxide (N₂O), may be the major reasons for climate change (Nowak et al., 2004);
hence, there is a strong interest in stabilizing the atmospheric abundance of CO₂ and other GHGs to mitigate the risks of global warming (Kerr, 2007; Kintisch, 2007; Kluger, 2007; Mikkelsen et al., 2010). CO₂ is the most prominent GHG in the Earth’s atmosphere. Among the different ways to reduce CO₂ emission and increase carbon capture and storage (e.g. chemical transformations, biological conversions, reforming and inorganic transformations, and manipulations of the soil carbon pool) (Lal, 2004; 2008; 2009), biotic carbon sequestration (Figure 1) is recommended by the Kyoto Protocol as one of the important and effective strategies to mitigate the effects of rising atmospheric CO₂ concentrations by enhanced carbon sequestration (Hill et al., 2007). The Kyoto Protocol is the first international environmental agreement to require binding greenhouse gas emission reductions by industrialized nations (http://en.wikipedia.org/wiki/Kyoto_Protocol). Compared to different techniques of carbon sequestration, biotic techniques are immediately applicable, natural, and cost-effective processes, with numerous ancillary benefits but a finite sink capacity (Lal, 2008). The increase in atmospheric CO₂ concentration is mainly from burning fossil fuel, thus, in the future CO₂ mitigation schemes have to match the scale of man-made CO₂ in the atmosphere. For example, if fossil fuel emissions from 1990 to 2100 are limited to 600 PgC, biotic carbon stocks must increase by 120 PgC to stabilize CO₂ concentrations at 450 ppm (Lashof and Hare, 1999); this trend to the utilization of biofuel (bioethanol, biodiesel, CH₄ gas, and H₂ cell) instead of fossil fuel (Ruan et al., 2008).

Biotic sequestration is a process that converts CO₂ into organic compounds, especially sugars, using the energy from sunlight (Smith, 1997), which enhances carbon sequestration and the storage capacity of ecosystems by plant photosynthesis. In plants, there are three types of photosynthetic CO₂ assimilation, and thus three categories of plants (Table 1, Table 2): (i) C₃ plants, in which atmospheric CO₂ is assimilated directly through the C₃ photosynthetic pathway. The majority of terrestrial plants, including many important crops such as rice (Oryza sativa L.), wheat (Triticum aestivum), soybean (Glycine max), and potato (Solanum tuberosum), are C₃ plants (Matsuoka et al., 2001); (ii) C₄ plants e.g. maize (Zea mays) and sugarcane (Saccharum officinarum), which evolved the C₄ photosynthetic pathway (Sage and Pearcy, 1987); and (iii) CAM (crassulacean acid metabolism) plants, in which the stomata are closed during the day and open only at night when the temperature decreases and humidity rises; at night, CO₂ is stored as malate in the large vacuoles and is released for photosynthesis during the day. While C₃ plants grow well in temperate climates, CAM plants such as stonecrops (Lithops spp.) and cactus adapt to extreme arid conditions, but their photosynthetic capacity is very low (Black, 1973). In contrast, C₄ plants such as maize and sugarcane adapt to high light, arid, and warm environments and achieve higher photosynthetic capacity and higher water- and nitrogen-use efficiencies compared to C₃ plants (Black, 1973). Both C₃ and CAM plants evolved from an ancestral C₃ species in response to changes in environmental conditions that caused a decrease in CO₂ availability. C₄ plants evolved in response to low atmospheric CO₂ concentrations, while CAM plants evolved either in response to the selection of increased water-use efficiency or for increased carbon gain (Ehleringer and Monson, 1993).

Photosynthesis is limited by multiple factors including photosynthetic electron transport, regeneration of CO₂ acceptor molecules in the reductive pentose phosphate cycle, the activity and substrate specificity of CO₂ fixing enzyme, the associated flow through the photorespiratory pathway, and environmental variables (e.g. temperature, CO₂ concentration and water availability) (Austin, 1989; Raines, 2006; Peterhansel et al., 2008a). In three distinct phases (carboxylation, reduction, and regeneration) of photosynthesis, several key enzymes directly influence its rate and efficiency (Singh and Malhotra, 2000), such as ribulose-1,5-bisphosphate (RuBP), fructose-1,
6-bisphosphatase (FBPase), and sucrose phosphate synthase (SPS). In C₃ plants, low ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity, photorespiration competing reactions catalysed by Rubisco and resulting in a loss of up to 50% of CO₂ fixation in ambient air, and variation of RuBP activity in stress environments (e.g. high temperature, drought and salinity) limit photosynthesis and carbon assimilation (Häusler et al., 2002). In contrast, C₄ plants, as CO₂ pumps (von Caemmerer and Furbank, 2003), possess a CO₂ concentrating mechanism, in which atmospheric CO₂ is bound to C₄-carbon compounds and is shuttled from the mesophyll cells where the fixation of bicarbonate occurs via phosphoenolpyruvate carboxylase (PEPC) into the gas-tight bundle-sheath cells in which the bound carbon is released again as CO₂ and enters the Calvin cycle. When this pathway was first discovered, conventional cross plant-breeding approaches were used to try to transfer C₄ traits into C₃ plants, but the results were unsuccessful (Brown and Bouton, 1993).

Recently, rapid development of molecular biology and transgenic technology has provided a set of available and effective tools for improving photosynthetic carbon assimilation in C₃ plants. On the one hand, although enzymes with desirable characteristics are available, they have not resulted in attempts to improve Rubisco enzymes to alter the catalytic properties of Rubisco either by amino acid substitutions in several distinct areas of the Rubisco large subunit (Spreitzer and Salvucci, 2002; Andersson and Taylor, 2003; Parry et al., 2003; Raines, 2006) or by random mutagenesis techniques (e.g., DNA shuffling and ‘accelerated evolution’ (Stemmer, 1994; Matsumura et al., 2005)). On the other hand, analysis of the photorespiratory pathway of transgenic plants suggests that C₃ photosynthesis is unlikely to be improved by direct manipulation of levels of enzymes in the photorespiratory pathway (Heineke et al., 2001; Winzer et al., 2001; Bauwe and Kolukisaoglu, 2003; Boldt et al., 2005). Hence, over the last 25 years, considerable efforts have been made to try to use genetic engineering and...
In particular, we outline the three types of C₄ pathway and the evolution of C₄ plants as a CO₂ pump. Then, we highlight the improvement of photosynthetic carbon assimilation in C₄ plants using genetic engineering, including (i) the enhancement of C₄ carbon assimilation in normal and stressed conditions (e.g. high temperature and drought) by improving RuBP activation using non-C₄ genes; and (ii) the overexpression of C₄ cycle photosynthetic genes in transgenic C₃ plants (e.g. tobacco (Nicotiana tabacum), potato and rice), such as PEPC, NADP–ME and PCK. Finally, we provide a prospect of the challenges for enhancing photosynthetic carbon assimilation of C₄ plants using genetic engineering for tackling global warming.

**Carbon assimilation of C₃, C₄, and CAM plants**

**CO₂ assimilation in C₃ plants**

In light-independent or dark reactions the enzyme Rubisco captures CO₂ from the atmosphere, in a process that requires newly formed NADPH, which is the reduced form of NADP⁺. This is termed the Calvin-Benson Cycle (Figure 2A), in which three-carbon sugars are released, and which combine later to form sucrose and starch. This is the primary carboxylating mechanism in plants and is comprised of 13 reactions catalyzed by...
The fixation or reduction of CO₂ is a process in which CO₂ combines with a five-carbon sugar, RuBP, to yield two molecules of a three-carbon compound glycerate 3-phosphate (GP), also known as 3-phosphoglycerate (PGA) (Figure 2A). GP, in the presence of ATP (adenosine 5'-triphosphate) and NADPH from the light-dependent stages, is reduced to glyceraldehyde 3-phosphate (G3P). This product is also referred to as 3-phosphoglyceraldehyde (PGAL) or even as triose phosphate. Triose is a 3-carbon sugar. Most (5 out of 6 molecules) of the GA-3P produced is used to regenerate RuBP so the process can continue. The 1 out of 6 molecules of the triose phosphates not “recycled” often condenses to form hexose phosphates, which ultimately yield sucrose, starch and cellulose. The sugars produced during carbon metabolism yield carbon skeletons that can be used for other metabolic reactions like the production of amino acids and lipids (http://en.wikipedia.org/wiki/Photosynthesis).

The pathway of CO₂ assimilation is strictly regulated by the key enzymes of the pathway, which are regulated by metabolites and light (Singh and Malhotra, 2000). Carbon in the form of dihydroxy acetone phosphate (DHAP) leaves chloroplasts through a phosphate translocator and is converted to sucrose in the cytosol. The major regulatory enzymes in the sucrose biosynthetic pathway include cytosolic FBPase and SPS (Daie, 1993; Chen et al., 2005), both of which catalyze irreversible reactions and have a tight allosteric regulation by cytosolic metabolites and in vivo intermediates. The rate of sucrose synthesis is also regulated by a molecular mechanism through changes in the amount of these regulatory proteins and/or post translational modifications of the pre-existing enzyme. Sucrose synthesized in the mesophyll cells is then translocated to various sink tissues through the phloem. Further transport of sucrose from phloem parenchyma cells to the sieve tubes can occur symplastically via plasmodesmata without involving translocators, or apoplastically. From the apoplast to companion cells, sucrose transport proceeds via proton symport driven by a proton gradient (Heldt, 2005).

The primary CO₂ assimilation step in the C₃ pathway is catalysed by Rubisco. However, Rubisco also reacts with O₂ at its catalytic site (oxygenase reaction), leading to photorespiration, which plays a role in protecting photosynthesis from photoinhibition (Osmond and Grace, 1995), but it wastes fixed carbon as released CO₂ and

Table 2. Differences in morphological and physiological characters (MPC) and photosynthetic carbon assimilation (PCA) between C₃ and C₄ plants, cited from Luo (2001) and Pan (2008).

<table>
<thead>
<tr>
<th>MPC</th>
<th>C₃ plants</th>
<th>C₄ plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant type</td>
<td>Typical temperate plants</td>
<td>Typical tropic or subtropical plants</td>
</tr>
<tr>
<td>Biomass (kg dry weight/ha·a)</td>
<td>22000 ± 300</td>
<td>39000 ± 17000</td>
</tr>
<tr>
<td>Leaf structure</td>
<td>Non Kranz type</td>
<td>Kranz type</td>
</tr>
<tr>
<td>Chloroplast (Chl) distribution</td>
<td>Mesophyll cell</td>
<td>Mesophyll cell and bundle sheath cell</td>
</tr>
<tr>
<td>Chl structure</td>
<td>Typical Chl</td>
<td>Mesophyll cell is typical Chl, Chl in big and more bundle sheath cell is no thylakoid meshed cell acts photorespiration, and fixes CO₂ by C₄ pathway; bundle sheath cell acts dark reaction but photorespiration</td>
</tr>
<tr>
<td>Chl function</td>
<td>Acting photoreaction and dark reaction</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll a/b</td>
<td>2.8 ± 0.4</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>Activity of PEP carboxylase</td>
<td>0.30–0.35 [pmol/(mg Chl min)]</td>
<td>16–18</td>
</tr>
<tr>
<td>Location and reaction of</td>
<td>Chl in mesophyll cell:</td>
<td>Chl in mesophyll cell:</td>
</tr>
<tr>
<td>photosynthesis</td>
<td>C₅ + CO₂ → 2C₃ + ATP + [H] → C₅ + (CH₂O) + H₂O</td>
<td>C₅ (PEP) + CO₂ → C₅</td>
</tr>
<tr>
<td>Photosynthetic rate [mg CO₂/(dm²·h)]</td>
<td>15–35</td>
<td>40–80</td>
</tr>
<tr>
<td>CO₂ compensation point (mg/L)</td>
<td>30–70</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Optimal temperature of</td>
<td>15–25</td>
<td>30–47</td>
</tr>
<tr>
<td>photosynthesis (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photorespiration</td>
<td>Strong photosynthesis occurring in mesophyll cell, resulting in loss of 20–40% CO₂ assimilation</td>
<td>Weak photosynthesis occurring in bundle sheath cell</td>
</tr>
<tr>
<td>Transpiration coefficient</td>
<td>450–950</td>
<td>250–350</td>
</tr>
<tr>
<td>(g H₂O/g dry weight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCA</td>
<td>Only Calvin-Benson Cycle (C₃ pathway)</td>
<td>C₄ pathway and Calvin-Benson Cycle (C₄ pathway)</td>
</tr>
<tr>
<td>Major enzyme of CO₂ assimilation</td>
<td>RuBP carboxylase</td>
<td>PEP and RuBP carboxylase</td>
</tr>
<tr>
<td>Primary receptor of CO₂</td>
<td>RuBP</td>
<td>PEP</td>
</tr>
<tr>
<td>Primary product of CO₂ assimilation</td>
<td>PGA with three-carbon</td>
<td>OAA with four-carbon</td>
</tr>
</tbody>
</table>

© 2012 Informa Healthcare USA, Inc.
decreases the efficiency of photosynthetic CO₂ assimilation in C₃ plants (Leegood et al., 1995). Under current atmospheric conditions (0.036% CO₂, 21% O₂), up to 50% of fixed carbon is lost by photorespiration (Ogren, 1984; Häusler et al., 2002; Miyao, 2003), and the loss of photosynthetic carbon fixation from photorespiratory processes increases substantially with high temperatures and in drought conditions (Ogren, 1984; Brooks and Farquhar, 1985; Sharkey, 1988).

**CO₂ assimilation in C₄ plants**

The C₄ pathway evolved in hot, arid regions in response to increasing O₂ levels as a mechanism to increase the CO₂ concentration at the site of Rubisco (Sage, 2004). This allows Rubisco to operate closer to its maximal carboxylation rate, reducing the oxygenation reaction and thereby reducing the carbon losses caused by photorespiration (Hatch, 1987). C₄ plants chemically fix CO₂ in the cells of the mesophyll by adding it to the three-carbon molecule phosphoenolpyruvate (PEP), a reaction catalyzed by an enzyme called PEPC (Cooper and Wood, 1971; O’Leary, 1982) and which creates the four-carbon organic acid, oxaloacetic acid (OAA) (Figure 2B). OAA or malate synthesized by this process is then translocated to specialized bundle sheath cells where the enzyme, Rubisco, and other Calvin cycle enzymes are located, and where CO₂ released by decarboxylation of the four-carbon acids is then fixed by Rubisco activity to the three-carbon sugar 3-phosphoglyceric acids. The physical separation of Rubisco from oxygen-generating light reactions reduces photorespiration and increases CO₂ fixation and thus the photosynthetic capacity of the leaf (Taiz and Zeiger, 2006). For C₄ plants, the location of the site of decarboxylation, its distance from the mesophyll interface, and the physical arrangement of chloroplasts and mitochondria in the bundle sheath cell are as important to the efficiency of the process as the properties of the bundle sheath cell wall (von Caemmerer and Furbank, 2003). C₄ plants can produce more sugar than C₃ plants in high light and temperature conditions. Many important crop plants are C₄ plants including maize, sorghum, sugarcane, and millet.

According to the use of different C₄-acid decarboxylases (Hatch et al., 1975), three sub-groups of C₄ plants have been characterized: the NADP-ME type carries out C₄ acid decarboxylation in the chloroplast, while the NAD-ME type decarboxylates in the mitochondria and the PCK type decarboxylates predominantly in the cytosol (e.g. *Urochloa panicoides*, Finnegan et al., 1999) (Table 3, Figure 3). The classification of species into these 3 sub-types is not as straightforward as originally thought, and particularly in the NADP-ME type there is
Table 3. Three types of C₄ pathway.

<table>
<thead>
<tr>
<th>Type</th>
<th>C₄ acid entering bundle sheath cells</th>
<th>Decarboxylate location</th>
<th>Decarboxylase</th>
<th>C₄ acid returning to mesophyll cell</th>
<th>Plant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP-ME</td>
<td>Malate</td>
<td>Chloroplast</td>
<td>NADP-ME</td>
<td>Pyruvate</td>
<td>Maize, sugar cane, sorghum, etc.</td>
</tr>
<tr>
<td>NAD-ME</td>
<td>Aspartate</td>
<td>Mitochondria</td>
<td>NAD-ME</td>
<td>Alanine</td>
<td>Bristlegrasses, <em>Portulaca oleracea</em>, etc.</td>
</tr>
<tr>
<td>PCK</td>
<td>Aspartate</td>
<td>Cytosol</td>
<td>PCK</td>
<td>Pyruvate and Alanine</td>
<td><em>Leymus chinensis</em>, <em>Sporobolus virginicus</em>, etc.</td>
</tr>
</tbody>
</table>

Figure 3. Three decarboxylation types C₄ photosynthesis [von Caemmerer and Furbank (2003) adapted from Furbank et al. (2000)]. The path of carbon flow and the location of the site of CO₂ release in the decarboxylation reaction is shown in the three biochemical sub-groups of C₄ plants. Metabolite flow between cells is denoted by heavy arrows. Key enzymes are numbered as follows: 1, phosphoenolpyruvate carboxylase; 2, NADP malate dehydrogenase; 3, NADP malic enzyme; 4, pyruvate Pi dikinase; 5, 3-phosphoglycerate kinase/glyceraldehyde 3-phosphate dehydrogenase; 6, aspartate aminotransferase; 7, NAD malate dehydrogenase; 8, NAD malic enzyme; 9, alanine aminotransferase; 10, phosphoenolpyruvate carboxykinase; 11, mitochondrial NADH oxidation.

some flexibility in the identity of the C₄ acid transported and the decarboxylase itself (Repo and Hatch, 1976; Meister et al., 1996; Wingler et al., 1999). In the NAD-ME type, there are also examples of diversity in biochemistry and structure. For example, some species have NAD-ME type biochemistry but a “classical” PCK-type anatomy (Prendergast et al., 1987).

The spatial separation between the mesophyll cells and bundle sheath cells has been thought to be a prerequisite for an efficient CO₂-concentrating mechanism. However, single cell C₄ photosynthesis was found in some aquatic plants, which lack a “Kranz”-anatomy typical of C₄ plants, and were identified as being capable of inducing a C₄-like metabolism, including (probably) *Elodea canadensis* (de Groote and Kennedy, 1977), *Hydroilla verticillata* (Holaday and Bowes, 1980; Salvucci and Bowes, 1981, 1983; Spencer et al., 1996; Magnin et al., 1997), and *Egeria densa* (Casati et al., 2000). In addition, the terrestrial chenopod *Borszczovia aralocaspica*, a halophyte with succulent leaves adapted to a semi-dry environment, is likely to carry out C₄ metabolism in only one cell type (Freitag and Stichler, 2000).

**CO₂ assimilation of CAM plants**

Crassulacean acid metabolism (CAM) is performed by about 6% of vascular plant species (Winter and Smith, 1996), such as *Crassula ovata*, cactus, and most succulents, mainly growing in habitats that are affected by drought, such as deserts and tropical forest canopies (Wild et al., 2010). In CAM plants, CO₂ uptake occurs at night when relative humidity is high, thus reducing water loss from open stomata and enabling the plants to survive in dry conditions (Figure 4A). The diurnal cycle of CAM can be divided into four phases (Osmond, 1978) (Figure 4B): The period of night-time CO₂ fixation catalysed by PEPC and the accumulation of malate in the vacuole is denoted as phase I. With the onset of light, phase II follows, characterized by a decline in PEPC fixation and, eventually, a certain amount of direct C₃-like fixation by Rubisco. Phase III starts with stomatal closure in late morning, when malate is decarboxylated and the released CO₂ is re-fixed by Rubisco. In the afternoon, stomata may re-open for direct CO₂ fixation by Rubisco in phase IV. The duration of the phases and amplitudes of CO₂ uptake differ widely between species and environmental
conditions and, in particular, additional direct fixation of CO₂ by Rubisco (phases II and IV) is highly variable (Dodd et al., 2002).

In phase I, CO₂ is attached to the C₃ molecule PEP derived from the breakdown of storage carbohydrates (usually starch or soluble sugars), which results in the formation of the C₄ molecule malate; however, in contrast to C₄ metabolism, CAM physically separates CO₂ fixation to PEP from the Calvin cycle, and only temporally separates these two processes. CAM plants need to maintain a high pool of easily available carbohydrates for photosynthesis. A low level of carbohydrates for PEP generation may lead to limited fixation of CO₂ by PEPC (Dodd et al., 2003), which, in turn, would further reduce carbohydrate stocks. Thus, the maintenance of CAM, and, in consequence, the ability to cope with unfavorable environmental conditions, depends on the availability of these carbohydrates. This necessity to maintain large carbohydrate stocks, together with the high energy demand caused by conversions between metabolites and intracellular transport processes, limits the productivity of CAM plants (Wild, 2010). However, some CAM plants exhibit growth rates close to C₃ plants (Nobel, 1991), therefore, there are highly coordinated control mechanisms that regulate the competition between carbohydrate storage and growth. By investigating the flux of carbon from PEPC and direct Rubisco fixation to different leaf carbon pools and to phloem sap over the diurnal cycle in CAM plant Kalanchoe daigremontiana, Wild (2010) showed (i) a high sucrose turnover, fed by nocturnal starch degradation and direct Rubisco fixation during the day; and (ii) a detailed dissection of the carbon fixation and mobilization pattern, revealed that direct fixation of Rubisco during light accounted for 30% of phloem sucrose, but only 15% of fixed carbon, which indicates that carbon from direct Rubisco fixation was preferentially used for leaf export.

Evolution of C₄ photosynthetic genes
C₄ photosynthetic genes may be specific for C₄ plants (Hatch, 1987), because the activities of the corresponding enzymes are low in C₃ plants and their kinetic properties are usually different from those of C₃ enzymes (Svensson et al., 1997; Dong et al., 1998). However, recent studies revealed that C₃ plants have at least two different types of genes, one encoding enzymes of “housekeeping” function and the other very similar to the C₄ genes of C₄ plants, though expression of the latter is very low or even undetectable in C₃ plants (Miyao, 2003). Hence, the C₄ genes may have evolved from a set of preexisting counterpart genes in ancestral C₃ plants, with modifications in the expression level in the leaves and kinetic properties of the enzymes (Ku et al., 1996). The C₄ genes in C₃ plants and their homologues in C₄ plants are designated as C₄-specific and C₄-like genes, respectively (Miyao, 2003); in addition to C₄-specific or C₄-like genes, both C₃ and C₄ plants have other homologous genes for the housekeeping function, which are designated as C₃-specific genes. Among C₄ enzymes and plant species, the number of homologous genes and the evolutionary origins of C₄-specific genes are different (Monson, 1999). Modifications of the C₄-like genes required for functioning in the C₄ pathway probably share common features in all the C₄-specific genes examined so far (Miyao, 2003).

Improvement of photosynthetic carbon assimilation in C₃ plants using genes from the non-C₄ pathway
In C₃ plants, the following features of Rubisco place it centre stage in attempts to improve their photosynthetic performance. First, low Rubisco activity limits light-saturated photosynthesis under present atmospheric conditions (Chen and Xu, 2007; Suzuki et al., 2009).
Second, the limitation to photosynthetic CO₂ assimilation in hot and/or dry environments is dominated by Rubisco because CO₂ availability is restricted and photorespiration is stimulated (Raines, 2006). Finally, Rubisco is not only an inefficient enzyme with a low turnover number (Raines, 2006), but it also catalyses two competing reactions: carboxylation and oxygenation. The oxygenation reaction directs the flow of carbon through the photorespiratory pathway (Figure 5), which can result in a loss of between 30–50% of photosynthetic carbon fixation (Ogren, 1984; Häusler et al., 2002; Miyao, 2003).

The low activity and the competing reactions catalysed by Rubisco are major limitations to photosynthetic carbon assimilation in C₃ plants, these limitations can be overcome by introducing Rubisco with a higher catalytic rate and/or better able to discriminate between gaseous substrates (Parry et al., 2007). However, the results of approaches that have tried to improve catalytic properties of Rubisco and reduce photorespiration using transgenic engineering have been modest (Heineke et al., 2001; Winzer et al., 2001; Bauwe and Kolukisaoglu, 2003; Boldt et al., 2005; Matsumura et al., 2005; Raines, 2006). In contrast, many studies have been conducted to improve photosynthetic carbon assimilation in C₃ plants using genes from non-C₄ plants (Table 4) (Chen and Xu, 2007; Feng et al., 2007; Feng et al., 2009; Kumar et al., 2009; Suzuki et al., 2009), some of which expressed the FBP/SBPase (Miyagawa et al., 2001; Tamoi and Shigeoka, 2005), ictB (Lieman-Hurwitz et al., 2003; 2005), and cytochrome c₆ genes (Chida et al., 2007); all showed positive effects on photosynthesis in transgenic C₃ plants.

Miyagawa et al. (2001) first reported the expression of a single plastid-targeted enzyme that improved carbon assimilation and growth in transgenic tobacco plants. Transgenic plants expressing a cyanobacterial FBP/SBPase targeted to chloroplasts showed enhanced photosynthetic efficiency and growth characteristics under atmospheric conditions (360 ppm CO₂). Compared to wild type (WT) tobacco, final dry matter, and photosynthetic CO₂ fixation of the transgenic plants were 1.5- and 1.24-fold higher, respectively. Transgenic tobacco also showed a 1.2-fold increase in initial activity of Rubisco compared to WT plants; levels of intermediates in the Calvin cycle and the accumulation of carbohydrates were also higher than those in WT plants.

Under many environmental conditions plant photosynthesis and growth are limited by the availability of CO₂ at the site of Rubisco (Austin et al., 1989). Lieman-Hurwitz et al. (2003; 2005) expressed ictB, a gene involved in HCO₃⁻-accumulation in Synechococcus sp. PCC7942, in Arabidopsis thaliana and tobacco plants. Transgenic plants exhibited significantly faster photosynthetic rates than the WT under limiting, but not under saturating CO₂ concentrations. Growth of transgenic A. thaliana plants under low humidity was considerably faster than that of the WT. There was no difference in the amount of Rubisco or the activity of the enzyme activated in vitro between the WT and transgenic plants. In contrast, the in vivo Rubisco activity, without prior activation of the enzyme, in plants grown under low humidity was considerably higher in ictB-expressing plants than in their WT. The CO₂ compensation point in transgenic plants was lower than in WT, suggesting a higher CO₂ concentration in close proximity to Rubisco. This may explain the higher activation level of Rubisco and enhanced photosynthesis and growth in the transgenic plants. These data indicate the potential use of ictB for stimulation of crop yield. Nelson (1988) and Austin (1989) reviewed there are no generally known instances where genetic improvement in crop yield has been attributed by improved photosynthesis. For example, in a few cases, notably maize, where selection for high photosynthetic rate has been made (Crosbie et al., 1981), there has been a considerable response to selection (about 1.5% per generation).

![Figure 5. The relationship between the C₃ cycle and the photorespiratory cycle (Raines, 2006). Rubisco is the initiating enzyme in both of these pathways, carrying out the carboxylase reaction to form 3-PGA, which then enters the C₃ cycle; alternatively, the oxygenase reaction takes place forming p-glycolate, which is then metabolized via the photorespiratory pathway. The activities of three enzymes in this cycle have been reduced via either antisense studies: (1) glycine decarboxylase, (2) serine hydroxymethyl transferase (SHMT) or an insertional mutant and (3) glycerate kinase. Ru-5P, ribulose 5-phosphate; Sed-7P, sedoheptulose 7-phosphate; Fru-6P, fructose 6-phosphate; DHAP, dihydroxyacetone phosphate; GA-3P, glyceraldehyde 3-phosphate; RuBP, ribulose bisphosphate.](image-url)
### Table 4. Improvement of photosynthetic carbon fixation in C₃ plants using genes from non-C₄ plants.

<table>
<thead>
<tr>
<th>Target C₃ plants</th>
<th>Gene introduced</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>FBP/SBPase</td>
<td>Compared with wild-type tobacco, final dry matter and photosynthetic CO₂ fixation of the transgenic plants were 1.5-fold and 1.24-fold higher, respectively. Transgenic tobacco also showed a 1.2-fold increase in initial activity of Rubisco compared with wild-type plants.</td>
<td>Miyagawa et al., 2001</td>
</tr>
<tr>
<td>Arabidopsis thaliana and Nicotiana tabacum</td>
<td>ictB, a gene involved in HCO₃⁻-accumulation in Synechococcus sp. PCC7942</td>
<td>Transgenic plants exhibited significantly faster photosynthetic rates than the wild-types under limiting (Arabidopsis: 7 µmol CO₂ m⁻² s⁻¹ for transforms vs. 5.5 µmol CO₂ m⁻² s⁻¹ for non-transforms; tobacco: 11 µmol CO₂ m⁻² s⁻¹ for transforms vs. 9 µmol CO₂ m⁻² s⁻¹ for non-transforms) but not under saturating CO₂ concentrations. The CO₂ compensation point in the transgenic plants that express ictB was lower than in the wild-types (Arabidopsis: 40 µL/L for transforms vs. 46 µL/L for non-transforms; tobacco: 48 µL/L for transforms vs. 57 µL/L for non-transforms), suggesting that the concentration of CO₂ in close proximity to Rubisco was higher.</td>
<td>Lieman-Hurwitz et al., 2003; 2005</td>
</tr>
<tr>
<td>Tobacco</td>
<td>FBP/SBPase</td>
<td>The photosynthetic CO₂ fixation and the final dry matter under atmospheric conditions in the transgenic plants were higher than that in the WT plants. Their results suggest that both FBPase and SBPase involved in the regeneration of RuBP seem to be one of the limiting factors that participate in the regulation of the carbon flow through the Calvin cycle and the determination of the partitioning of carbon to end products.</td>
<td>Tamoi and Shigeoka, 2005</td>
</tr>
<tr>
<td>Rice</td>
<td>OsBP-73, a rice gene, encoding a novel DNA-binding protein with a SAP-like domain</td>
<td>The 29.3% decrease of leaf net photosynthetic rate in the transgenic rice with silenced OsBP-73 gene is related to both RuBP carboxylation and RuBP regeneration limitations, and the latter is a predominant limitation to photosynthesis.</td>
<td>Chen and Xu, 2007</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>An algal cytochrome c₆</td>
<td>At 60 d after planting, the plant height, leaf length and root length of the transformants were 1.3-, 1.1- and 1.3-fold those in the wild-type plants, respectively; the CO₂ assimilation capacity of the transgenic plants was 1.3-fold that of the WT. The results demonstrate that the growth and photosynthesis of Arabidopsis plants could be enhanced by the expression of the algal cytochrome c₆ gene.</td>
<td>Chida et al., 2007</td>
</tr>
<tr>
<td>Rice</td>
<td>SBPase</td>
<td>Transgenic plants accumulating SBPase in chloroplasts resulted in enhanced tolerance to salt stress at the young seedlings stage. Moreover, CO₂ assimilation in transgenic rice plants was significantly more tolerant to salt stress than in wild-type plants. Under salt stress, SBPase maintained the activation of Rubisco by providing more regeneration of the acceptor molecule RuBP in the soluble stroma and by preventing the sequestration of Rubisco activase to the thylakoid membrane from the soluble stroma, and, thus, enhanced the tolerance of photosynthesis to salt stress.</td>
<td>Feng et al., 2007</td>
</tr>
<tr>
<td>Rice</td>
<td>SBPase antisense under the control of the maize ubiquitin promoter.</td>
<td>Low N in addition to a 34% decrease in SBPase activity was sufficient to diminish photosynthesis and limit biomass production. Decreased SBPase activity may have reduced the N use efficiency of photosynthesis and growth and alter biomass allocation.</td>
<td>Feng et al., 2009</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>A chimeric activase comprised of the tobacco activase, but containing an Arabidopsis Rubisco recognition domain</td>
<td>Transgenic lines of rca mutant expressing a chimeric activase showed higher rates of photosynthesis (6.9 µmol CO₂ m⁻² s⁻¹) than the WT (6.4 µmol CO₂ m⁻² s⁻¹), and after a short exposure to higher temperatures (38°C for 2h) and they also recovered better, when they were returned to the normal temperature (22°C for 2h). Moreover, under extended exposure to moderately elevated temperature, the transgenic lines had higher biomass (6 g) and seed yield (202 mg) when compared with the WT plants (4.1 g and 50 mg, respectively).</td>
<td>Kumar et al., 2009</td>
</tr>
<tr>
<td>Rice</td>
<td>An overexpression of RBCS-sense</td>
<td>Although the overexpression of RBCS-sense led to an enhancement of Rubisco protein content in the uppermost (5.5 µmol m⁻² for transgenic plants vs. 4.5 µmol m⁻² for WT), fully expanded leaves, it does not result in increased photosynthetic rates or plant biomass, because of an apparent down-regulation in its activation state.</td>
<td>Suzuki et al., 2009</td>
</tr>
</tbody>
</table>
however, after five generations of selection, the lines with a high photosynthetic rate gave similar yields to the base population (Crosbie and Pearce, 1982). Similar conclusions have been reached in studies on soybean (Secor et al., 1982; Ford et al., 1983), and peas (Hobbs, 1986). In contrast, in Populus, high photosynthetic rate, estimated by a 14C method, was positively related to tree biomass (Isebrands et al., 1988). In contrast, there are some evidences of the positive effects of overexpressing C4 genes in C3 plants on photosynthetic rate and yield (Kurek et al., 2007; Kershanskyaya and Teixeira da Silva, 2010). For example, transgenic wheat plants exhibited a higher photosynthetic capacity (up to 40%) and a higher grain yield (up to 20–50%), compared to untransformed plants (Zhang et al., 2010).

Feng et al. (2007) showed that overexpression of SBPase was an effective method for enhancing salt tolerance in rice. This is because transgenic rice plants accumulating SBPase in chloroplasts resulted in enhanced tolerance to salt stress at the young-seedling stage. Moreover, CO2 assimilation in transgenic rice plants was significantly more tolerant to salt stress than in WT plants. Under salt stress, SBPase maintained the activation of Rubisco by providing more regeneration of the acceptor molecule RuBp in the soluble stroma and by preventing the sequestration of Rubisco activase (RCA) to the thylakoid membrane from the soluble stroma, and, thus, enhanced the tolerance of photosynthesis to salt stress.

The limitation imposed by Rubisco on C3 carbon fixation is exacerbated in high light and temperature conditions (Krapp et al., 1994; Stitt and Schulze, 1994), although growth in elevated CO2 can have a compensatory effect (Masle et al., 1993). Plant photosynthesis declines when the temperature exceeds its optimum range, particularly in temperate species like A. thaliana. Recent evidence (Kurek et al., 2007) indicates that the reduction in photosynthesis is linked to Rubisco deactivation due to the inhibition of RCA under moderately elevated temperature. To test the hypothesis that thermostable RCA can improve photosynthesis under elevated temperature, Kurek et al. (2007) used gene shuffling technology to generate several A. thaliana RCA1 (short isoform) variants exhibiting improved thermostability. WT RCA1 and selected thermostable RCA1 variants were introduced into an Arabidopsis RCA deletion line. In a long-term growth test at either constant 26°C or daily 4 h 30°C exposure, the transgenic lines with the thermostable RCA1 variants exhibited higher photosynthetic rates, improved development patterns, higher biomass, and increased seed yields compared to the lines expressing WT RCA1 and a slight improvement compared to untransformed Arabidopsis plants. Their results provide clear evidence that RCA is a major limiting factor in plant photosynthesis under moderately elevated temperature and a potential target for genetic manipulation to improve crop plant productivity under heat stress conditions.

On the other hand, at moderately high temperature, the decrease in photosynthetic rate may be attributed to a reduced ability of RCA to achieve optimum activation of Rubisco, leading to reduced RCA. In order to overcome this problem, Kumar et al. (2009) transformed the Arabidopsis rca (regulation carboxylation activity or RCA gene) mutant with a more thermostable, chimeric activase where a Rubisco recognition domain in the more thermostable tobacco activase was replaced with that from Arabidopsis. Transgenic lines expressing this activase showed higher photosynthetic rate than the WT after a short exposure to higher temperature and they also recovered more efficiently when they were returned to normal temperature. Moreover, under extended exposure to moderately elevated temperature of 30°C, the transgenic lines had higher biomass and seed yield, compared to WT plants.

**Overexpression of C4 cycle photosynthetic genes in transgenic C3 plants**

Based on (i) the limited factors of photosynthesis in C3 plants and (ii) high photosynthesis efficiency, high rates of biomass accumulation, and high water and N-use efficiency in C4 plants, biotechnologists have long been intrigued by the overexpression of different enzymes of the C4 pathway in C3 plants (Edwards et al., 2001; Leegood, 2002; Häusler et al., 2002; von Caemmerer and Furbank, 2003). Hence, individual or multiple enzymes (PEPC, PPDK, PCK, NADP-ME and NADP-MDH) of the C4 pathway have been overexpressed in different C3 plants (e.g. tobacco, potato, rice and Arabidopsis) (Table 5, Table 6). In this section, we highlight the success and limitations by transformation of individual or multiple genes, respectively.

**Single gene transformation**

Overexpression of a single enzyme (PEPC, PPDK, PCK and NADP-ME) of the C4 pathway has been reported in C3 plants of tobacco, potato, wheat, rice and Arabidopsis (Table 5). The efficient fixation of atmospheric CO2 by PEPC is a prerequisite for the insertion of a single cell C4 cycle in C3 plants. The cDNAs of the C4 type PEPC sequences for cloning were first obtained from maize (Izui et al., 1986) and Flaveria trinervia (Poetsch et al., 1991). Hudspeth et al. (1992) expressed the PEPC gene of maize in transgenic tobacco (N. tabacum). Where transcription was under the control of a maize PEPC gene promoter, a low level of aberrantly large PPC transcript was detected. Higher levels of maize PEPC transcript of the correct size were obtained with a chimeric gene construct containing a tobacco (Nicotiana plumbaginifolia) chlorophyll a/b binding protein gene promoter. The PEPC activity in the leaves of these transgenic plants was up to 2-fold higher than that of non-transformed plants. Biochemical analyses of these plants indicated that the transgenic plants had significantly elevated levels of titratable acidity and malic acid. However, these biochemical differences did
Table 5. Introduction single C₄ enzymes into C₃ plants.

<table>
<thead>
<tr>
<th>Target C₃ plants</th>
<th>Enzyme genes induced (C₄ plants)</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>PEPC (Maize)</td>
<td>Transgenic plants showed 2-fold higher PEPC activities than that of control plants (97 vs. 48 Chl-h), significantly elevated levels of titratable acidity (16 vs. 10 µeq/g fresh wt) and malic acid (8.9 vs. 4.5 µmol/mg Chl), but no physiological changes with respect to photosynthetic rate or CO₂ compensation point.</td>
<td>Hudspeth et al., 1992</td>
</tr>
<tr>
<td></td>
<td>PPDK (Mesembryanthemum crystallinum)</td>
<td>Transgenic plants have 125–166% of PPDK activity relative to WT, produced more seeds per seed capsule (149 for transformants vs. 100 for WT) and heavier seed capsules than the WT (13.21 g/100 seed capsules for transformants vs. 11.99 g/100 seed capsules for WT).</td>
<td>Sheriff et al., 1998</td>
</tr>
<tr>
<td>Potato</td>
<td>PPDK (Maize)</td>
<td>PPDK activity in the leaves of transgenic potatoes was up to 5.4-fold higher than that of control potato plants (WT and treated control plants), but only a small effect on the total photosynthetic characteristics of the transgenic plants.</td>
<td>Ishimaru et al., 1998</td>
</tr>
<tr>
<td></td>
<td>PEPC (Corynebacterium glutamicum)</td>
<td>Elevated CO₂/O₂ ratios in the mesophyll are concomitant with a more favoured carboxylation/oxygenation ratio of Rubisco.</td>
<td>Häusler et al., 1999</td>
</tr>
<tr>
<td></td>
<td>PEPC (Orynebacterium glutamicum)</td>
<td>Overexpression of PEPC gene resulted in a 3- to 6-fold induction of endogenous cytosolic NADP-ME and an increase in the activities of NAD-ME (3-fold).</td>
<td>Häusler et al., 2001</td>
</tr>
<tr>
<td></td>
<td>NADP-ME (Flaveria pringlei)</td>
<td>There were no changes in enzyme activity.</td>
<td>Häusler et al., 2001</td>
</tr>
<tr>
<td>Rice</td>
<td>PEPC (Maize)</td>
<td>Most transgenic rice plants showed high-level expression of the maize gene; the activities of PEPC in leaves of some transgenic plants (0.04 to 6.76 µmol/mg/min) were 2- to 3-fold higher than those in maize (2.25 µmol/mg/min). Under atmospheric conditions, the photosynthetic rates of transgenic plants were comparable to those of the two untransformed rice cultivars.</td>
<td>Ku et al., 1999; 2000</td>
</tr>
<tr>
<td></td>
<td>PPDK (Maize)</td>
<td>Transgenic plants have higher photosynthetic rates (up to 35%) than untransformed plants, which is at least in part due to an enhanced stomatal conductance and a higher internal CO₂ concentration.</td>
<td>Ku et al., 2000</td>
</tr>
<tr>
<td></td>
<td>PCK (Urochloa panicoides)</td>
<td>20% of the radioactivity was incorporated into 4C compounds (malate, oxaloacetate, and aspartate) in excised leaves of transgenic plants, as compared with about 1% in excised leaves of control plants, indicating that the ectopic expression of PCK in rice chloroplasts was able to change the carbon flow in mesophyll cells into a C₄-like photosynthetic pathway.</td>
<td>Suzuki et al., 2000</td>
</tr>
<tr>
<td></td>
<td>NADP-ME (Maize)</td>
<td>A 20- to 70-fold increase in NADP-ME activity may have caused abnormal chloroplasts with agranal thylakoid membranes. They postulate a high level of chloroplastic NADP-ME activity could strongly affect the development of chloroplasts.</td>
<td>Takeuchi et al., 2000</td>
</tr>
<tr>
<td></td>
<td>PPKD (Maize)</td>
<td>The intron (s) or the terminator sequence of the maize gene, or a combination of both, was necessary for high-level expression.</td>
<td>Fukayama et al., 2001</td>
</tr>
<tr>
<td></td>
<td>NADP-ME (Maize)</td>
<td>In transformants, the accumulation of the maize C₄-specific NADP-ME led to bleaching of leaf color and growth hindrance in rice plants under natural light. These deteriorative effects resulted from enhanced photoinhibition of photosynthesis due to 30-fold increase of NADP-ME activity relative to non-transformants inside the chloroplast by the action of the maize enzyme.</td>
<td>Tsuchida et al., 2001</td>
</tr>
<tr>
<td></td>
<td>PEPC (Maize)</td>
<td>PEPC did not contribute significantly to the photosynthetic CO₂ fixation of transgenic rice plants. Rather, it slightly lowered the CO₂ assimilation rate (7.3%). Overproduction of PEPC did not directly affect photosynthesis significantly, but it suppressed photosynthesis indirectly by stimulating respiration in the light.</td>
<td>Fukayama et al., 2003</td>
</tr>
<tr>
<td></td>
<td>PEPC (Maize)</td>
<td>The transgenic rice (0.25U/mg protein) showed 14- to 60-fold higher PEPC activity than control plants (0.02U/mg protein), which facilitates provision of carbon to the anaplerotic pathway, biosynthesis of amino acids and prevents the accumulation of starch.</td>
<td>Suzuki et al., 2006</td>
</tr>
</tbody>
</table>
not produce any significant physiological changes with respect to the photosynthetic rate or CO₂ compensation point.

Ishimaru et al. (1998) overexpressed a C₄ maize PPDK gene in C₃ transgenic potato. PPDK activity in the leaves of transgenic potatoes was up to 5.4-fold higher than that of the control plants (WT and treated control plants). In the transgenic plants, PPDK activity in the leaves was negatively correlated with pyruvate content and was positively correlated with malate content. A significant increase in the δ¹³C value was observed in the transgenic plants, suggesting a certain contribution of PEPC as the initial acceptor of atmospheric CO₂. Their results suggested that elevated PPDK activity may alter carbon metabolism and lead to a partial operation of C₄-type carbon metabolism. However, since parameters associated with CO₂ gas exchange were not affected, the altered carbon metabolism had only a small effect on the total photosynthetic characteristics of the transgenic plants.

Fukayama et al. (2001) introduced the C₄-Pdk gene encoding the C₄ enzyme PPDK of maize (Zea mays cv Golden Cross Bantam) into the C₃ plant, rice (Oryza sativa cv Kitaake). When the intact maize C₄-Pdk gene, containing its own promoter and terminator sequences and exon/intron structure, was introduced, the PPDK activity in the leaves of some transgenic lines was greatly increased, in one line reaching 40-fold more than that of the WT plants. In a homozygous line, the PPDK protein accounted for 35% of total leaf-soluble protein or 16% of total leaf nitrogen. In contrast, introduction of a chimeric gene containing the full-length cDNA of the maize PPDK fused to the maize C₄-Pdk promoter or the rice Cab promoter only increased PPDK activity and protein level slightly (not exceeding 5-fold). Their results indicate that the intron(s) or the terminator sequence of the maize gene, or a combination of both, is necessary for high-level expression.

Zhang et al. (2010) also introduced the intact maize C₄-Pdk gene into rice (Oryza sativa L. indica “IR64”). Expression of C₄-Pdk in most transgenic rice lines resulted in the increase of CO₂ assimilation rates compared to untransformed control plants. Most of the transformants showed higher photosynthetic activities than the WT plants. In addition, the photosynthesis rates of some transgenic IR64 lines also increased in the greenhouse. Molecular analysis revealed that the intact maize C₄-Pdk gene integrated in the rice genome and affected the phenotypes of plants, particularly tillers, and enhanced the yield of transgenic IR64 rice plants in the greenhouse.

Kershanskaya and Teixeira da Silva (2010) introduced the maize C₄-specific PEPC gene into wheat. Their results showed that transgenic plants exhibited a higher photosynthetic capacity (up to 40%), an increase in PEPC enzyme activity (up to 6-fold), the appearance of a C₄ Kranz leaf anatomy structure, and a higher grain yield (up to 20–50%), compared to untransformed plants.

So far, there has been significant progress in the overexpression of the key enzymes of C₄-type biochemistry in transgenic C₃ plants, but the above efforts to overexpress the individual enzymes of C₄ photosynthesis in C₃ plants (rice, tobacco and potato) have produced modest results, and it is still uncertain whether this approach will be sufficient to improve the photosynthetic rate.

### Double transformation

Although overproduction of a single C₄ enzyme can alter the carbon metabolism in C₃ plants, only few examples

<table>
<thead>
<tr>
<th>Target C₃ plants</th>
<th>Enzyme genes induced (C₄ plants)</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>PEPC (Maize)</td>
<td>Transgenic plants exhibit a higher photosynthetic capacity (up to 40%) than untransformed plants; high grain yield increased up to 25–50% in transgenic plants in comparison with WTs.</td>
<td>Kershanskaya and Teixeira da Silva, 2010</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>NADP-ME</td>
<td>The transformants did not show any differences in morphology and development when grown in long days; however, dark-induced senescence progressed more rapidly in transgenic plants compared to the WT; malate and fumarate are important forms of fixed carbon that can be rapidly metabolized under stress conditions in Arabidopsis.</td>
<td>Fahnenstich et al., 2007</td>
</tr>
<tr>
<td>Wheat</td>
<td>PEPC (Maize)</td>
<td>Compare to WT (21.22 µmol CO₂ m⁻² s⁻¹), the photosynthesis rate of most of transgenic lines (21.26–26.80 µmol CO₂ m⁻² s⁻¹) was increased.</td>
<td>Zhang et al., 2010</td>
</tr>
<tr>
<td>PPDK (Echinochloa)</td>
<td>Enzyme activity was elevated 1- to 11-fold. However, no appreciable change demonstrated in carbon assimilation of the transgenic upland rice though increased photo-inhibition was noted under high light intensity.</td>
<td>Wang and Li, 2008</td>
<td></td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>PPDK (Maize)</td>
<td>Enzyme activity increased, in one line reaching 40-fold more than that of the WT plants. Furthermore, PPDK transgenic rice produced 22% more grains than the WT plants.</td>
<td>Jiao, 2008</td>
</tr>
<tr>
<td>PPDK (Maize)</td>
<td>The transformants showed enhanced photosynthesis rate (24.2–25.4 µmol m⁻² s⁻¹ vs. 19.6 of control plants) at high temperature (35–41°C) but without any improvement of yield.</td>
<td>Bandypadhyay et al., 2007</td>
<td></td>
</tr>
</tbody>
</table>

© 2012 Informa Healthcare USA, Inc.
in the greenhouse showed positive effects on photosynthesis. Transgenic C₃ plants overproducing multiple enzymes are now being produced for improving their photosynthetic performance (Table 6), including the combinations of PEPC and NADP-ME (Lipka et al., 1999; Häusler et al., 2001), as well as PEPC and PPDK (Streatfield et al., 1999; Matsuoka et al., 2000; Ku et al., 2001; Yuan et al., 2007; Jiao, 2008; Zhang et al., 2008).

Table 6. Introduction multiple C₄ enzymes into C₃ plants.

<table>
<thead>
<tr>
<th>C₃ plants</th>
<th>Enzyme genes induced (C₄ pathway)</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>PEPC (Orynebacterium glutamicum) and NADP-ME (Flaveria pringlei)</td>
<td>Photorespiration in transgenic tobacco plants appeared to be diminished most in single PEPC or NADP-ME overexpressors rather than in double transformants (PEPC and NADP-ME).</td>
<td>Häusler et al., 2001</td>
</tr>
<tr>
<td>Potato</td>
<td>PEPC and NADP-ME</td>
<td>Double transformants with an additional 3–5-fold over-expression of Flaveria pringlei NADP-malic enzyme in the chloroplast showed a temperature-dependent decrease in the electron requirement for CO₂ assimilation, suggesting a slight suppression of photorespiration.</td>
<td>Lipka et al., 1999</td>
</tr>
<tr>
<td></td>
<td>PCK and PEPC</td>
<td>Transformants of two enzymes genes resulted in improved photosynthetic properties and relief of pleiotropic effects.</td>
<td>Peterhänsel et al., 2008b</td>
</tr>
<tr>
<td>Rice</td>
<td>Maize PEPC and PPDK</td>
<td>The photosynthetic rates of plants with both maize enzymes are up to 35% higher than those of untransformed plants. The grain yield is about 10–30% higher in PEPC and 30–35% higher in PPDK transgenic rice plants relative to untransformed plants. These results suggest that introduction of C₄ photosynthesis enzymes into rice has a good potential for enhancing the crop’s photosynthetic capacity and yield.</td>
<td>Ku et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The transgenic rice plants expressing both PEPC and PCK showed up to 55-fold higher PEPC activity than the control plants and PCK activity comparable to the transgenic rice plants expressing only PCK, but elevated PEPC activity in combination with PCK activity contributed little to C₄-like carbon flow.</td>
<td>Suzuki et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The photosynthesis rate and PEPC enzyme activity of transgenic homozygous lines increased by 19–40% and 1.5 to 4.9-fold, respectively compared with those of the WT, indicating that these transgenic rice lines are of the great potential values in increasing photosynthesis for hybrid rice breeding.</td>
<td>Yuan et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The activities of PEPC and PPDK increased up to 110- and 40-fold more, respectively, than those of nontransgenic rice. High expression of C₄ enzymes did not result solely from the high expression activity of the maize gene, since the introduction of a maize PPDK cDNA fused to the maize Pdk promoter or rice Cab promoter did not lead to high expression of PPDK. In some transgenic rice plants carrying the intact maize gene, the level of PPDK protein amounted to 35% of total leaf-soluble protein. The high expression of each C₄ enzyme altered metabolism slightly but did not seem to increase the photosynthetic efficiency of transgenic rice leaves.</td>
<td>Matsuoka et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Untransformed rice possessed all the C₃ photosynthetic enzymes but their activities are very low. PCR indicated that the PEPC and PPDK genes from maize were transformed into common rice. The PEPC and PPDK activities of the C₄ enzymes in PEPC+PPDK transgenic rice were 18 and 3-fold of non-transformants.</td>
<td>Zhang et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Overproduction individually of PPDK, MDH or ME did not affect the rate of photosynthetic CO₂ assimilation. The reduction in CO₂ assimilation in PEPC overproduction lines remained unaffected by overproduction of PPDK, ME or a combination of both; however, it was significantly restored by the combined overproduction of PPDK, ME, and MDH. Transgenic rice plants overproducing the four enzymes showed slight stunting. Comparison of transformants overproducing different combinations of enzymes indicated that overproduction of PEPC together with ME was responsible for stunting, and that overproduction of MDH had some mitigating effects.</td>
<td>Taniguchi et al., 2008</td>
</tr>
</tbody>
</table>
Lipka et al. (1999) transformed two potato lines using NADP-ME-cDNA constructs, one of which already overexpressed the PEPC from *Corynebacterium glutamicum*. Both genes were under the control of the constitutive 35S CaMV promoter. Increased levels of NADP-ME were found in chloroplasts of transformants. Expression of both genes led to a significantly reduced electron requirement for apparent CO₂ assimilation (e/A) at higher temperature. At low temperatures (15°C) 11 electrons per CO₂ were assimilated (e/A) in controls, single (PEPC or NADP-ME) and double (PEPC and NADP-ME) transformation. However, when the leaf temperature was raised to 36°C, the electron requirement of the double transformation (15 e/A) was 65% of controls or single transformation (23 e/A). Thus, the temperature-dependent increase in electron requirement was reduced in the double transformation, suggesting a suppression in the oxygenation reaction of Rubisco.

The introduction of a fully operational C₄ cycle might ultimately provide a clue as to whether C₃ photosynthesis can be improved or not (Häusler et al., 2002; Yuan et al., 2007). In transgenic rice plants overexpressing *Urochloa* PCK in combination with maize PEPC, PEPC added little to the effects already obtained with PCK alone (Suzuki et al., 2006). Transgenic lines of hybrid rice expressing PEPC and PPDK genes from maize showed that the photosynthesis rate and the PEPC enzyme activity of the transgenic homozygous lines increased by 19–40% and 1.5 to 4.9-fold, respectively, compared with those of the WT plants, indicating that the transgenic rice lines have great potential value in increasing photosynthesis for hybrid rice breeding (Yuan et al., 2007).

Four enzymes, namely, the maize C₄-specific PEPC, PPDK, the sorghum NADP-MDH, and the rice C₃-specific NADP-ME, were overexpressed in the mesophyll cells of rice plants independently or in combinations (Taniguchi et al., 2008). Overproduction individually of PPDK, NADP-MDH or NADP-ME did not affect the rate of photosynthetic CO₂ assimilation, while in the case of PEPC it was slightly reduced. The reduction in CO₂ assimilation in lines overexpressing PEPC remained by overexpression of PPDK, NADP-ME, or their combination, although it was significantly restored to levels comparable to or slightly higher than those of non-transgenic rice by the combined overexpression of PPDK, NADP-ME, and NADP-MDH. The extent of the restoration of CO₂ assimilation was more marked at higher CO₂ concentrations; however, overexpression of the four enzymes in combination did not act to concentrate CO₂ inside the chloroplast, and transgenic rice plants overproducing the four enzymes showed slight stunting. A comparison of transformants overexpressing different combinations of enzymes indicated that overexpression of PEPC together with NADP-ME was responsible for the stunting, and that overexpression of NADP-MDH had some mitigating effects.

**Factors that affect the expression levels and efficiency of C₄ enzymes in C₃ plants**

Expression of a transgene is hampered by many mechanisms including positional effects (Helmin, 1998), silencing (Chandler and Vaucheret, 2001) and rearrangement (Hiei et al., 1994) of the transgene or factors associated with the transformation protocol (Karami, 2008). For example, rearrangements occurred frequently during gene transfer mediated by *Agrobacterium tumefaciens* in a study in which C₄ enzymes were overproduced (Miyao, 2003). In the next section, we discuss the factors influencing the expression levels and efficiency of C₄ enzymes in C₃ plants.

First, phylogenetic distance may hamper the expression of genes from C₄ plants in the leaves of C₃ plants (Häusler et al., 2002). Not only incorrect initiation and termination of transcription, but also incorrect splicing could occur when genes from monocots are introduced into dicots (Goodall and Filipowicz, 1991). For example, the intact maize C₄-specific PEPC gene was not expressed at high levels in tobacco leaves, because of incorrect transcription initiation (Hudsphett et al., 1992). A major problem has been in generating transgenic lines with enzyme activities sufficient to support the high fluxes necessary to sustain the C₄ pathway (Häusler et al., 2002).

Second, conventional techniques for overproduction of transgenes, namely, the introduction of a chimeric gene containing cDNA for a C₄ enzyme, fused to a strong promoter alone or together with enhancer sequences, only can increase the several fold activity of C₄ enzymes in the leaves of C₃ plants exceeding that of non-transformants (Matsuo et al., 2001).

Finally, at present, it still remains obscure if foreign PEPC is expressed in guard cells of these transformants (Miyao et al., 2003). A research group claims that the photosynthetic rate under saturating light can be greatly increased by overexpression of the maize PEPC in transgenic rice plants (Jiao et al., 2001); however, their results need to be reconsidered, because the correlation between the photosynthetic rate and the level of the PEPC protein in transgenic rice leaves has not yet been confirmed. In contrast, some groups reported negative effects on photosynthesis, and the photosynthetic rate was slightly lowered by overexpression (Agarie et al., 2002). It is more likely that the suppression of photosynthesis results from the enhanced respiration by elevated PEPC (Miyao et al., 2003).

**Challenges and prospects**

**Challenges of improving carbon assimilation of C₃ plants using genetic engineering**

To improve the photosynthetic performance of C₃ plants for global warming, botanists and plant breeders have been interested in enhancing the low activity of Rubisco, decreasing photorespiration, in which up to 50% of the fixed carbon may be lost, and introducing enzymes of the C₄ pathway into C₃ plants.
Overexpression of the non-C₄ genes FBP/SBPase, ictB and cytochrome c₅ showed positive effects on photosynthetic efficiency and growth characteristics in C₃ plants. However, over the last 28 years, efforts to over-express individual or multiple C₄ enzymes in C₃ plants, specifically rice, tobacco, potato, and Arabidopsis, have produced mixed results, and it is still uncertain whether this approach will be sufficient to improve the photosynthetic rate (Sheehy et al., 2000). In addition, it seems unlikely that attempts to introduce single cell CO₂-concentrating mechanisms will be successful without simultaneously introducing some of the structural characteristics of C₄ photosynthesis (Matsuoka et al., 2001), that is, a compartment in which CO₂ could be concentrated (Leegood, 2002).

Under saturating CO₂ conditions, Rubisco does not limit photosynthesis (Masle et al., 1993; Makino et al., 2000; Long et al., 2004), and the limitation of photosynthesis is shifted to the RuBP regeneration capacity of the C₃ cycle (Harrison et al., 1998; Haake et al., 1999; Henkes et al., 2001; Raines, 2003; Miyagawa et al., 2001; Lefebvre et al., 2005). Hence, in the future, in a high CO₂ world, Rubisco will not be limiting, and it is likely to be enzymes of the regenerative phase of the cycle that will be targets for improving C₃ carbon fixation (Raines, 2006). FBP/SBPase enzymes of the C₄ cycle have been identified as being important control points, and two examples of over-expression of these enzymes have demonstrated that photosynthesis can be increased in these plants, leading to increased yield. However, what has not yet been tested is the impact of these manipulations on the photosynthesis and yield of plants grown in elevated CO₂ conditions.

Overproduction of C₄ enzymes in C₃ plants can be achieved by introducing appropriate gene constructs (Mann, 1999; Surridge, 2002; Miyao, 2003). So far, most approaches have aimed at introducing the highest possible activities of the respective enzymes in transgenic C₃ plants (Häusler et al., 2002). This is reasonable because these enzymes are expressed to very high levels in the leaves of C₄ plants, for example, PEPC activity in maize is 1186 Chl-h, but only 48 Chl-h in tobacco (Hudspeth et al., 1992); Total NADP-ME activity in Flaveria trinervia (C₄) is 737.9 mU mg⁻¹, but only 14.3 mU mg⁻¹ for Flaveria aangustifolium (C₃) and 13.1 mU mg⁻¹ for Flaveria pringlei (C₃) (Häusler et al., 2002). It is also necessary to screen a number of transgenic plants to obtain the desired expression level of a C₄ enzyme and to confirm the enzyme location in the leaves of C₃ plants. However, whether this pathway can operate with desirable effects on C₄ photosynthesis or not is a matter of controversy (Edwards, 1999; Häusler et al., 2002; Leegood, 2002), and a high expression of the introduced enzyme is not necessarily linked with high in vivo activities. Considering the C₄ pathway operating in a single cell found in some aquatic organisms (for a review see Leegood, 2002), it might be possible that the C₄-like pathway could support C₃ photosynthesis under some stress conditions such as drought, in which CO₂ availability is limited. It appears to be useful and perhaps even necessary to engineer more of the C₄-cycle enzymes and/or the respective promoters to be better adapted to the specific requirements of a C₄-like cycle in a ‘C₃ environment.’ Thus, it raises further questions as to whether the C₄ genes encoding the PPDK, NADP-ME and PCK enzymes should be introduced in one genome in addition to PEPC either by transgene-breeding or by multigene transformation to achieve an overall increase in yield under natural field conditions (Bandyopadhyay et al., 2007). However, Taniguchi et al. (2008) considered that rice is not a good plant material for an approach to introduce the C₄-like pathway with respect to increased production, because (i) overproduction of a single C₄ enzyme (PEPC, PPDK, NADP-MDH or NADP-ME) did not improve photosynthesis of rice; (ii) overproduction of the maize PEPC inhibited photosynthesis through stimulation of respiration in the light and reduction of Rubisco activity (Fukayama et al., 2003); (iii) the combinations of C₄ enzymes examined, only overproduction of all the four enzymes had some positive effects on photosynthesis, and only to a limited extent; and (v) transgenic rice plants overproducing the four enzymes showed stunting.

Hence, the mixed results of overexpression of C₄ enzymes in C₃ plants do not confirm or eliminate the possibility of improving photosynthesis of C₃ plants by this approach. Furthermore, it is important for the improvement of photosynthetic carbon assimilation in C₃ plants to (i) find an alternative approach to reducing photorespiration, such as the introduction of pyrenoids or carboxysomes into the chloroplasts of C₃ plants (Leegood, 2002), the movement of the location of photorespiratory glycone metabolism to the bundle-sheath (Monson and Rawsthorne, 2000; Winzer et al., 2001), and the improvement of forms of Rubisco, notably those from rhodophyte algae in which the relative specificity for CO₂ compared to O₂ is higher than that of the higher plant Rubisco (Whitney et al., 2001); (ii) understand the complex factors that regulate the development of the different cell types in C₃ plants, in which anatomical characteristics should be incorporated, although this may only be controlled by a few genes, which have not yet been identified; (iii) determine whether photosynthesis could be substantially improved by changing the relative level of expression of the introduced enzymes; (iv) develop an efficient PEPC system towards increasing the productivity of C₃ plants; (v) conduct more field studies to evaluate whether there will be differences in productivity with increasing photosynthesis rate under normal planting densities and natural field conditions.

In addition, improvement of photosynthetic carbon assimilation of C₄ plants using genetic engineering has only been reported in tobacco, potato, rice, and Arabidopsis. For global warming, drying and salinization (Rozema and Flowers, 2008), scientists have long been developing xero-halophytes used for desertic or
marginal lands where no other plants are able to grow and to save good quality water for human consumption, provide human nutrition and green biofuel, reduce CO₂ emission, and assimilate and sequester carbon into biomass. Several tolerant plants with potential interest for agriculture and environmental management have already been identified, such as Atriplex halimus (Lutts et al., 2004), Kosteletzya virginica (Ruan et al., 2008), and Salicornia bigelovii (Zerai et al., 2010). However, most orphan xero-halophyte plants have relatively no genetic information compared to conventional crops with known genetic information (e.g. genetic transformation system, sequences, QTLs, etc.). Hence, to improve photosynthetic carbon assimilation of xero-halophytes, sometime in the future, as an alternative to improvement of carbon assimilation using genetic engineering, enhance carbon fixation by environmental, physiological, and biological regulations will have great potential and we will highlight this topic in another review.

Declaration of interest

This work was jointly funded by the Keystone Project of the Agriculture Science and Technology Research Item of the Scientific and Technological Committee (No. 2007207005) of Liaoning Province of China, the Research Project of Higher Education of Education Commission of Liaoning Province of China (2009A150) and the Fundamental Research Funds for the Central Universities (DC10020102). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


