Regulation of Photosynthesis in C₃ and C₄ Plants: A Molecular Approach

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INTRODUCTION

Most plants use the C₃ pathway of photosynthesis, also called the photosynthetic carbon reduction cycle (PCR), shown in Figure 1A. C₃ plants have a single chloroplast type that performs all of the reactions that convert light energy into the chemical energy that is used to fix CO₂ and to synthesize the reduced carbon compounds upon which all life depends. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes primary carbon fixation, in which a five-carbon sugar phosphate, ribulose-1,5-bisphosphate (RuBP), and CO₂ are converted to two molecules of the three-carbon compound 3-phosphoglycerate (hence the name C₃). Phosphoglycerate is then phosphorylated and reduced by the products of the light reactions of photosynthesis (ATP and NADPH) to produce triose phosphate (TP). TP can be exported from the chloroplast via the chloroplast envelope phosphate (Pi) transporter to the cytosol and used in the synthesis of sucrose, which is then translocated throughout the plant (see Sonnewald et al., 1994), or it can be retained within the chloroplast for starch synthesis or recycling to RuBP. Rubisco also catalyzes the fixation of O₂ in a process known as photorespiration, which competes directly with fixation of CO₂. At air levels of CO₂, for every three CO₂ molecules fixed by Rubisco to form 3-phosphoglycerate, approximately one O₂ molecule is fixed, producing 3-phosphoglycerate and 3-phosphoglycolate (Figure 1A). Because 3-phosphoglycolate cannot be used in the PCR cycle, it must be recycled to phosphoglycerate via the photorespiratory pathway, expending ATP and NADPH. This competition between O₂ and CO₂ and the energy costs associated with recycling phosphoglycolate largely determine the efficiency of C₃ photosynthesis in air (Hatch, 1988; Woodrow and Berry, 1988).

The C₄ pathway is a complex adaptation of the C₃ pathway that overcomes the limitation of photorespiration and is found in a diverse collection of species, many of which grow in hot climates with sporadic rainfall. The C₄ pathway effectively suppresses photorespiration by elevating the CO₂ concentration at the site of Rubisco using a biochemical CO₂ pump. C₄ plants have two chloroplast types, each found in a specialized cell type. Leaves of C₄ plants show extensive vascularization, with a ring of bundle sheath (B) cells surrounding each vein and an outer ring of mesophyll (M) cells surrounding the bundle sheath. The development of this so-called Kranz anatomy and the cell-specific compartmentalization of C₄ enzymes are important features of C₄ photosynthesis (Hatch, 1988, and references therein). CO₂ fixation in these plants is a two-step process. Atmospheric CO₂ is initially fixed in the cytosol of M cells by phosphoenolpyruvate carboxylase (PEPC) to form the four-carbon dicarboxylic acid oxaloacetate (hence the name C₄), which is converted to malate or aspartate (Figure 1B). These C₄ acids then diffuse into the inner ring of B cells, where they are decarboxylated in the chloroplasts. The CO₂ produced is then refixed by Rubisco. The mechanism of decarboxylation in B chloroplasts varies among the three different C₄ types. We confine our discussion to the most extensively studied type, the NADP-malic enzyme (ME) type, which is named for its B cell decarboxylating activity.

The key feature of C₄ photosynthesis is the compartmentalization of activities into two specialized cell and chloroplast types. Rubisco and the C₃ PCR cycle are found in the inner ring of B cells. These cells are separated from the mesophyll cells and from the air in the intercellular spaces by a lamella that is highly resistant to the diffusion of CO₂ (Hatch, 1988). Thus, by virtue of this two-stage CO₂ fixation pathway, the mesophyll-located C₄ cycle acts as a biochemical CO₂ pump to increase the concentration of CO₂ in the bundle sheath an estimated 10-fold over atmospheric concentrations. The net result is that the oxygenase activity of Rubisco is effectively suppressed and the PCR cycle operates more efficiently. C₄ plants show higher rates of photosynthesis at high light intensities and high temperatures due to the increased efficiency of the PCR cycle (Hatch, 1988). In favorable environments, C₄ plants outperform C₃ plants, making them the most productive crops and the worst weeds. Maize, sugarcane, sorghum, and amaranth are examples of C₄ crops, and nutgrass (Cyperus rotundus), crabgrass (Digitaria sanguinalis), and barnyard grass (Echinochloa crusgalli) are some of the worst C₄ weeds.

Although the pathways of C₃ and C₄ photosynthesis are well established and the properties of individual enzymes are reasonably well understood, there remain several areas of
Figure 1. Simplified Schemes of the C3 and C4 Photosynthetic Pathways.

(A) C3 pathway (photosynthetic carbon reduction or PCR cycle), emphasizing the enzymes that have been manipulated using molecular genetic techniques. Carbon initially fixed by Rubisco is phosphorylated and reduced by the products of the light reactions (ATP and NADPH). The reduced three-carbon sugar-phosphate (triose phosphate, TP) can be either exported from the chloroplast for sucrose synthesis via the chloroplast envelope Pi transporter (Pi TRANS) or retained for starch synthesis or recycling to ribulose bisphosphate, the C3 acceptor for the Rubisco enzyme. CA, carbonic anhydrase, catalyzes $\text{HCO}_3^{-} \rightarrow \text{CO}_2$; RUBISCO, ribulose-1,5-bisphosphate carboxylase-oxygenase, catalyzes ribulose bisphosphate + CO$_2$ $\rightarrow$ 2X 3-phosphoglycerate or ribulose bisphosphate + O$_2$ $\rightarrow$ 3-phosphoglycerate + phosphoglycolate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase, catalyzes 1,3-diphosphoglycerate + NADPH $\rightarrow$ glyceraldehyde 3-phosphate + NADP$^+$; FBPase, fructose 1,6-bisphosphatase, catalyzes fructose bisphosphate $\rightarrow$ fructose 6-phosphate + Pi; PRK, phosphoribulokinase, catalyzes ribulose 5-phosphate + ATP $\rightarrow$ ribulose bisphosphate + ADP.

(B) C4 pathway, simplified to describe only the NADP-malic enzyme type, which transports malate from the mesophyll to the bundle sheath chloroplasts. Compartmentation of enzymes between the two cell types is shown. CO$_2$ (in the form of bicarbonate) is fixed by the enzyme PEPC to form the C4 acid oxaloacetate (OAA), which is reduced by NADPH from the light reactions to form malate (MAL), the C4 acid that is transported to the bundle sheath cells. Malate is decarboxylated in the bundle sheath cells, where the CO$_2$ released is fixed by the PCR cycle in much the same way as in C3 plants. The three-carbon compound pyruvate (PYR) diffuses back to the mesophyll, where it is phosphorylated by ATP to regenerate the carbon acceptor phosphoenolpyruvate (PEP). PEP, phosphoenolpyruvate carboxylase, catalyzes phosphoenolpyruvate + HCO$_3$ $\rightarrow$ oxaloacetate; MDH, malate dehydrogenase, catalyzes oxaloacetate + NADPH $\rightarrow$ malate + NADP$^+$; NADPME, NADP-malic enzyme, catalyzes malate + NADP$^+$ $\rightarrow$ pyruvate + NADPH; PWK, pyruvate orthophosphate dikinase, catalyzes pyruvate + ATP + Pi $\rightarrow$ phosphoenolpyruvate + AMP + PPi.
mous leaf cells in C₃ plants (Muller et al., 1980; Eckes et al., 1985). A signal from the developing chloroplast is required for high-level transcription of Cab and RbcS genes, even when light signals are fully activated (Oelmuller, 1989; Taylor, 1989b). This chloroplast signal may be at least partially responsible for spatial regulation of nuclear genes coding for chloroplast proteins.

Three main features distinguish C₄ plants from C₃ plants: (1) the differentiation of the two cell and chloroplast types, (2) the presence of an additional set of genes, and (3) a mechanism regulating the cell-specific expression of these additional genes. Very little is known about how the differentiation of the two cell types is regulated. The position of cells relative to the vein is known to determine fate, with those in close association with the vein becoming B cells and those at least one cell removed from the vein becoming M cells (Nelson and Langdale, 1992).

Genes encoding C₄ enzymes are, in most cases, members of small gene families. Other members of each gene family code for isozymic forms that perform nonphotosynthetic functions; these non-C₄ genes are usually expressed at low levels in a wide range of cells. C₄ enzymes are found at high levels in either B or M cells. Therefore, genes encoding C₄ enzymes must have evolved new programs of gene expression.

Two experimental approaches have been used to study the mechanisms regulating the cell-specific expression of C₄ gene. One is the use of gene and antibody probes to localize mRNAs and proteins at various stages in leaf development. These studies have shown that light induces high-level expression of C₄ genes, whereas cell-specific expression of some C₄ genes is controlled by a light-independent developmental program. However, for other genes, light controls both high-level expression and cell specificity. For instance, Sheen and Bogorad (1985) found that transcripts for both RbcS and rbcL (a chloroplast gene that codes for the Rubisco large subunit, LSU) are present in both B and M cells of etiolated maize leaves. Light is necessary to induce high levels of both transcripts and to suppress their accumulation in M cells. In contrast, light does not affect the spatial pattern of C₄ mRNA accumulation in amaranth leaves (Wang et al., 1993). However, RbcS and rbcL transcripts and proteins can be detected in both cell types at early stages of development but then become restricted to B cells (Wang et al., 1992). Accumulation of transcripts encoding C₄ enzymes appears to be cell specific in both plants (Nelson and Langdale, 1989), although Wang et al. (1992) detected transcripts coding for PEPC in both cell types of amaranth. However, as they pointed out, it is possible that the B cell transcripts encode a nonphotosynthetic isoform.

A complementary approach is to identify cis-acting DNA sequences responsible for cell-specific expression and to use these sequences to unravel the mechanisms that control high-level, cell-specific expression. Until recently, the lack of an efficient, stable transformation system for any C₄ plant has slowed progress using this approach. Therefore, one strategy has been to study the expression of C₄ gene promoters in transgenic C₃ plants to identify sequences responsible for high-level, organ-specific expression.

P. Westhoff and colleagues have used this strategy to determine how the C₄ members of the gene family encoding PEPC differ from members coding for nonphotosynthetic isoforms. Sequence comparisons indicated that in the C₄ dicot Flaveria trinervia, these Ppc genes fall into four subfamilies (Hermans and Westhoff, 1990). The PpcA subfamily codes for the C₄ isomerase, and its members are expressed at high levels in M cells. The C₃ species F. pringlei also has PpcA genes, but these are expressed at low levels in leaves, roots, and stems. The PpcA genes of these two species are similar to one another and can fuse to Ppc other subfamilies with the same species. Stockhaus et al. (1995) determined that the F. trinervia PpcA gene has unique cis-acting sequences that are at least partially responsible for its high-level expression in leaves by comparing the expression in transgenic tobacco of gene fusions in which 5' regions from F. trinervia and F. pringlei PpcA genes were fused to the β-glucuronidase (gusA) reporter gene. Sequences between -2118 and -500 of the F. trinervia PpcA promoter conferred high-level reporter gene expression in tobacco leaves, primarily in the palisade parenchyma, whereas promoter sequences from the F. pringlei PpcA conferred lower level gusA expression in roots and stems and very low expression in leaves. Whether the preferential expression of the F. trinervia PpcA gene in tobacco palisade parenchyma is due to M cell–specific control remains to be seen.

Similar experiments by M. Matsuoka and colleagues revealed that cis-acting sequences from the 5' regions of the maize RbcS, Pepc, and PpdK (which codes for M pyruvate orthophosphate dikinase, PPdK) genes confer light-regulated reporter gene expression in transgenic rice plants (Matsuoka et al., 1993, 1994). In all three cases, leaf M cells preferentially expressed the reporter gene, despite the fact that RbcS is B cell specific in maize. Although rice leaves have B cells, these contain only a few chloroplasts, which may account for the relatively greater expression in M cells. Novel cis-acting sequences at the 5' ends of some C₄ genes are therefore an important component of the mechanism that controls high-level leaf-specific and light-regulated expression of these genes.

Transient expression studies of C₄ gene promoter constructs introduced into leaf cells of C₄ plants have also provided clues about cell-specific regulation. Schaffner and Sheen (1992) showed that 5' sequences from a maize C₄ Pepc gene that are not present in a closely related non-C₄ gene confer high-level light-regulated expression in maize leaf protoplasts. Using microprojectile bombardment of maize leaf sections, Bansal and Bogorad (1993) have identified separate sites in the upstream region of a maize Cab gene that control light responses and cell specificity. Although Cab expression is not strictly cell specific in most C₄ plants, this particular gene is expressed primarily in M cells. This cell preference appears to be due to the combination of enhanced expression in M cells and suppression of B cell activity. Transient
expression experiments (T. Nelson, personal communication) have shown that 5' sequences of RbcS genes from *F. trinervia* (C4) and *F. pringlei* (C3) specify different expression patterns in *F. trinervia* leaves. These results imply that, for at least some C4 genes, the cis-acting sequences controlling cell specificity are located at the 5' end of the gene and are found only in the genes from C4 species.

To study further the mechanisms controlling C4 gene expression and the regulation of C4 enzyme activities (see following sections), we have developed an Agrobacterium-mediated transformation system for the C4 dicot *Flaveria bidentis* (Chitty et al., 1994). This system is reasonably fast and efficient, giving transformed plants 15 to 20 weeks after plant cocultivation, and it has already provided some interesting insights. In *F. bidentis*, two genes code for chloroplastic forms of ME, one of which, *MeA*, encodes the C4 form, which is expressed at high levels and in a light-regulated fashion in B cells (J.S. Marshall, J.D. Stubbs, and W.C. Taylor, unpublished data). *F. bidentis* plants stably transformed with a series of 5' *MeA* sequences fused to *gusA* show very low GUS activity in leaves but high levels in meristems and moderately high levels in stems and anthers (J.S. Marshall, J.A. Chitty, J.D. Stubbs, and W.C. Taylor, unpublished data). The longest of these constructs had 2.2 kb of 5' noncoding sequence, whereas the shortest had 0.39 kb. However, when 5.8 kb of sequence at the 3' end of *MeA* was added to the longest 5' construct, high-level leaf expression was attained. Preliminary analysis of primary transgenic plants suggests that this expression occurs primarily in B cells.

In *Flaveria* species, PPdK is encoded by a single gene, *Pdk* (E. Rosche and P. Westhoff, personal communication; C.J. Chastain, M. Matsuoka, and W.C. Taylor, unpublished data). *Pdk* encodes two different isoforms, a prevalent chloroplastic form located in M cells and a presumably nonchloroplastic form, the transcript of which is found in all organs at very low levels. Primary transformants with 1.5 kb of *Pdk* 5' sequence fused to *gusA* show high levels of GUS activity in leaves (W.C. Taylor, J.A. Chitty, and E. Rosche, unpublished observations). Transcription of the mRNA encoding the nonchloroplastic form of PPdK is driven by a promoter located in the large 6-kb intron of the *Pdk* gene; similar results have been obtained with the maize *Pdk* gene, which also encodes both a C4 isoform and a nonchloroplastic form (Giackin and Grula, 1990; Sheen, 1991).

These studies show that environmental cues and developmental programs that use positive and negative regulatory mechanisms control the accumulation of mRNAs coding for photosynthetic proteins. This regulation affects the timing of protein synthesis and its cellular localization, but it does not always directly control the quantities of proteins. Regulation of protein quantity also occurs at the stage of assembly of multimeric complexes in the chloroplast. For example, when synthesis of LSU is inhibited, unassembled SSU polypeptides are rapidly degraded so that stoichiometric amounts of both Rubisco subunits accumulate (Schmidt and Mishkind, 1983). The assembly process is poorly understood, especially its quantitative aspects. How the quantity of each enzyme is determined as the C3 and C4 pathways are assembled during chloroplast development is unknown. Regulation of enzyme quantity is also important in the mature leaf cell, because part of the plant's response to environmental changes can be to change absolute amounts of enzymes as well as their activities.

### METABOLIC ENGINEERING OF PHOTOSYNTHESIS

The response of photosynthetic rate to environmental parameters has been well characterized for a wide range of plants (for review, see Woodrow and Berry, 1988). From the kinetic characteristics of Rubisco (Figure 1), it has been possible to construct comprehensive models of photosynthesis that accurately predict the response of carbon fixation in C3 plants to particular ambient CO2 concentrations (von Caemmerer and Farquhar, 1981). In the case of C3 plants in air and saturating light, it is now clear that the photosynthetic rate is largely, although not entirely, governed by the amount and kinetic characteristics of Rubisco. The remaining fractional control of photosynthetic rate is distributed among the other enzymes, and the degree of limitation by each step presumably varies with environmental conditions, such as light regimes, in a manner that is difficult to assess (Figure 2). This kind of regulation occurs via covalent modification and allosteric effects on enzyme activity and in the short term (that is, hours) does not involve changes in the amount of enzyme.

Figure 1 illustrates the complexity of regulation in the photosynthetic system, showing potentially regulatory enzymes in both the C3 and C4 pathways. In C4 plants, determining which enzymes control flux is further complicated by the additional complexity of cell specialization. Superimposed upon the regulation of the PCR cycle enzymes is regulation of the enzymes involved in the CO2 concentrating mechanism of the C4 pathway. From in vitro studies, we know that the activities of a number of enzymes in the PCR cycle are capable of responding to changes in light and could potentially limit photosynthetic flux. This response occurs either indirectly, via changes in the stromal pH and Mg2+ concentration, both of which increase on illumination, or directly, by reduction/oxidation of the enzyme via the thioredoxin system, a signal transduction pathway responsive to the redox state of photosystem I (Figure 3; Buchanan, 1991). In the case of Rubisco, activation is mediated by a specific activating protein (Rubisco activase; Salvucci et al., 1985), which senses chloroplast energy status. Such a complex set of light-responsive regulatory mechanisms is necessary for two reasons. First, a crude on/off switch is required to prevent "futile cycles" occurring between respiratory and catabolic processes and the photosynthetic pathway, which share the same biochemical intermediates. Second, the major environmental variable that plants experience is short-term change in light intensity (light flecks, for example, are common in many closed canopy crops, forests, and grasslands). It appears that a multienzyme control mechanism has evolved...
Enzyme activity can be reduced to below wild-type levels by antisense suppression or elevated to above wild-type levels by overexpression. The effect on photosynthetic flux depends on whether the enzyme (a) is essentially “non-limiting” over a range of enzyme activities, (b) has control over flux but “co-limits” along with the activity of other enzymes, or (c) is classically “limiting.”

for conservation of metabolites within the PCR cycle and for intrinsic stability of the photosynthetic system during such transients, when the photosynthetic flux can change 10-fold or more in seconds (see Woodrow and Berry, 1988; Geiger and Servaites, 1994).

Although we have amassed a great deal of information on the regulatory properties of enzymes in vitro, evidence for their individual contributions to controlling photosynthetic flux in vivo is largely circumstantial. This is because it is often difficult to extrapolate regulatory properties and kinetic characteristics of enzymes in vitro to the cellular environment of the intact plant. Estimation of inhibitor/activator concentrations and substrate levels within cellular compartments in vivo and the effect of the high protein concentration present in cells can become insurmountable problems for such studies (see Ashton, 1982). Recombinant DNA technology and plant genetic transformation have provided us with excellent tools to get around some of these obstacles. Using the techniques of gene suppression and overexpression, it is possible to alter the amount of a single enzyme in a transgenic plant, thus generating a series of mutants with a range of enzyme activities from below to above wild-type levels. Depending on the importance of this enzyme in determining photosynthetic rate, the phenotypic effects of changes in the level of expression may vary widely (see Knight and Gray, 1992). Figure 2 shows the expected response of photosynthetic rate to varying enzyme levels in three hypothetical cases: (1) in which the enzyme is present in considerable excess; (2) in which the enzyme is “co-limiting,” along with other enzymes in the pathway; and (3) in which the enzyme is

![Figure 2: The Response of Photosynthetic Carbon Fixation to Changes in Enzyme Activity.](image)

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(A) Thioredoxin mediated enzyme activation

![Thioredoxin-mediated enzyme activation diagram](image)

(B) Phosphorylation/dephosphorylation regulation of PPDK

![Phosphorylation/dephosphorylation regulation of PPDK](image)

(C) Phosphorylation/dephosphorylation regulation of PEPC

![Phosphorylation/dephosphorylation regulation of PEPC](image)

Figure 3. Three Examples of Enzyme Regulation by Covalent Modification.

(A) Regulation by the thioredoxin system. An enzyme is activated when a disulfide bridge on the protein is reduced by the regulatory protein thioredoxin-m (ThR-m). Thioredoxin’s redox state is determined by the redox state of ferredoxin (Fd), the terminal electron acceptor of photosystem I, and consequently is responsive to the rate of electron transport and light intensity.

(B) Phosphorylation regulation of PPDK. PDKR, the PPDK regulatory protein, catalyzes both the dephosphorylation of inactive enzyme (E-P) to active enzyme (E) and the reverse reaction. The relative rates of these reactions are believed to respond to the energy status of the chloroplast.

(C) Phosphorylation regulation of PEPC. In contrast to PPDK, PEPC is activated when phosphorylated by a protein kinase and becomes insensitive to the metabolic inhibitor malate. The “inactivated” dephosphorylated form still has activity but is sensitive to inhibition by malate.
“limiting” for photosynthetic flux. A quantitative assessment of an enzyme’s role in each class can be made using control analysis (Kacser and Burns, 1973). This mathematical treatment of biochemical regulation assigns a “control coefficient” between 0 and 1, with 0 indicating no control over flux by the enzyme and 1 indicating that the enzyme is limiting. Results from studies on diverse organisms indicate that control of flux through a pathway is frequently shared between a number of steps.

Metabolic engineering can be used to control not only the quantity of enzyme present in a transgenic plant but also its “quality.” Both site-directed mutagenesis and expression of heterologous enzymes allow the significance of the regulatory properties of an enzyme to be investigated in vivo. Removing the amino acids responsible for light regulation of an enzyme, for example, is a powerful tool in assessing the importance of this property to the plant. The following sections outline some of the approaches used to alter enzyme quantity and quality; a unifying theme throughout is the role of individual enzymes in controlling photosynthetic rate in vivo.

Antisense Suppression of Key Photosynthetic Enzymes

C3 Pathway

By far the most widely used approach to the genetic manipulation of photosynthesis to produce transgenic plants with reduced levels of key photosynthetic enzymes. “Metabolic engineering” of photosynthesis was first reported by Rodermel et al. (1988), who transformed tobacco with a full-length antisense RNA construct targeted to the SSU of Rubisco, using a constitutive promoter. These transormants showed a substantial reduction in RbcS transcript levels as well as in Rubisco protein levels and enzyme activity (Rodermel et al., 1988; Quick et al., 1991). However, Rubisco activity could be reduced by up to 40% before even a marginal effect on photosynthesis would be observed. In contrast, similar experiments by Hudson et al. (1992) resulted in a range of phenotypes in which both photosynthesis and growth were adversely affected. Careful analysis of these plants has revealed that when plants are grown at high light and at atmospheric CO2 concentrations, Rubisco activity exerts a high degree of control over photosynthetic carbon flux and under these conditions can be considered a limiting enzyme (Hudson et al., 1992). The apparent inconsistencies between these observations appear to relate mostly to the light intensities used for growth of transformants, that is, environment cabinet illumination in the former case (Quick et al., 1991) and full sunlight in the latter (Hudson et al., 1992). These transgenic plants have far wider uses than in the study of photosynthetic flux. For example, because Rubisco is also the major protein present in leaves, it is extremely important in the nitrogen relations and nutritive value of higher plants, making these transformants valuable in studying nitrogen use and allocation (Masle et al., 1993). In addition, these plants have also been used to study the relationship between photosynthesis and growth and regulation of stomatal aperture (Evans et al., 1994).

The list of photosynthetic enzymes whose expression has been reduced in C3 plants is steadily growing, as indicated in Table 1. In most cases, soluble stromal enzymes of photosynthetic carbon metabolism have been targeted: Rubisco, Rubisco activase, fructose-1,6-bisphosphatase (FBPase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoribulokinase (PRK), and carbonic anhydrase. More recently, membrane proteins, such as translocators (chloroplast Pi translocator) and thylakoid membrane proteins (Reiske Fe-S center, ATPase, and the 10-kD polypeptide of photosystem II [PSII]), have also been manipulated. As discussed earlier, the range of phenotypes observed in these experiments can vary enormously. Many enzymes, such as FBPase (Kosmann et al., 1994), GAPDH (Price et al., 1995a), and the Pi translocator (Riesmeier et al., 1993), seem to fall into the intermediate or “co-limiting” category. For example, a 70% decrease in FBPase activity resulted in only a 20% decrease in maximum photosynthetic rate (Kosmann et al., 1994). For some enzymes, such as the thylakoid PSII 10-kD polypeptide (whose function is unknown; Stockhaus et al., 1990) and chloroplastic carbonic anhydrase (Price et al., 1994), little or no phenotype is evident when their levels are reduced by antisense suppression, suggesting that these proteins fall into the “nonlimiting” category.

The interpretation of the phenotypic effects of an antisense construct on photosynthesis and growth of the transformed plants is not always straightforward. The importance of growth conditions in influencing phenotype should not be underestimated, as demonstrated by the effect of light intensity during growth on the interpretation of the Rubisco antisense experiments described previously. It also appears that in many instances, the relationship between steady state mRNA levels and protein levels in transgenic plants expressing antisense RNA may not be simple. Attempting to reduce levels of the 23- and 33-kD polypeptides of the PSII oxygen-evolving complex and the Reiske Fe-S center of the cytochrome b6/f complex, Palomares et al. (1993) recently reported up to a 90% reduction in target mRNA levels in transgenic plants without a discernible effect on protein levels or phenotype. In contrast, Price et al. (1995b) produced slow-growing transgenic tobacco with reduced Reiske Fe-S and electron transport rates; however, a 93 to 94% reduction in message level gave only a 60 to 66% reduction in protein. The phenotype was often unstable and could be ameliorated by high growth irradiance, suggesting that low mRNA levels are not limiting under some conditions or that the endogenous sense transcript can “swap” out the antisense mRNA at high irradiance. Similar nonlinear relationships of protein level to transcript level have also been observed in plants transformed with an antisense gene targeted to the chloroplast envelope Pi translocator (Riesmeier et al., 1993). If this proves to be a widespread phenomenon, it may be difficult in some cases to produce a series of mutants with a wide range of enzyme activities.
Table 1. Antisense Suppression of Key Photosynthetic Enzymes in C3 Plants

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Target Enzyme</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>Rubisco</td>
<td>Reduced maximal rate of photosynthesis and growth (if enzyme activity below 60% of wild-type levels)</td>
<td>Quick et al. (1991)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Rubisco</td>
<td>As above, but more severe over the entire range of Rubisco activities</td>
<td>Hudson et al. (1992)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Rubisco activase</td>
<td>Photosynthesis and growth impaired at air CO2 levels; requires high CO2 for growth</td>
<td>Mate et al. (1993)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>PRK</td>
<td>Severe photoinhibition and stunting even at low light</td>
<td>G.S. Hudson (personal communication)</td>
</tr>
<tr>
<td>Potato</td>
<td>Pi translocator</td>
<td>Maximal photosynthesis reduced 40 to 60% by 20 to 30% decrease in translocation; early growth retarded; stores more starch</td>
<td>Riesmeier et al. (1993)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Reiske Fe-S</td>
<td>Photosynthesis inhibited and linearly related to Fe-S content at high light; slow growth</td>
<td>Price et al. (1995b)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>ATP synthase</td>
<td>Photosynthesis inhibited</td>
<td>Price et al. (1995b)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>GAPDH</td>
<td>Photosynthesis unaffected until GAPDH less than 20% of wild-type levels</td>
<td>Price et al. (1995a)</td>
</tr>
<tr>
<td>Potato</td>
<td>FBPase</td>
<td>Maximal photosynthesis impaired when FBPase below 40% of wild-type levels; growth impaired when FBPase below 15% of wild-type levels</td>
<td>Kosmann et al. (1994)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Carbonic anhydrase</td>
<td>No phenotype with CA below 2% of wild-type levels; small effect on CO2 conductance</td>
<td>Price et al. (1994)</td>
</tr>
<tr>
<td>Potato</td>
<td>10-kD polypeptide</td>
<td>No phenotype even when less than 3% of wild-type levels are attained</td>
<td>Stockhaus et al. (1990)</td>
</tr>
</tbody>
</table>

Because it is desirable for biochemical analysis of flux control to produce such a series (Figure 2; see Woodrow and Berry, 1988), this could limit the usefulness of the antisense technique for some applications.

C4 Pathway

Use of the antisense approach in studying C4 photosynthesis has so far been impeded by the lack of efficient and reliable genetic transformation systems for C4 plants, and attempts to use this approach to study C4 photosynthesis have only recently been reported. The key regulatory enzymes unique to the C4 pathway are shown in Figure 1B. PPdK, PEPC, and NADP-dependent malate dehydrogenase (NADP-MDH) are all localized in M cells, with PPdK and NADP-MDH located in the chloroplast and PEPC in the cytosol. The extractable activity of PPdK is only just sufficient to account for observed rates of photosynthesis, and the activity of all three enzymes in vivo is increased markedly by illumination (Hatch, 1988), suggesting that these enzymes are potentially rate limiting.

Rubisco is also a good candidate for the antisense approach because, in C4 plants, Rubisco levels are already reduced by up to 50% on a chlorophyll basis as compared with C3 plants. Less Rubisco is required in these plants due to the CO2-concentrating function of the pathway, which increases effective carbon flux through Rubisco (see Hatch, 1988). This reduction in the amount of Rubisco required improves the nitrogen use efficiency of C4 plants considerably over their C3 counterparts. It is not known whether a further reduction in Rubisco activity via antisense RNA suppression could limit flux through C4 photosynthesis.

So far, antisense constructs targeted to the B cell enzyme Rubisco and the M cell enzymes PPdK and NADP-MDH have been transformed into F. bidentis. F. bidentis plants transformed with a full-length antisense construct targeted to RbcS mRNA show a range of Rubisco activities, from 10 to 100% of wild type (Furbank et al., 1994). mRNA levels were reduced roughly in proportion to protein levels, as was seen in similar experiments with C3 plants (Rodermark et al., 1988). Plants with reduced Rubisco levels show a stunted phenotype, with
proportionally reduced photosynthetic capacity at high light and over a range of CO₂ concentrations. Interestingly, there appears to be a strong regulatory mechanism linking Rubisco activity to the rate of the mesophyll C₄ cycle. Although the levels of mesophyll enzymes were unaffected in the transformants, down-regulation of the mesophyll C₄ cycle turnover was observed in intact leaves, presumably via down-regulation of enzyme activity. The biochemical basis for such a mechanism is currently unknown, and further physiological and biochemical analysis of the progeny of these transformants is under way. These plants will be a valuable tool for understanding photosynthetic regulation in C₄ plants because, unlike the C₃ case, the role of Rubisco activity in determining photosynthetic rate in C₄ plants under varying environmental conditions has not been extensively modeled.

Analysis of F. bidentis transformants containing other photosynthetic antisense constructs is still in its early stages. NADP-MDH has been reduced by as much as 60% from wild-type levels (S.J. Trevanion, R.T. Furbank, and A.R. Ashton, unpublished observations); in these plants, there is a commensurate reduction in the light-saturated rate of photosynthesis but only a slight effect on growth.

In the case of PPdk, antisense transformants show up to a 90% reduction in both enzyme activity and transcript levels (Furbank et al., 1994). Transformants containing a full-length antisense construct fall into two groups: in one, enzyme activities range from 10 to 20% of wild type; in the other, enzyme activities are normal. No transformants showed an intermediate level of PPdk, although some plants transformed with an 0.8-kb antisense construct corresponding to the 5' end of the Pdk cDNA show a reduction in Pdk transcript levels but no reduction in extractable enzyme activity. The phenotype of plants with 20% or less PPdk activity is severe. Regenerants are incapable of photo-autotrophic growth in air and can be maintained only on exogenous sucrose or in an atmosphere containing 1% CO₂. Plants deprived of exogenous sucrose show rapid and severe photoinhibition in air, even at very low light intensity. One explanation for the all-or-none nature of the phenotype in these experiments could be that the relationship between transcript level and protein level is nonlinear or possibly even sigmoidal. Thus, when the transcript level drops below a critical threshold, a catastrophic falloff in protein levels may occur.

Site-Directed Mutagenesis and “Overexpression”

High-level expression of a native or heterologous protein to increase flux through a pathway, divert flux, or change the regulatory properties of an enzyme is a powerful tool for understanding regulation of photosynthesis. So far, these techniques have been more commonly applied to the pathways of sucrose and starch biosynthesis than to photosynthesis per se (Sonnewald et al., 1994; Stitt, 1994). A classic example of this approach is the expression of a bacterial ADP-glucose pyrophosphorylase in plants to “short circuit” regulation of this step and increase starch accumulation (see Martin and Smith, 1995, this issue). Site-directed mutagenesis to alter the kinetic properties of Rubisco has been moderately successful in vitro; however, the heteropolymeric nature of the protein and the fact that LSU is chloroplast encoded have prevented expression of the mutated holoenzyme in vivo. It is also generally accepted that overexpression of genes in plants is more difficult than antisense suppression due to cosuppression (Napoli et al., 1990; van der Krol et al., 1990) and the possible inactivation of the overexpressed protein by endogenous regulatory systems (for example, Sonnewald et al., 1994).

In the past two decades, it has become apparent that a major form of enzyme regulation involves covalent modification of specific amino acid residues either by the formation of disulfide bridges or by protein kinase-mediated phosphorylation (Figure 3). This type of regulation in plants responds directly to light (via the thioredoxin system) or to cellular energy reserves such as ATP. With sequences of more photosynthetic enzymes appearing in genetic databases almost daily, this area of enzyme regulation is particularly amenable to study by expression of recombinant protein both in vitro and in vivo. For example, NADP-MDH is a light-activated enzyme in the C₄ pathway (Figure 1) that undergoes reductive activation in the light via photosynthetic electron transfer and the thioredoxin system (Buchanan, 1991). Reduction of a disulfide bridge between two cysteine residues in NADP-MDH by reduced thioredoxin activates the enzyme (Figure 3). NADP-MDH protein is homologous to the NAD-dependent nonphotosynthetic form of the enzyme, but it also has C- and N-terminal extensions that have been implicated in conferring the unique regulatory properties of the photosynthetic enzyme. Using site-directed mutagenesis, the pairs of cysteine residues responsible for redox activation at both the N and C termini of the sorghum enzyme have been identified (see Issakidis et al., 1994). Mutant forms of this enzyme that are not inactivated by oxidation and thus should not be inactivated in the dark have been produced. *Flaveria* and tobacco have been transformed with these constructs to examine the role of light activation of this enzyme in vivo (S.J. Trevanion, A.R. Ashton, and I. Issakidis, personal communication).

Two other key enzymes in C₄ photosynthesis are regulated by covalent modification: PPdk and PEPC (Figures 1 and 3). PEPC, the primary CO₂-fixing enzyme of the C₄ pathway, is “activated” in vivo when phosphorylated by a specific protein kinase (for review, see Budde and Chollet, 1988). This activation does not result in an increase in the Vₘₐₓ of the enzyme but manifests itself by a decrease in sensitivity to the inhibitor malate. In the sorghum protein, phosphorylation of a serine residue is responsible for this activation. Substitution of this serine in a recombinant enzyme expressed in *Escherichia coli* prevents this activation (Duff et al., 1993). This region of the amino acid sequence appears to be highly conserved among PEPC enzymes from many sources, suggesting that this
regulatory mechanism may be universal. So far, no transformation experiments have been performed with a recombinant enzyme in higher plants; therefore, it is not possible to assess the importance of this regulatory mechanism in vivo. The signal transduction chain that controls the activity of the protein serine kinase in response to environmental conditions remains somewhat of a mystery, as does the identity of the kinase itself.

PPdK, which is responsible for regenerating PEP, the acceptor for CO₂ fixation in the mesophyll chloroplast, has long been recognized as a light-activated enzyme (Figure 1; reviewed in Hatch, 1988). It is now known that, in contrast to PEPC, this enzyme is inactivated by phosphorylation of a threonine residue (Ashton and Hatch, 1983; Ashton et al., 1984). This residue is not the site that is phosphorylated during catalysis, which is a nearby histidine residue. The enzyme that catalyzes the regulatory phosphorylation of PPdK, the PPdK regulatory protein, is unusual in that it uses ADP rather than ATP as the phosphate donor. In addition, this same enzyme catalyzes the removal of the phosphate group, reactivating PPdK by phosphorolysis rather than by hydrolysis (Figure 3; Ashton et al., 1984). It is not clear how the activity of the PPdK regulatory protein itself is controlled, although high pyruvate levels appear to block inactivation (Burnell et al., 1986). The regulatory protein is not abundant in leaves, and purification to homogeneity has proven elusive (Smith et al., 1994), as has the isolation of its isoforms.

The gene coding for PPdK has now been cloned from a number of species (see previous discussion). Preliminary work has been done expressing the native enzyme from maize in E. coli (A.R. Ashton and R.T. Furbank, unpublished observations) and from representatives of the Flaveria genus (J.N. Burnell, personal communication). By mutagenesis of the regulatory threonine and expression of the recombinant protein in E. coli and higher plants, it should be possible to examine the regulatory significance of phosphorylation.

One of the greatest challenges in the area of photosynthesis is to use the large body of information available in the literature on the enzymes of the pathway and their regulation to understand and perhaps improve photosynthetic performance in whole plants. As discussed earlier, the major barrier to this research has been the difficulty of performing biochemical manipulations in vivo in a precise and interpretable fashion. Early indications suggest that metabolic engineering, coupled with more traditional biochemical and physiological approaches, may provide the means to achieve this aim.

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