Annu. Rev. Plant Physiol. Plant Mol. Biol. 1999. 50:187–217

C_4 GENE EXPRESSION

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KEY WORDS: photosynthesis, bundle sheath, mesophyll, transcription, signal transduction

ABSTRACT

C_4 plants, including maize, *Flaveria*, amaranth, sorghum, and an amphibious sedge *Eleocharis vivipara*, have been employed to elucidate the molecular mechanisms and signaling pathways that control C_4 photosynthesis gene expression. Current evidence suggests that pre-existing genes were recruited for the C_4 pathway after acquiring potent and surprisingly diverse regulatory elements. This review emphasizes recent advances in our understanding of the creation of C_4 genes, the activities of the C_4 gene promoters consisting of synergistic and combinatorial enhancers and silencers, the use of 5′ and 3′ untranslated regions for transcriptional and posttranscriptional regulations, and the function of novel transcription factors. The research has also revealed new insights into unique or universal mechanisms underlying cell-type specificity, coordinate nuclear-chloroplast actions, hormonal, metabolic, stress and light responses, and the control of enzymatic activities by phosphorylation and reductive processes.

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1040-2519/99/0601-0187$08.00

INTRODUCTION

The majority of plants assimilate atmospheric CO_2 through the C_3 pathway of photosynthesis by using ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO or RBC). Under high light intensity, high temperature, and arid environmental conditions, RBC's low CO_2 affinity and inability to distinguish O_2 from CO_2 result in low photosynthetic capacity and significant energy waste through photorespiration in C_3 plants (35, 36, 43, 48, 49, 67) (Figure 1). The so-called C_4 plants have evolved the C_4 cycle pathway that serves as a CO_2 pump. By integrating the two CO_2 assimilation pathways consecutively in the spatially cooperative mesophyll (MC) and bundle sheath cells (BSC), C_4 plants can achieve high photosynthetic efficiency, especially under conditions that cripple C_3 plants (35, 36, 43, 48, 49, 67). Studies of the molecular basis of C_4 photosynthesis have enhanced our understanding of fundamental and complex biological processes and provided information that can be exploited for potential agricultural applications.

Research in molecular biology of C_4 photosynthesis has been initiated from the study of chloroplast genes in maize 20 years ago (78, 152). Significant progress has been made in understanding the molecular basis of C_4 photosynthesis by using multiple model systems. Because each system evolved independently (27, 28, 35–37, 48, 67, 128), these studies offer a wealth of information about how plants use diverse and creative molecular mechanisms to

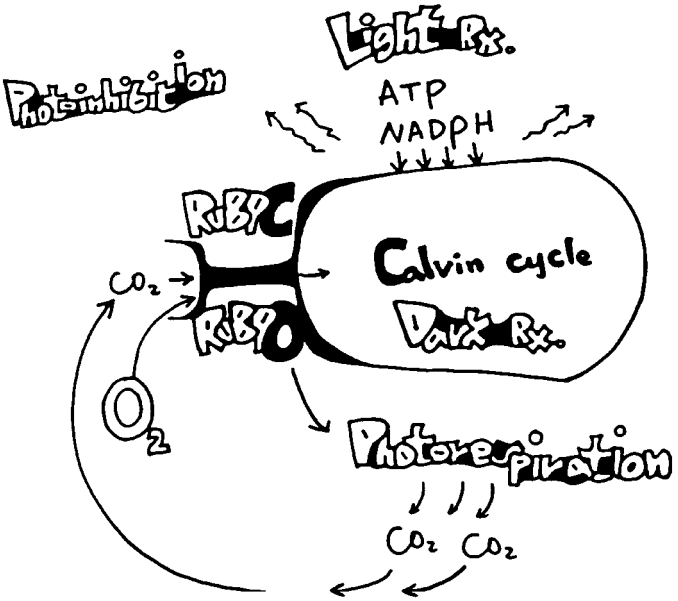

Figure 1 Rubisco is the bottleneck of C_3 photosynthesis. Ribulose-1,5-biphosphate carboxylase (RuBPC) and oxygenase (RuBPO).

acquire and regulate new genes. The elucidation of biochemistry, physiology, and leaf anatomy underlying the operation of C_4 photosynthesis constitutes a great accomplishment in plant biology (35, 36, 43, 48, 49, 67). The link between leaf development and gene expression has been extensively investigated (28, 29, 73, 98). Excellent reviews on some aspects of C_4 gene evolution and regulation are available (29, 43, 67, 73, 86, 88, 98, 134). More general coverage of C_4 photosynthesis is presented by a special issue of the *Australian Journal of Plant Physiology* (Volume 24, No 4, 1997), and a new book, *The Biology of C_4 Plants* (edited by R Sage & RK Monson, 1998). This review emphasizes the unique contributions in the understanding of molecular mechanisms underlying C_4 gene evolution and regulation. The most recent studies using transgenic plants, transient expression, and genetic approaches are summarized and discussed. Other aspects include nuclear-chloroplast coordination, stress and UV-B responses, and the integrated views of signal transduction from hormonal and metabolic regulations to enzymatic controls by protein phosphorylation that were first discovered in C_4 plants, but may turn out to be universal regulatory mechanisms in higher plants.

DIFFERENTIAL GENE EXPRESSION AND C_4 PHOTOSYNTHESIS

Genes Involved in C_4 Photosynthesis

Despite their diverse origins, all C_4 plants exhibit characteristic features of two morphologically and biochemically distinct photosynthetic cell types, MC and BSC, in leaves or culms (28, 29, 35, 36, 43, 48, 49, 67, 73, 98, 134, 148). The assimilation of CO_2 is first carried out in MC through the C_4 cycle pathway. Carbonic anhydrase (CA) and phosphoenolpyruvate carboxylase (PEPC) are responsible for the hydration and fixation of CO_2 to oxaloacetate (OAA). This process is highly efficient and insensitive to O_2. The CO_2 acceptor PEP is generated from pyruvate by pyruvate orthophosphodikinase (PPDK). OAA is then converted to malate by NADP malate dehydrogenase (MDH) or to aspartate by aspartate aminotransferase (AST). Either malate or aspartate is transferred to BSC where CO_2 is released by NADP- or NAD-malic enzyme (ME) or PEP carboxykinase (PCK), and reassimilated by RBC through the Calvin cycle in a CO_2-enriched environment, thus avoiding photorespiration (Figure 2) (43, 48, 50, 49, 67, 98).

Many nuclear genes encoding the enzymes in the C_4 cycle pathway have been isolated from maize, amaranth, *Flaveria*, sorghum, and *Eleocharis vivipara* (1, 9, 14, 16, 45, 51, 54, 64, 76, 79, 80, 86, 93, 94, 97, 105, 120, 125, 126, 134, 141, 157). By using purified MC and BSC or in situ hybridization and immunolocalization, it has been demonstrated that these C_4 genes display MC- or BSC-specific expression patterns at both the protein and transcript levels in the mature leaves of all C_4 plants examined (Table 1). In addition, nuclear genes encoding the conserved photosynthetic enzymes or proteins involved in the Calvin cycle, photosystem II, and oxygen-evolving complex have been identified and shown to be differentially or preferentially expressed in MC or BSC (Table 1) (14, 118, 121, 159). Nuclear run-on experiments have indicated that MC-specific expression is mainly regulated at the transcriptional level, whereas BSC-specific expression is likely controlled at both transcriptional and posttranscriptional levels in maize leaves (109; J Sheen, unpublished data).

Cell-Type Specific Regulation of Diverse Genes

Besides photosynthesis genes, the expression of diverse genes involved in nitrogen and sulfate assimilation, amino acid metabolism, metabolite transport, and the biosynthesis of starch and sucrose also exhibits cell-type specific patterns (15, 20, 35, 36, 48, 66, 82, 99, 134, 138, 139, 159). The most striking example is the MC-specific expression of genes encoding nitrate reductase (NR), nitrite reductase (NiR), and other related functions for nitrate assimilation (134).

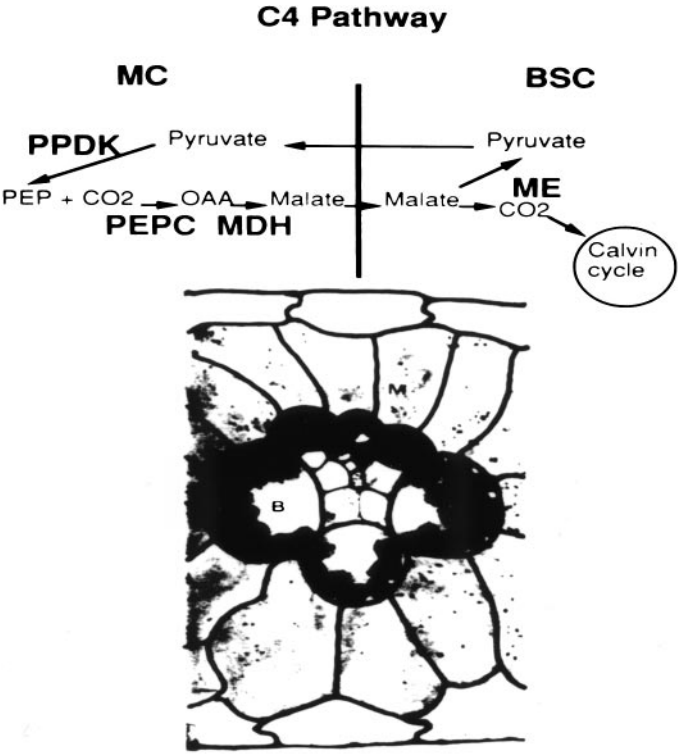

Figure 2 A simplified NADP-ME-type C_4 cycle pathway in maize leaves. The bundle sheath cells (BSC or B) accumulate starch and are darkly stained by iodine. Mesophyll cells (MC or M) are located between BSC and epidermal layers. Pyruvate orthophosphodiknase (PPDK), phosphoenolpyruvate carboxylase (PEPC), and NADP-malate dehydrogenase (MDH) are present in MC, whereas NAPD-malic enzyme (ME) is expressed in BSC.

Moreover, the genes encoding ferredoxin-dependent glutamate synthase (Fd-GOGAT) and glutamine synthase (GS2) for ammonia metabolism are preferentially induced by nitrate signals in MC but not in BSC. The differential induction of these genes in MC may accommodate the physiological specialization of each cell type for nitrogen metabolism (134). Recently, extensive differential screening has been performed in sorghum and maize to identify large number of genes that are specifically expressed in MC or BSC (Table 1). Sequence analysis of these genes has uncovered new candidates (e.g. BSC-specific *Pck* in maize) that will likely broaden our understanding of the physiological functions and gene regulation in MC and BSC (153, 159; T Furumoto & K Izui, personal communication).

Table 1 Regulation of nuclear genes involved in C_4 photosynthesis

Genes[a,b]	Cell type	Induction	Repression	Species
C4Pdk	M[c]	L[c], N, UV-A UV-B	S[c], A, gly ABA	Maize, *F. t.*[d], *A. h.*, Sorghum, *E. v.*
C4Ppc	M	L, N	S, A	Maize, *F. t.* *A. h.*, sorghum
C4Mdh	M	L	—	Maize, *F. t.*, sorghum
C4Cah	M	L, N	—	Maize, sorghum
Cab	M	L	S, A	Maize, sorghum
PsbO	M	L	—	Maize, sorghum
PsbP	M	L	—	Maize, sorghum
PsbQ	M	L	—	Maize, sorghum
PsbR	M	—	—	Sorghum
PsbS	M	—	—	Sorghum
PsbT	M	—	—	Sorghum
PsbW	M	—	—	Sorghum
GapB	M	—	—	Sorghum
PetF	M	—	—	Sorghum
PetH	M	—	—	Sorghum
Tpi	M	—	—	Sorghum
RbcS	B	L	S, A, ABA	Maize, *F. t.*, *A. h.*
Pck	B	—	—	Maize
NADPMe	B	L, UV-B	S, A	Maize, *F. t.*, sorghum
NADMe	B	L	—	*A. h.*
Rca	B	—	—	Sorghum
Prk	B	—	—	Sorghum
FbaC	B	—	—	Sorghum
TklC	B	—	—	Sorghum
Rpe	B	—	—	Sorghum
Omt	B	—	—	Sorghum

[a]References: 1, 9, 14, 17, 86, 97, 112, 116, 118, 119, 120, 121, 126, 134, 141, 157.

[b]Genes not found in the text: *Psb*: photosystem II; *GapB*: NADP-glyceraldehyde-3-phosphate dehydrogenase; *PetF*: ferredoxin; *PetH*: ferredoxin-NADP-oxidoreductase; *Tpi*: triosephosphate isomerase; *Rca*: rubisco activase; *Prk*: phosphoribulokinase; *Fba*: fructose-1,6-bisphosphatase aldolase; *Tkl*: transketolase; *Rpe*: ribulose-5-phosphate 3-epimerase; *Omt*: 2-oxoglutarate/malate translocator.

[c]M: mesophyll; B: bundle sheath; L: red and/or blue light; N: nitrogens; S: sugars; A: acetate; gly: glycerol; ABA: abscisic acid.

[d]*F. t.*: *Flaveria trinervia*; *A. h.*: *Amaranthus hypochondriacus*; *E. v.*: *Eleocharis vivipara*.

Leaf Development and Differential Gene Expression

Most C_4 genes examined show strict expression in leaves or leaf-like structures but not in non-photosynthetic roots and stems. Antibodies and cDNA probes for photosynthetic enzymes and genes were used as cell-specific markers by in situ hybridization and immunolocalization to follow leaf development from primodia or regenerating calli to mature leaves in maize (2, 28, 29, 71, 72, 73, 74, 97, 98). The expression of C_4 genes shows temporal and spatial regulation

patterns that match the developmental stage and age gradients of leaves. Clonal analysis of photosynthetic BSC and MC in maize leaves suggests that MC development is dependent on position rather than lineage (71). The photosynthetic competence of MC and BSC is tightly coupled to vein development and is superimposed by light regulation. Light signaling not only enhances C_4 gene expression but also reduces the expression of *RbcS* and *RbcL* in MC (75, 119, 120). A current model points to unknown regulatory signals generated from veins for the control BSC and MC differentiation and C_4 pattern gene expression (28, 73, 98).

In the NAD-ME type of C_4 dicot *Amaranthus hypochondriacus*, it has been shown that posttranscriptional regulation determines initial C_4 gene expression patterns in developing cotyledons and leaves (9). Transcripts for *RbcS*, *RbcL*, *Ppc*, and *Pdk* accumulate in the shoot meristems and leaf primordia. RBCS and RBCL proteins, but not the C_4 enzymes, are detected in early tissues. Protein accumulation of the C_4 enzymes occurs only when the leaf vascular system begins to differentiate (101). Distinct from maize, light is not required for the cell-type-specific expression of *RbcS* and *RbcL* genes and other C_4 genes in amaranth (154). In the three-color leaves of *A. tricolor*, both transcription and translation are responsible for the lack of photosynthetic capacity in the red and yellow regions of leaves (92). There is a tight coordination between the basipetal C_3 to C_4 transition in *Rbc* gene expression and the basipetal sink-to-source transition in amaranth (155). In the developing cotyledons of C_4 dicot *Flaveria trinervia*, the expression of C_4 genes is light-dependent and more similar to that in monocot maize but not dicot amaranth, which suggests independent evolution of different C_4 plants (125).

C_4 GENE EVOLUTION AND REGULATION

Most C_4 genes have closely related homologs displaying low ubiquitous expression in C_4 and C_3 plants, suggesting that C_4 genes are recent products of gene duplications. Drastic modifications in regulatory sequences could have accommodated the changes in abundance and localization for C_4 photosynthesis. The lack of consensus regulatory sequences among C_4 genes documents the remarkable co-evolution of diverse genetic modifications. The molecular mechanisms for C_4 gene evolution have been deduced from comparisons of the C_4 genes with their non-photosynthetic counterparts in the same C_4 plant or with their orthologs in closely related C_3 plants.

Pdk Genes

The maize *C_4Pdk* gene shares almost all of its coding sequence with the ancestral gene (*cyPdk1*) encoding a cytosolic PPDK (Figure 3). The first exon

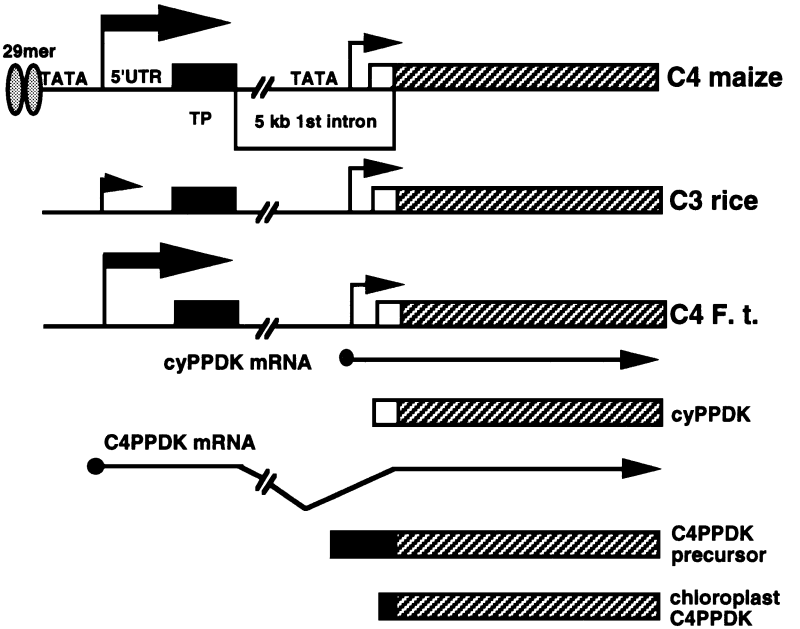

Figure 3 Dual promoters regulate *Pdk* expression in C_4 and C_3 plants. The *black box* represents the chloroplast transit peptide (TP), the *white box* is the unique N terminus of the cytosolic PPDK (cyPPDK), and the *hatched box* covers the shared coding sequence by C_4PPDK and cyPPDK. *Arrows* indicate transcription.

of *C_4Pdk* encoding a chloroplast transit peptide is separated from the first exon of *cyPdk1* and the rest of the shared coding region by a 5-kb intron, which includes repetitive sequences. The promoter of *cyPdk1* is located in the first intron of *C_4Pdk* and has ubiquitous activity. In contrast, the promoter of *C_4Pdk* directs leaf-specific and light-inducible transcription in MC. Another family member, *cyPdk2*, in maize shares similarity with *cyPdk1* up to the TATA box of the promoter. It was proposed that the creation of *C_4Pdk* could be mediated through repetitive sequences by unequal recombination that resulted in a new intron and brought a transit peptide and a new promoter to fulfill the need of a C_4 gene (45, 113). However, recent characterization of the *Pdk* gene in the C_3 monocot rice revealed similar dual promoters, gene structure, and expression patterns (56, 86). Furthermore, the single-copy *Pdk* gene in the C_4 dicot *F. trinervia* has strikingly similar structure (Figure 3) and shows high sequence similarity to the *Pdk* gene in the C_3 dicot *F. pringlei* (103). Therefore, the major difference between the C_4 and C_3 *Pdk* is at the strength of the first promoter as the transcripts for the chloroplast PPDK is expressed at very low level in C_3 rice and *F. pringlei* (56, 103).

Ppc Genes

At least three classes of *Ppc* genes have been found in maize and sorghum. The single copy *C_4Ppc* gene shows a unique and high level of expression in MC (32, 54, 64, 76, 110, 145). The comparison of the more divergent sequences upstream of the coding regions among three maize *Ppc* genes revealed that *C_4PpcZm1* and *PpcZm3B* are very similar but their 5′ flanking sequences diverge before the TATA box. The observation leads to the suggestion that the *C_4Ppc* gene could be generated from an ancestral *Ppc* gene after the unequal recombination near the TATA box bringing regulatory elements required for the new expression patterns and high activity (110). Note that the two maize *cyPdk* genes also share similar sequences up to the TATA box but show distinct expression levels. Perhaps the regions near the transcription initiation site are hot spots for DNA recombination responsible for the generation of distinct expression patterns and levels among homologous genes in maize. The most informative sequence comparison between the C_4 and C_3 *Ppc* orthologs was performed in dicot *F. trinervia* (C_4) and *F. pringlei* (C_3). There are four classes of *Ppc* genes in each plant. The *PpcA* genes from these two species are 96% identical but expressed differently. Thus the major events during the evolution of the *C_4PpcA* gene occurred at the promoter level (38, 51, 52, 130, 157).

Me Genes

Two closely related NADP-ME genes have been isolated from *F. bidentis* and maize (84, 105). Surprisingly, both *Me* genes encode chloroplast transit peptides and similar sequences, but show different expression patterns. Although both *Me* genes are found in C_3 *F. pringlei*, the expression of its C_4 *Me* ortholog (*Me1*) is not observed (84). In the C_4 dicot amaranth, NAD-ME but not NADP-ME is used for decarboxylation in the mitochondria of BSC. The amaranth C_4 NAD-ME shares more similarity with human NADP-ME than with maize and thus may represent another origin and process for C_4 gene evolution (9, 79).

Mdh Genes

Although two distinct NADP-MDH genes have been found, the expression of only one *Mdh* gene is MC-specific and light-inducible, and serves in C_4 photosynthesis in maize and sorghum (80, 94). In various *Flaveria* species, however, there appears to be only a single *Mdh* gene, suggesting that a pre-existing gene has been re-regulated without gene duplication during the evolution from C_3 to C_4 plants (93).

Cah Genes

The cytosolic and chloroplast forms of CA have been characterized in plants (3, 16, 50, 67, 81). The cytosolic CA is MC-specific and important for C_4 photosynthesis. Two *Cah* cDNAs have been isolated in maize, sorghum, and

F. bidentis, but only one *Cah* gene shows enhanced and MC-specific expression in illuminated leaves and likely encodes the C_4 gene (16, 81, 159). As all *Cah* genes are closely related in C_4 and C_3 plants, simple genetic alternation is likely required for the C_4 function.

Other Photosynthesis Genes

The evolution of C_4 plants also included the altered cell-type specificity for the existing genes involved in the Calvin cycle and photosystem II. As in C_3 plants, RBCS and chlorophyll *a/b* binding proteins (CAB) are encoded by multigene families in maize, amaranth, and *Flaveria* (9, 39, 118, 119, 126). The six members of the maize *Cab* genes show complex light and cell-type expression patterns (118). However, unlike the C_4 genes whose expression pattern is distinct from their closely related homologs, all *RbcS* genes exhibit a similar BSC-specific expression pattern in C_4 plants (9, 39, 119, 126).

C_4 GENE EXPRESSION IN TRANSGENIC PLANTS

To identify, characterize, and definitively prove that any putative regulatory elements associated with C_4 genes are functional and can account for the unique expression patterns and levels in C_4 plants, it is essential to develop reliable transformation methods (21, 24, 63, 83, 102, 131, 141, 157). Despite technical difficulties, successful studies of C_4 gene regulation in transgenic *Flaveria* species and maize have recently been achieved (21, 24, 63, 83, 102, 131, 141, 157).

C_4 Dicot Flaveria

The most extensive transgenic analyses have been carried out in the C_4 dicot *F. bidentis* based on a facile transformation procedure and the use of the ß-glucuronidase reporter gene (GUS) (21). The transgenic plants were analyzed by histochemical and cell separation techniques (21, 141, 157). The 5′ flanking region (2185 bp) of the *F. trinervia* C_4*PpcA1* gene is sufficient to reproduce MC-specific, developmental, and basipetal expression patterns. The promoter region (2583 bp) of the orthologous *PpcA1* gene from *F. pringlei* (C_3) directs reporter gene expression mainly in vascular tissues of leaves and stems and at low levels in MC. These experiments are the first to demonstrate that *cis*-acting elements are responsible for the C_4 expression patterns that require synergistic cooperation between distal (-2074 to -1501) and proximal (-570 to $+1$) regions (131, 157). Similarly, the 5′ region (1491 bp) of the C_4*Pdk* gene directs MC specificity and light induction in seedlings and mature leaves (102, 141).

Complex regulatory mechanisms have been unveiled from the study of the C_4*Me1* gene (83, 141). Transgenic plants were obtained with a set of chimeric

constructs using the 5′ (up to 2361 bp) and 3′ (up to 5900 bp) regions of the *F. bidentis C4Me1* gene fused to GUS. Although the 5′ region determines BSC specificity, the 3′ region contains enhancer-like elements and confers high-level expression in leaves. This interaction of the 5′ and 3′ sequences appears to be specific to C_4 *F. bidentis* because the same construct does not direct significant expression in transgenic C_3 tobacco. Although the 3′ region (900 bp) of the *C4Me1* gene can serve as a transcriptional terminator with a heterologous promoter in tobacco protoplasts, it does not enhance the promoter activity (83). It remains to be determined whether the 3′ region affects transcription, mRNA stability, or translation. Independent transgenic *F. bidentis* plants have also been generated with the 5′ sequences of the *F. trinervia C4Me1* gene and the C_3 *F. pringlei* ortholog. The 5′ region of the *C4Me1* gene is sufficient for the basipetal and BSC-specific expression patterns, whereas the 5′ sequence of the C_3 *Me1* gene directs expression in all cell types and the expression is turned off basipetally (T Nelson, personal communication). In maize, the *C4Me1* promoter is active in MC (112). A nuclear run-on experiment with MC and BSC shows *C4Me* transcription in both cell types, implying posttranscriptional control for BSC specificity (J Sheen, unpublished data). These different results point to the possibility that the *C4Me1* genes might have evolved by very different mechanisms even in two very closely related C_4 species, *F. trinervia* and *F. bidentis*.

The promoter of a C_4 *F. trinervia RbcS* gene gives a BSC-specific pattern very similar to that of the *F. trinervia C4Me1* promoter in transgenic *F. bidentis*, whereas the promoter of a C_3 *F. pringlei RbcS* gene shows expression in both MC and BSC (T Nelson, personal communication). Thus there appear to be C_4-specific *cis*-acting elements for the expression of the *F. trinervia RbcS* gene in BSC. However, analyses of the *RbcS* promoters in maize and rice MC and the *RbcS* expression patterns in C_4/C_3 *Flaveria* hybrids imply the involvement of C_4-specific *trans*-acting factors (87, 109, 127). In the transgenic C_4-like *F. brownii*, a C_3 petunia *RbcS* gene can direct a high level of leaf-specific expression, although the cell-type pattern is not known (85). In addition, translation enhancer function of the 5′ (47-bp) and 3′ (130-bp) UTR sequences of the *RbcS* gene from amaranth can be detected when fused to GUS in transgenic *F. bidentis*. (AC Corey & JO Berry, personal communication). Thus the regulation of *RbcS* involves complex and diverse mechanisms in C_4 plants.

In addition to the analyses of gene regulation, the C_4 *F. bidentis* transformation system provides an unprecedented opportunity for the genetic manipulation of key photosynthetic enzymes in C_4 plants (42, 43). Antisense transformants for *C4Pdk* and *RbcS* genes have been generated that can reduce C_4PPDK to 40% and RBC to 15% of the wild-type levels. Co-suppression has been used for the *C4Mdh* gene to achieve lower than 2% of the wild-type C_4MDH

activity. Under saturating illumination, RBC levels have the most significant effect on photosynthetic rates, but C_4MDH has a very minor impact. These valuable transgenic lines will aid our understanding and improvement of C_4 photosynthesis.

C_4 Monocot Maize

The first study of the *C_4Ppc* promoter in transgenic C_4 monocot maize has been accomplished (63). The 5′ flanking region (1.7 kb) of the maize *C_4Ppc* gene is sufficient to direct GUS expression in a MC-specific and light-inducible fashion identical to the endogenous gene. Similar to C_4 dicots, the adoption of DNA regulatory elements for C_4-specific gene expression is a crucial step in the *C_4Ppc* gene evolution in maize. The transgene is repressed by metabolic signals and is blocked completely when the biogenesis of chloroplasts is inhibited under light (63). The regulatory mechanisms of C_4 genes need to be integrated into the universal signaling network that modulates photosynthesis genes in both C_4 and C_3 plants (26, 40, 60, 140, 144). The results from transgenic maize studies fully support previous observations using isolated maize mesophyll protoplasts which do not dedifferentiate in culture and behave as bona fide MC (110, 112).

C3 Dicot Tobacco and C3 Monocot Rice

Several studies of C_4 gene expression in transgenic C3 dicot tobacco and C3 monocot rice provide valuable insights into the nature of the regulatory elements acquired by the C_4 genes. The 5′ flanking regions of the *C_4Pdk* (-1032 to $+71$), *C_4Ppc* (-1212 to $+78$), and *RbcS* (-444 to $+66$) genes from maize have been introduced into transgenic rice (86, 87, 90, 91). The promoters of *C_4Pdk* and *C_4Ppc* display MC specificity, light inducibility, and high activity that are characteristic of the C_4 genes. Moreover, similar DNA-binding activities for *cis*-acting elements in the *C_4Pdk* and *C_4Ppc* promoters have been detected in the nuclear extracts of maize and rice (56, 87, 89, 91, 162, 163). Very high levels of C_4PPDK and C_4PEPC activities have also been ectopically expressed in transgenic rice (88), opening an avenue to genetically manipulate the C_4 cycle pathway in C_3 plants. However, the *RbcS* promoter of the C_4 maize does not support the BSC-specific expression pattern in transgenic rice. It is suggested that differences in *trans*-acting factors exist between C_4 maize and C_3 rice for the regulation of *RbcS* genes (87). The C_4 expression pattern of the *F. trinervia C$_4$Ppc* promoter in C_3 transgenic tobacco reinforces the concept that the evolution of this C_4 gene needs alternations only in the *cis*-acting elements. However, the evolution and regulatory mechanisms of the *C_4Me* gene are unique to C_4 plants and cannot be reproduced in C_3 transgenic tobacco (83).

TRANSIENT EXPRESSION ANALYSES OF GENES INVOLVED IN C_4 PHOTOSYNTHESIS

Many regulatory mechanisms can be more efficiently investigated by transient expression assays using cellular systems where gene expression patterns are faithfully retained. Maize mesophyll protoplasts currently represent the most sophisticated system for the study of gene expression and signal transduction relevant to photosynthesis in higher plants (22, 59, 109, 110, 112–114, 116, 163). Rice, tobacco and Arabidopsis protoplasts have also been applied (47, 87, 109). However, the protoplast system has a limitation as BSC are not easily isolated with high activity. An alternative transient expression assay is to transform whole tissues of maize and *Flaveria* with DNA-coated microprojectiles (4, 5; T Nelson, personal communication). Histochemical analysis of intact tissues or fluorometric assay of tissue extracts based on GUS activity is used in this transient expression assay.

Pdk Genes

The analysis of the three maize *Pdk* gene promoters in mesophyll protoplasts identified distinct *cis*-acting elements for leaf, stem, and root expression. Deletion analysis revealed that a 300-bp region of the *C_4Pdk* promoter (-347 to -44) shows strong leaf specificity, developmental regulation, and light inducibility even when it is fused to a heterologous basal promoter in etiolated and greening protoplasts. The same promoter sequences are important for sugar, acetate, and glycerol repression (112, 113). Deletion and site-directed mutational analyses have identified at least six distinct functional elements (K To & J Sheen, unpublished data). The most significant *cis*-acting element is a 29mer (-286 to -258) with a pair of 7-bp inverted repeats (Table 2). The promoter activity is reduced tenfold when this 29mer is deleted. Most significantly, only the nuclear extract isolated from greening leaves but not from etiolated and green leaves or roots can form a complex with the ^{32}P-labeled 29mer by a gel mobility shift assay (K To & J Sheen, unpublished data). However, this 29mer alone cannot activate transcription when fused to a heterologous basal promoter, which suggests that there is a synergistic cooperation between this element and other regulatory elements.

Another important finding is the dependence of the upstream elements on the 5′UTR elements ($+1$ to $+139$) for a high level of transcriptional activity that distinguish the *C_4Pdk* promoter from the *c_yPdk1* promoter (113; P Leon & J Sheen, unpublished data). Extensive deletion and mutational analyses of this 5′UTR region have defined at least four functional CT and CA repeats whose deletion decreases the *C_4Pdk* promoter activity 80-fold (Table 2). As the *Pdk* gene in C_3 rice only differs from the *C_4Pdk* gene in maize at the transcriptional

Table 2 *Cis*-acting regulatory elements of genes involved in C_4 photosynthesis

Genes[a]	Regions	Sequences[b]	Species
C_4Pdk	5′ flanking	−33 TATAA −29	Maize
	5′ flanking	−286 CTGTAGC–GCTACAG −258 (29mer)	Maize
	5′ flanking	−308 AGTGGAGTCGTGCCGCGTGT −289	Maize
	5′ UTR	CCCCCTCTCC (CT repeats)	Maize
	5′ UTR	CACTCGCCACACACA (CA element)	Maize
	5′ flanking	−1212 to +279	F. t.[c]
C_4Ppc	5′ flanking[d]	−119 CCATCCCTATTT −107	Maize
	5′ flanking	CCCTCTCCACATCC (C14 repeats)	Maize
	5′ flanking	AAAAAGG (29A repeats)	Maize
	5′ flanking[d]	−2187 to −1	F. t.
C_4Me	5′ flanking	−2038 TO +323, −311 TO +83	F. b.[c]
	3′ flanking	900 bp, 5900 bp	F. b.
RbcSZm1	5′ flanking[d]	−24 CTATATATGCCGTCGGTG −7	Maize
	5′ flanking[d]	−105 GAACGGTGGCCACT-CCACA −83	Maize
	5′ flanking[d]	−123 CCGGGTGCGGCCAC −110	Maize
	5′ flanking[d]	−154 GCGCGCGT −147	Maize
	5′ flanking[d]	−179 GATAAG −174	Maize
	5′ flanking[d]	−588 to −183 (silencer)	Maize
	3′ UTR	281 b	Maize
RbcSZm3	5′ flanking[d]	−31 CTATATATGCCGTCGGTG −14	Maize
	5′ flanking[d]	−102 GTCCTGTCCTGTACT-GTCCT −81	Maize
	5′ flanking[d]	−133 GAACGGTGGCCACT-CCACA −111	Maize
	5′ flanking[d]	−151 CCGGGTGCGGCCAC −138	Maize
	5′ flanking[d]	−125 GATAAG −210	Maize
RbcS-m3	5′ flanking[d]	−885 to −229 (silencer), −907 to −455	Maize
	3′ UTR[d]	289 b, +720 to +957	Maize
RbcS	5′ & 3′ UTR	47 b and 130 b	Amaranth
Cab-m1	5′ flanking[d]	−1026 to −850, 359 to +14	Maize
	5′ flanking[d]	−949 AATATTTTTTCT −937	Maize
CabZm1	5′ flanking[d]	−158 CGCGCCAAGTGTTCCAG −142	Maize
	5′ flanking[d]	−184 CCTCA-TGAGG −166	Maize
CabZm5	5′ flanking	−34 TATTTA −29 (TATA box)	Maize
	5′ flanking	−59 GATAAG −54 (I box)	Maize
	5′ flanking	−90 CCAAT −86 (CAT box)	Maize
	5′ flanking	−103 CACCTCCGGCGA −92	Maize
	5′ flanking	−115 ATCCGCCCACCT −104	Maize

[a]References: 4, 83, 89, 102, 109, 110, 112, 113, 114, 123, 131, 151.

[b]The *cis*-acting regulatory elements were defined by deletion, site-directed mutagenesis, or gain-of-function analyses.

[c]*F. t.*: *Flaveria trinervia*, *F. b.*: *Flaveria bidentis*.

[d]Numbers are based on the translation initiation site (+1).

activity, this specific 5′UTR may underlie the major evolutionary changes in the maize *C_4Pdk* gene (Figure 3). It is not clear why the two promoters show similar activity in maize mesophyll protoplasts, as reported by another group (56, 88). The differences could be attributed to protoplast activity and plasmid constructs. Interestingly, this 5′UTR can function at only one position but can interact with multiple promoters in leaves, roots, and stems in dicot or monocot and in C_3 or C_4 plants (P Leon & J Sheen, unpublished data). This is another example that a C_4 gene utilizes conserved machinery to enhance gene expression (12, 143). Distinct from the *C_4Pdk* promoter, the *c_yPdk1* and *c_yPdk2* promoters are expressed ubiquitously. The sequences upstream of the TATA box of the two promoters are divergent and responsible for a 20-fold difference in promoter strength (113). Deletion analysis of the *c_yPdk1* promoter has identified distinct *cis*-acting elements for leaf or root/stem expression, suggesting the use of combinatorial *cis*-acting elements for tissue-specific controls (113). Functional analysis of the maize *C_4Pdk* promoter (-327 to $+211$) has also been performed in maize green leaves by microprojectile bombardment (89). The sequence between -308 and -289 is important for the promoter activity in green leaves. Protein binding activity has been found with a shorter element (-301 to -296) using nuclear extracts isolated from green leaves but not from etiolated leaves or roots (89). This *cis*-acting element may be more important for the maize *C_4Pdk* promoter activity in green than in greening leaves (K To & J Sheen, unpublished data). In maize mesophyll protoplasts, the rice *c_yPdk* promoter is more active than the rice promoter for the chloroplast PPDK (56, 86–88).

Ppc Genes

The regulatory elements of the *Ppc* promoters have been well characterized in maize by using transfected mesophyll protoplasts. Among the three maize *Ppc* promoters examined, only the *C_4Ppc* promoter shows leaf-specific, light, and developmental regulation (110). The 5′ flanking sequences are sufficient to give C_4 expression patterns in isolated mesophyll protoplasts and in transgenic maize plants (63). The importance of two redundant *cis*-acting elements (A29 and C14) has been demonstrated by deletion and hybrid promoter analyses (Table 2). The C14 and A29 direct repeats act as enhancers in maize or rice protoplasts (87, 109). Two groups have shown that proteins in the maize and rice leaf nuclear extracts can bind to these elements (162, 87). A short promoter (-119 to $+1$) retains 20% of the full promoter activity. The significance of the TATTT sequence is confirmed by deletion and site-directed mutagenesis. The *C_4Ppc* and *C_4Pdk* promoters in maize do not share common regulatory elements, which indicates independent evolution.

Despite the identification of numerous *cis*-acting elements important for C_4 gene expression in maize, very little is known about the properties of the

corresponding *trans*-acting factors. A recent study has focused on the function of a conserved zinc-finger binding protein Dof1 (DNA-binding with one finger) in the regulation of the maize *C_4Ppc* promoter (160, 161, 163). Dof proteins represent a new family of transcription factors carrying a conserved motif for DNA-binding, which seem to be ubiquitous in plants. The consensus AAAAGG core sequence motif bound by Dof1 is part of the A29 enhancer identified previously in the maize *C_4Ppc* promoter. Although Dof1 is constitutively expressed in the nuclei of etiolated and greening mesophyll protoplast, it only binds to DNA and activates transcription in greening protoplasts assayed with a synthetic promoter. In fact, Dof1 can only specifically activate the *C_4Ppc* promoter but not the maize *RbcS* and *C_4Pdk* promoters. Although Dof1 exhibits a ubiquitous expression pattern in maize, the function of Dof1 is leaf specific. The activity of Dof1 in other tissues is likely inhibited by a competitive transcriptional repressor Dof2 that is only expressed in roots and stems (163).

RbcS Genes

Nuclear run-on experiments and promoter analyses in maize mesophyll protoplasts and transfected leaves have indicated the involvement of both transcriptional and posttranscriptional regulation of *RbcS* genes (109, 151). Although *RbcS* transcripts do not accumulate in the MC of greening and green maize leaves, the promoters of *RbcSZm1* and *RbcSZm3* are very active and light-inducible in transfected mesophyll protoplasts. The C_4 maize and C_3 wheat *RbcS* promoters share three of five functional *cis*-acting elements (Table 2) that are distinct from the *cis*-acting elements found in the dicot *RbcS* promoters except the universal I box (GATAAG). The activity of the upstream regulatory elements absolutely depends on the specific TATA box, suggesting their synergism in function (109, 114). Further investigations show that the monocot *RbcS* promoters (maize, wheat, and rice) are only active in maize and wheat protoplasts regardless of their C_4 or C_3 origin, whereas the dicot *RbcS* promoters (pea, tobacco, and Arabidopsis) are only active in the dicot tobacco protoplasts (6, 109, 142, 143, 144). Deletion analyses have uncovered upstream silencers (-588 to -183 for *RbcSZm1* and -885 to -229 for *RbcSZm3*) and mRNA destablization elements (281 b in *RbcSZm1* and 289 b in *RbcSZm13*) in the 3′UTR that may mediate the repression of maize *RbcS* in MC (109; P Leon & J Sheen, unpublished data). Based on an in situ transient expression assay in which each blue spot represents a group of MC or BSC, the *RbcS-m3* promoter activity is also detected in both MC and BSC. However, further studies have identified the 5′ upstream region (-907 to -455) and the 3′ region ($+720$ to $+957$) that act together to partially suppress *RbcS-m3* expression in MC of illuminated dark-grown seedlings. As the 3′ region is equally active when located

upstream of the *RbcS-m3* promoter, its photoregulated suppression is mediated by transcriptional control (100, 151).

Cab Genes

At least six members of the *Cab* gene family are differentially expressed in MC and BSC of the maize leaves (4, 118). Using the microprojectile-based in situ transient expression assay, the regulatory *cis*-acting elements of the MS-specific *Cab-m1* have been determined based on GUS reporter activity. Upstream sequences (-1026 to -850) containing four *cis*-acting elements (Table 2) and the core promoter (-359 to $+14$) are identified by deletion and gain-of-function experiments to be important for *Cab-m1* regulation in maize leaves. The core promoter directs high expression in MC and the upstream elements inhibit expression in BSC (4, 5, 123).

Deletion and mutational analyses have been carried out with the distinct *CabZm1* and *CabZm5* promoters using the maize mesophyll protoplast transient assay (59, 112; H Huang & J Sheen, unpublished data). The two promoters share extensive sequence similarity in the proximal elements from the TATA box, I box to the CCAAT box, but use different upstream regulatory elements (Table 2) that determine high level expression and light responses. The *CabZm5* promoter is active both in the dark and light, but the *CabZm1* promoter is only active under light. At least two upstream *cis*-acting elements have been defined in the *CabZm1* promoter that act synergistically (Table 2). In addition, mutations that alter the sequences of TATA, I, or CCAAT boxes in both promoters eliminate their activity. Unlike the strict monocot and dicot specificity of the *RbcS* promoters, the monocot (maize, wheat, and rice) *Cab* promoters can function in dicot tobacco or Arabidopsis mesophyll protoplasts but they exhibit more than tenfold higher activity in maize mesophyll protoplasts. Similarly, the *Cab* promoters from pea, tobacco, and Arabidopsis show significant activity and light regulation in maize protoplasts (22; J Sheen, unpublished data). Only minor changes are expected in the C_4 plants to adjust the expression patterns of various *Cab* gene members in MC and BSC.

CHLOROPLAST GENE REGULATION IN C_4 PLANTS

The functional coordination between the nucleus and the chloroplast is evident in C_4 plants as the differential expression of the nuclear-encoded RBCS in BSC is accompanied by a matching specificity of the chloroplast-encoded RBCL. Moreover, dimorphic chloroplasts are pronounced in maize with agranal chloroplasts in BSC that have greatly reduced photosystem II (*psb*) activity encoded by both nuclear and chloroplast genes. RNA and protein blot analyses with purified MC and BSC from maize show that the transcripts and proteins encoded

by *psbA, B, C, D,* and *E/F* are predominant in MC but those encoded by *RbcL* are BSC specific. The differences are more than tenfold in mature greening (after 24 to 96 h of illumination) and green leaves (119, 122). However, in vitro transcription and slot blot assays using chloroplasts isolated from the same BSC and MC preparations reveal little difference in the transcription activity of *RbcL* and *psb* genes (J Sheen, unpublished data). Thus, posttranscriptional regulation plays a crucial role in the differential expression of chloroplast genes in BSC and MC of maize leaves. In dark-grown etiolated leaves, *RbcL* transcripts but not the protein can be detected in both MC and BSC. Illumination enhances its expression in BSC but causes repression in MC (75, 119). This result has been confirmed by in situ hybridization (75). The differential expression of *RbcL* and *psbA* genes studied by Kubicki et al was less significant in maize (threefold) than in sorghum (tenfold), perhaps due to differences in experimental procedures (68). In amaranth, the differential expression of *RbcL* in BSC and MC is also controlled at the posttranscriptional level (11). The signals and molecular mechanisms for the coordinated mRNA degradation in the nucleus and chloroplast in BSC and MC remain unknown (140).

REGULATION OF C_4 GENE EXPRESSION BY DIVERSE SIGNALS

Nitrogen and Cytokinin

Nitrogen is a limiting factor in photosynthetic capacity. C_4 plants not only have higher efficiency than C_3 plants for the utilization of light and water, but also use nitrogen more efficiently, perhaps owing to low photorespiration that affects carbon and nitrogen metabolism (99, 134). Although the effect of nitrogen on photosynthesis is well known, the underlying mechanisms remained illusive. The first evidence that nitrogen signals control C_4 gene expression was discovered in maize. In nitrogen-starved maize seedlings, nitrate, ammonium, or glutamine can induce the accumulation of transcripts encoding *C_4Ppc* and *Cah* in leaves (132–135). Both transcriptional and posttranscriptional mechanisms are involved. The signaling process does not require de novo protein synthesis but involves calcium and protein phosphorylation (106). Recent studies have demonstrated that nitrogen signals are sensed by roots and stimulate the accumulation of cytokinin, which activates C_4 gene expression in leaves (107, 134). This study illustrates the first link between nitrogen and cytokinin signaling in controlling photosynthesis in higher plants. Recent advances include the identification of early cytokinin-inducible genes in maize (*ZmCip1*) and Arabidopsis (*ARR3-7* and *IBC6&7*) (13, 57, 107, 137). Sequence analyses show that these proteins possess similarity to the response-regulators in the bacterial

two-component signaling systems. It is proposed that *ZmCip1* could be a signaling component between cytokinin and transcription of *C_4Ppc*, *C_4Pdk*, and *Cah* in maize leaves (107, 134).

Metabolites

Metabolic repression of C_4 gene transcription has been extensively demonstrated in maize mesophyll protoplasts using C_4 gene promoters (59, 112). The studies revealed that the *C_4Pdk* promoter is specifically repressed by sucrose, glucose, fructose, acetate, and glycerol. Inhibition of positive elements but not activation of negative elements causes transcriptional repression. The examination of six other maize promoters has uncovered the global effects of sugars and acetate in gene repression. As these seven maize promoters are controlled by distinct *cis*-acting elements, no consensus sequences responsible for the sugar repression can be identified. Studies performed in C_3 plants support the concept that sugar repression of genes involved in photosynthesis is a universal phenomenon in higher plants. Biochemical, pharmacological, and molecular approaches have been taken to elucidate the sugar sensing and signaling mechanisms using the maize mesophyll protoplast system. The results have demonstrated the uncoupling of sugar signaling from sugar metabolism via glycolysis, and the involvement of protein kinases and phosphatases. Protein synthesis is not required. There is no evidence to suggest a role of calcium, cAMP, cGMP, or other second messengers in the signaling process (59, 60; J Jang & J Sheen, unpublished data). The implication that hexokinase is a sugar sensor has been supported by both biochemical experiments in transient expression assays and by genetic manipulation in transgenic plants (58, 59). Metabolic repression overrides light and developmental control and may serve as a mechanism for feedback regulation of photosynthesis and source-sink interactions in plants. The coupling of differential *Rbc* gene expression in BSC and source-sink transition discovered in amaranth may be an especially intriguing case of metabolic control (155).

Stress and Abscisic Acid

The amphibious leafless sedge *E. vivipara* develops Kranz anatomy and conducts C_4 photosynthesis under terrestrial conditions, but develops C_3-like traits and operates C_3 photosynthesis when submerged in water (147, 148). As the transition from water to land could signal a water-deficient condition for this unique plant, the anatomical development and gene expression could represent an adaptational response to water stress. Abscisic acid (ABA) is a stress hormone that is involved in the determination of leaf identity in some heterophyllic aquatic plants and the induction of crassulacean acid metabolism (CAM) in some succulent plants (148). The effect of ABA was tested in submerged

E. vivipara and shown to fully induce Kranz anatomy, C_4 gene expression, and C_4 photosynthesis. These experiments provide a new example for hormonal control of the development of the C_4 cycle pathway. Although the development of Kranz anatomy and C_4-type of gene expression are always tightly associated, recent studies suggest that the two processes can be uncoupled. In the transition region of newly sprouting culms after the terrestrial culm is submerged, the C_4 pattern of *RbcS* and *Ppc* expression can still be maintained when the development of BSC is repressed (146).

In contrast, ABA and environmental stress signals repress the expression of genes involved in C_4 photosynthesis. For example, the *RbcS* and *C_4Pdk* promoters are repressed significantly by ABA in maize (116, 117). This ABA signaling pathway is mediated by calcium-dependent protein kinases (CDPKs) and can be blocked by multiple protein phosphatases 2C (115, 116). It has been well documented that ABA and other stresses repress photosynthetic gene expression in C_3 plants (30, 65, 156, 158). Despite their recent evolution, the C_4 genes are subject to regulation of the conserved signaling pathways in plants. In the common ice plant, NaCl stress triggers complex responses in gene expression, including the induction of genes involved in CAM but repression of *RbcS* expression (25, 30).

Because of their ubiquitous expression patterns, it has been speculated that most ancestral genes for the C_4 genes have housekeeping functions, but their low expression may suggest that these functions may not be essential. A different explanation is suggested by the finding that the rice *cyPdk* gene is dramatically induced by low oxygene, ABA, and PEG, implicating a physiological function in stress responses in roots. The activities of PPDK, PEPC, and MDH are similarly induced in stressed rice plants (96). As plants cope with changing environments, their ability to alter carbon metabolism through gene expression offers great flexibility and adaptability for survival. Future experiments will confirm whether this stress response is conserved in plants.

Light

The most fascinating discoveries in the regulatory mechanisms for C_4 photosynthesis are the distinct posttranslational controls of the C_4 enzyme activities by light. The light activation of C_4PEPC requires protein phosphorylation. The use of protoplasts and biochemical tools has revealed a complex signaling cascade that includes cytosolic alkalination, calcium release, CDPK activation, protein synthesis, and the activation of a specific PEPCPK that is calcium-independent (34, 44, 62, 129, 149, 150). Although first discovered in C_4 plants, similar regulatory mechanisms are functional in diverse physiological contexts involving PEPC (44, 77, 150). Another light-regulated C_4 enzyme is C_4PPDK. C_4PPDK

undergoes a diurnal light-dark regulation of activity mediated by an unusual bifunctional regulatory protein (RP). RP inactivates PPDK in the dark by an ADP-dependent phosphorylation and activates PPDK in the light by dephosphorylation of the same Thr (456 in maize) (19, 48, 49). The light activation of the C_4 NADP-MDH is mediated by another distinct mechanism, reductive process during which disulfides are reduced into dithiols by reduced thioredoxin (48, 95). The isolation of genes encoding PEPCPK and RP, and the identification of regulatory residues by site-directed mutagenesis coupled to enzymatic activity assays are the focus of current research.

The expression of most C_4 photosynthesis genes is controlled by light perceived by red, blue, and UV light photoreceptors (17, 100, 117). The study of the maize *C_4Pdk* promoter in mesophyll protoplasts has defined a distinct blue light signaling pathway that is insensitive to green and red light, and uncoupled from the phytochrome or cryptochrome action mediated by calcium (6, 18, 26, 55, 61, 100, 117, 123). This is a direct and fast light response that does not depend on the presence of chloroplasts. The requirement of low fluence light (activated by 0.1 μE/m^{2}s of white light and saturated by 2.8 μE/m^{2}s of blue light) separates it from the blue/UVA pathway activated by high-intensity white light (100 μE/m^{2}s) (18, 23, 41, 61, 117, 124). A similar response is observed with the *RbcS* and *CabZm1* promoters but not the *C_4Ppc* promoter. Inhibition of PP1 activity by okadeic acid blocks this signaling process, which is also found in barley (23, 114). However, PKs seem to be required as well because two PK inhibitors, K252a and staurosporine, can also abolish this blue light response (K To & J Sheen, unpublished data). It will be interesting to determine whether any of the isolated cryptochrome genes (18) and the light-activated PKs (53, 108) are involved in this unique blue light signaling process.

Unlike the *C_4Pdk, RbcS* and *CabZm1* promoters, the activity of the *C_4Ppc* promoter is not detected in etiolated maize mesophyll protoplasts even under illumination (110). It is proposed that activation of this promoter by light is indirect and tightly coupled to the light induction of chloroplast biogenesis. In transgenic maize, it takes 6 h of illumination to activate this promoter and the inhibition of chloroplast protein synthesis eliminates its activity completely (63). This indirect light effect also contributes to the activity of the *RbcS* and *CabZm1* promoters. As Dof1 only activates the *C_4Ppc* promoter, distinct transcription factors are likely involved in transmitting the chloroplast signals to the *RbcS* and *CabZm1* promoters (40, 140).

Using the transient in situ expression assay, it has been shown that the *RbcS-m3* reporter gene is stimulated in BSC via a red/far-red reversible phytochrome signaling pathway. However, blue light is required for its suppression

in MC in illuminated maize leaves (100). Although the quality of light for the activation of the maize *Cab-m1* promoter in MC is not determined, the 54-bp fragment (−953 to −899) has been reported to mediate its photoregulation through calcium and calmodulin (6, 123, 136).

In amaranth, extensive studies indicate that translation initiation and elongation control the accumulation of RBC proteins during dark-to-light and light-to-dark transitions, respectively (7–10). Recent experiments have identified two 47-kDa proteins that specifically interact with the *RbcL* 5′UTR in light-grown but not in etiolated amaranth plants based on polysome heel printing, gel retardation, and UV cross-linking (DJ McCormac & JO Berry, personal communication). Whether these RNA binding proteins are universal for the regulation of RBCL translation but are modified to respond to different regulatory signals in diverse plants has not been determined.

Recent studies have described an effect of UV-B radiation on C_4 gene expression (17, 33). The transcript, protein, and enzymatic activity of C_4ME can all be increased by a short exposure (2–60 min) to low dose (0.5-2 μE/m^{2}s) of UV-B but not UV-A in maize. In addition, a low level of red light (10 μE/m^{2}s) exhibits similar effects as UV-B radiation. A 5-min exposure to far-red light (100 μE/m^{2}s) following UV-B or red light treatment largely reverses the induction. Furthermore, the maize *C_4Pdk* promoter can be activated by UV-A and UV-B radiation in maize mesophyll protoplasts (J Sheen, unpublished data). These surprising results support a novel physiological function of the C_4 cycle pathway in UV responses and deserve further investigation. It is proposed that the induction of the C_4 enzymes may contribute to repair of UV-induced damage by providing reductive power, increasing pyruvate for respiration, and providing substrates for lipid synthesis and membrane repair (17, 33).

GENETIC ANALYSIS OF C_4 GENE REGULATION

Maize Mutants

To dissect the pathway leading to dimorphic photosynthetic cell differentiation, a powerful genetic approach has been taken to isolate maize mutants that exhibit perturbed leaf development (28, 29, 46, 70, 104, 111). Many *bundle sheath defective* (*bsd*) mutants that display specific disruption in BSC have been identified and characterized (46, 70, 104). The primary effect of the *bsd1* (allelic to *goden 2, g2*) mutation is on plastid biogenesis and gene expression (*RbcS, RbcL, C_4Me*) specifically in BSC in a light-independent manner (70). The *G2* gene has been cloned by using Suppressor-mutator as a molecular tag (46). The leaf-specific expression pattern matches its function in leaves. G2 is the first member of a plant protein family with a TEA DNA-binding domain conserved from yeast, fly, to mammals. It has a functional nuclear localization

signal and a proline-rich region that can act as a transcription activation domain. Although G2 expression is detected in both MC and BSC, it is proposed that G2 might be regulated posttranscriptionally or act with BSC partners to serve its BSC-specific function. However, in the C3-type sheath leaves (where *Rbc* is expressed in both MC and BSC), G2 seems to be important for *Rbc* expression in both MC and BSC (46). The BSC-specificity of this mutant could be due to its regulation of photosynthetic functions more essential in BSC. It will be informative to determine whether the expression of other genes encoding Calvin cycle enzymes and plastid-encoded genes are also affected by the *bsd1-g2* mutation. The only known MC-specific maize mutant is *high chlorophyll fluorescence3* (*hcf3*) that displays MC-specific defect in the photosystem II thylakoid complex that is less important in BSC (28).

The *bsd2* mutant shows specific defect in RBC protein translation, accumulation, or assembly. The expression of BS-specific ME and plastid biogenesis in the dark are normal. The BS-specific defects of chloroplasts under light and the ectopic accumulation of *RbcL* transcripts in MC may be secondary effects due to the lack of RBC for Calvin cycle activity (104). Another interesting maize mutant that shows preferential disruption in BSC is the *leaf permease1* (*lpe1*) mutant (111). The cloned *Lpe1* sequence indicates that it is a membrane protein sharing homology to the bacterial and eukaryotic pyrimidine and purine transporters or permeases (111). The BS preferential nature of the phenotype may be a consequence of the greater dependence of BSC on Lpe1-related metabolites. How Lpe1 affects early chloroplast development with a root- and dark-specific expression pattern is an intriguing question. As proposed, its effect should have occurred at the very early stage of plastid development in leaves. It is not clear whether the *lpe1* mutant exhibits aberrant phenotypes in roots or other organs.

Amaranth Mutants and Flaveria C_3-C_4 Hybrids

By screening plants able to grow under high CO_2 but showing symptoms of stress or chlorosis following exposure to normal air, some *Amaranthus edulis* mutants have been isolated. Further characterization confirms that these mutants show severely reduced PEPC or lack NAD-ME that are essential for C_4 photosynthesis. These mutants are very valuable for further investigation of the role and regulation of PEPC, NADP-ME, and photorespiration in C_4 photosynthesis (31, 69).

Taking advantage of the sexual compatibility between *Flaveria* C_4 and C_3 species and gene-specific molecular markers, it has been possible to genetically identify *cis*-acting elements and *trans*-acting factors that are important for the C_4 gene expression patterns (127). A cross between a C_4 *F. palmeri* and a non-C_4 *F. ramossisima* has been carried out. The F_1 hybrids and F_1 backcross segregates have been analyzed for *RbcS* and *Pdk* gene expression by using PCR markers, in

situ hybridization, and immunolocalization. The results suggest that the BSC-specific trait of *RbcS* expression in the C_4 *F. palmeri* is recessive and may be controlled by one or few *trans*-acting factors. On the other hand, the C_4 *Pdk* gene expression trait is dominant and mainly mediated by *cis*-acting elements (127). The result of the C_4 *Pdk* gene expression in the C_4/C_3 hybrids is consistent with the similar analyses performed in transgenic C_4 and C_3 plants. However, the regulation of *RbcS* genes in BSC seems to be complex in different C_4 plants.

SUMMARY

The studies of C_4 gene expression have illustrated the complexity and flexibility of molecular mechanisms and the diversity of signal transduction pathways that are engaged in gene regulation in plants. It is clear that all C_4 genes originated from closely related ancestral genes by acquiring new *cis*-acting regulatory elements that are surprisingly divergent in sequences. The need of C_4-specific *trans*-acting factors is also evident for *C_4Me* and *RbcS* expression. C_4 photosynthesis research was initiated 30 years ago by the discovery of the unconventional CO_2 fixation pathway. New findings including the dimorphic BSC and MC in C_4 leaves, the three decarboxylation pathways, and the light regulation of C_4 enzymes by phosphorylation followed. The recent application of molecular, cellular, transgenic, and genetic approaches has revealed novel C_4 gene structure, specific cell-cell communications, complex transcriptional and posttranscriptional regulations, new DNA-binding transcription factors, and innovative signal transduction pathways.

The powerful combination of transgenic and transient expression tools will bring new insights into the molecular mechanisms of C_4 gene regulation. The genetic crosses and molecular analyses of the *Flaveria* C_3/C_4 hybrids provide new means to sort out the regulatory components involved in the C_4 gene expression patterns. The protoplast transient expression system is most useful for the elucidation of signal transduction pathways. Novel mutant screens for uncommon MC phenotypes and variations in leaf morphology and anatomy may uncover genetic components controlling C_4 photosynthesis and leaf development. With facile transformation methods and the knowledge of C_4 gene structure and regulation, genetic manipulation of the C_4 cycle pathway in C_4 and C_3 plants can now be fully enjoyed.

ACKNOWLEDGMENTS

This review is dedicated to MD Hatch for scientific inspiration and to my mother for spiritual inspiration. I thank my colleagues A Schäffner, H Huang, P Leon, K To, J-C Jang, A Kausch, and S Yanagisawa for numerous contributions. Special thanks to WC Taylor, M Matsuoka, JO Berry, L Bogorad, G Edwards, M Ku,

R Chollet, C Chastain, H Sakakibara, T Shugiyama, O Ueno, T Izui, T Nelson, N Dengler, E Kellogg, P Westhoff, L Mets, and G-P Shu for providing information. Work in the Sheen lab has been supported by the USDA photosynthesis program, NSF Integrated Plant Biology, and Hoechst AG.

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