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Update on Photosynthetic Gene Expression

Molecular Genetics of Crassulacean Acid Metabolism[1]

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Most higher plants assimilate atmospheric CO_2 through the C_3 pathway of photosynthesis using ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). However, when CO_2 availability is reduced by environmental stress conditions, the incomplete discrimination of CO_2 over O_2 by Rubisco leads to increased photorespiration, a process that reduces the efficiency of C_3 photosynthesis. To overcome the wasteful process of photorespiration, approximately 10% of higher plant species have evolved two alternate strategies for photosynthetic CO_2 assimilation, C_4 photosynthesis and Crassulacean acid metabolism. Both of these biochemical pathways employ a "CO_2 pump" to elevate intracellular CO_2 concentrations in the vicinity of Rubisco, suppressing photorespiration and therefore improving the competitiveness of these plants under conditions of high light intensity, high temperature, or low water availability. This CO_2 pump consists of a primary carboxylating enzyme, phospho*enol*pyruvate carboxylase. In C_4 plants, this CO_2-concentrating mechanism is achieved by the coordination of two carboxylating reactions that are spatially separated into mesophyll and bundle-sheath cell types (for review, see R.T. Furbank, W.C. Taylor [1995] Plant Cell 7: 797–807; M.S.B. Ku, Y. Kano-Murakami, M. Matsuoka [1996] Plant Physiol 111: 949–957). In contrast, Crassulacean acid metabolism plants perform both carboxylation reactions within one cell type, but the two reactions are separated in time. Both pathways involve cell-specific changes in the expression of many genes that are not present in C_3 plants.

Unlike C_3 and C_4 plants, CAM plants assimilate atmospheric CO_2 into C_4 acids predominantly at night and subsequently refix this CO_2 to the level of carbohydrates during the following day (Fig. 1). To accomplish this nocturnal CO_2 uptake, stomata of CAM plants are opened at night and kept closed during most of the day. This strategy allows CO_2 uptake from the atmosphere to occur when evapotranspiration rates are low and permits daytime photosynthetic carbon fixation by the carbon reduction cycle to occur behind closed stomata, resulting in minimal water loss and reduced photorespiration. Thus, CAM plants exhibit water use efficiency rates 5 to 10 times higher than C_4 and C_3 plants, resulting in a considerable competitive advantage in water-limited environments such as deserts or epiphytic microhabitats. The CAM adaptation can also be found in some aquatic plants that grow in environments with low daytime CO_2 concentrations (Keeley, 1983), suggesting that water-use efficiency per se was apparently not the primary driving force for the evolution of CAM. Instead, the stress of CO_2 limitation brought about by either daytime stomatal closure or low CO_2 availability in aquatic habitats may have provided the common selective pressure responsible for the evolution of CAM in both terrestrial and aquatic plants (Ehleringer and Monson, 1993).

Aside from conducting the bulk of atmospheric CO_2 assimilation at night, CAM plants are further distinguished from C_3 or C_4 plants by a number of unique features (Ting, 1985). First, CAM plants accumulate organic acids, predominantly malic acid, at night, and reciprocal accumulation of storage carbohydrates such as starch, glucans, or soluble hexoses occurs during the day. Second, to accommodate these large diurnal changes in C_4 acids and carbohydrates, CAM plants display high amounts of PEPC for nighttime CO_2 fixation and possess active C_4 acid decarboxylation enzyme systems to provide CO_2 for daytime refixation via the photosynthetic carbon reduction cycle. Third, PEPC is activated at night by phosphorylation and inactivated during the day by dephosphorylation, a pattern opposite to that found in C_3 or C_4 plants. In addition to these unique biochemical characteristics, CAM plants have evolved a number of distinct anatomical and morphological features. These may include chlorenchymatous mesophyll tissues consisting of large, thin-walled cells containing prominent vacuoles, varying degrees of succulence in photosynthetic leaves or stems, small stomata, and low stomatal frequencies to limit water loss. CAM plants often have a large, nonchlorophyllous mesophyll or other water-storage cell types that do not participate in CAM but contribute to survival during prolonged periods of drought. In addition, the root systems of CAM plants exhibit low root:shoot ratios to limit water loss to the soil, yet

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Abbreviations: enolase, 2-phospho-D-glycerate hydrolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase; PPDK, pyruvate orthophosphate dikinase.

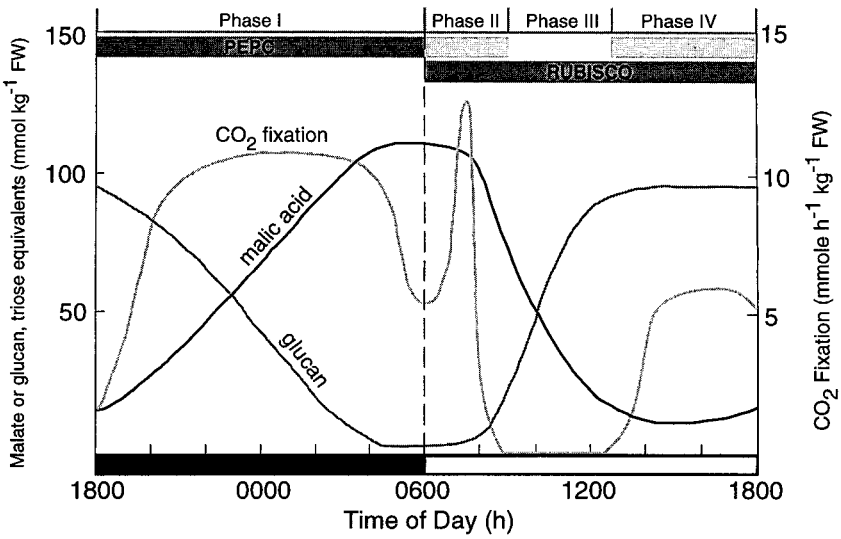

Figure 1. The diurnal cycle of CAM photosynthesis. The day-night cycle of the CAM pathway observed in the mesophyll cells of a typical well-watered constitutive CAM plant can be divided into four distinct phases. The bulk of CO_2 fixation occurs at night (phase I) by the action of PEPC. At the beginning of the light period (phase II), the combined actions of Rubisco and PEPC result in a burst of CO_2 fixation. Daytime malate decarboxylation leads to high internal CO_2 concentrations resulting in stomatal closure and a drop in net CO_2 fixation (phase III). Toward the end of the light period (phase IV), PEPC may be catalytically active, but makes no significant contribution to malate formation. Nocturnal malic acid accumulation fluctuates in a reciprocal diurnal manner with daytime glucan accumulation. Modified with permission from Winter and Smith (1996). FW, Fresh weight.

retain the ability to readily take up water when rewetted following drought (Nobel and North, 1996).

THE CAM PHOTOSYNTHETIC CYCLE

The key feature of CAM is the temporal separation and differential control of two major carboxylating enzymes within a single cell. The temporal sequence of CAM can be divided into four phases based on stomatal behavior, modes of CO_2 uptake and fixation, and C_4 acid and carbohydrate accumulation over the course of the diurnal cycle (Osmond, 1978) (Fig. 1). At night (phase I) PEPC catalyzes fixation of atmospheric CO_2 from PEP, resulting in an increase in malic acid and its accumulation in the vacuole, and accompanied by a reciprocal decrease in storage carbohydrates (glucans or soluble sugars) (Fig. 2A). CO_2 fixation slows toward the end of the dark period as malic acid accumulation reaches a maximum, which inhibits PEPC activity. Upon illumination, the concerted action of PEPC and Rubisco during phase II results in a transient burst of CO_2 fixation into both C_4 acids and C_3 products. Stomata close gradually and fixation of atmospheric CO_2 into C_4 acids ceases. During the day (phase III), malic acid is decarboxylated to produce CO_2 and C_3 carbon backbones for carbohydrate synthesis and C_3 photosynthesis (Fig. 2B). Declining vacuolar malic acid reserves slow malate decarboxylation and the internal CO_2 concentration declines, leading to stomatal opening under favorable environmental conditions. In the beginning of phase IV, fixation of exogenous CO_2 proceeds via typical C_3 photosynthesis. Toward the end of this phase PEPC becomes active again and CO_2 assimilation by Rubisco diminishes, completing the cycle.

Similar to C_4 plants, CAM plants can be grouped into subtypes based on the decarboxylating enzymes employed during phase III. Unlike C_4 plants, however, CAM plants cannot clearly be classified on the basis of three C_4-acid-decarboxylating systems (NADP-ME, NAD-ME, and PEPCK), since many contain substantial activities of both cytoplasmic NADP-ME and mitochondrial NAD-ME. Pyruvate generated by malate decarboxylation in NAD(P)-ME plants requires PPDK activity in the chloroplast for gluconeogenic recovery of PEP (Fig. 2B). PEPCK-like CAM plants display high cytoplasmic levels of PEPCK activity, but have low ME activities and completely lack PPDK activity (Winter and Smith, 1996). With the basic principles of CAM well established, we will summarize recent progress in the molecular genetic analysis of CAM, with emphasis on the regulation of this unique metabolic adaptation to environmental stress.

PERMUTATIONS OF CAM

A striking feature of CAM is the extreme physiological and metabolic plasticity in the extent to which nocturnal CO_2 fixation contributes to net carbon gain. Variations range from species that appear "nearly C_3" to those that exhibit obligate CAM, including species that swing into and out of CAM depending on changes in the environment or developmental events. One mode, "CAM cycling," is characterized by diurnal fluctuations in C_4 acids in the absence of daytime stomatal closure and little or no net nocturnal carbon assimilation (Ting, 1985). By refixing respiratory CO_2 at night, plants that perform CAM cycling are thought to be poised to enter full CAM rapidly when necessary due to environmental stress conditions. Another variation, "CAM idling," occurs under extreme environmental stress conditions when stomata remain closed day and night, yet diurnal fluctuations in organic acids continue as a result of refixation of respiratory CO_2 (Bastide et al., 1993). CAM idling may help to preserve photosynthetic enzyme activities until favorable growth conditions return. In addition to cycling or idling modes, facultative CAM plants may shift from C_3 to CAM in response to age or environmental conditions such as photoperiod, high salinity, or water deficit.

The shift from C_3 photosynthesis to CAM is usually accompanied by dramatic changes in physiology, biochemistry, and gene expression. In facultative CAM plants, activities of glycolytic and gluconeogenic and C_4 acid metab-

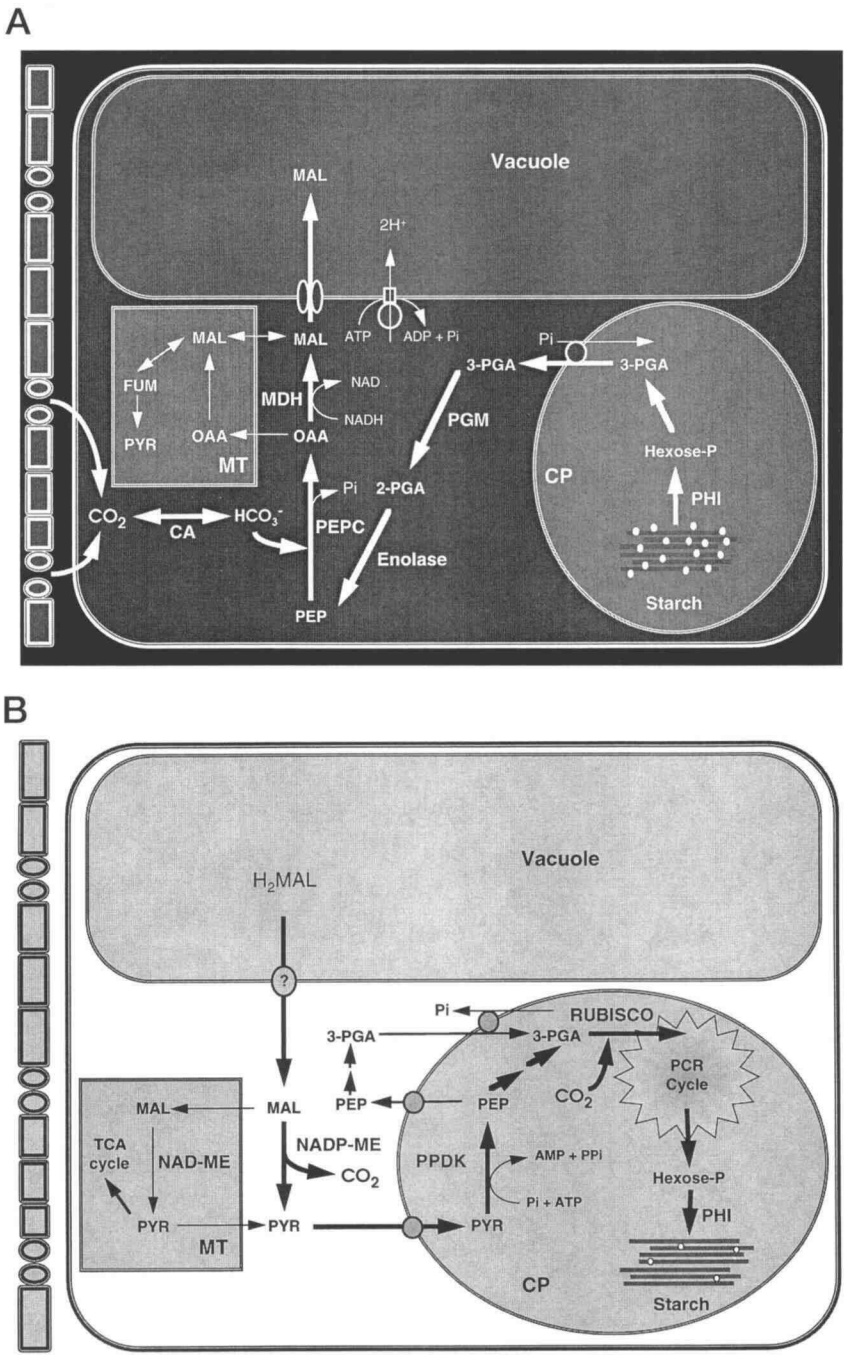

Figure 2. Metabolite flux during CAM. Carbon flow in a cell performing CAM (ME type) during the diurnal cycle. During phase I (A), nocturnal CO_2 uptake through open stomata and glycolytic breakdown of storage carbohydrates result in the formation of PEP, which serves as the substrate for CO_2 fixation by PEPC, leading to oxaloacetate (OAA) production and reduction to malate by MDH. PEP is supplied from glycolytic 3-phosphoglycerate formation and export from the chloroplast via the chloroplast Pi transporter. Malate is transported into the vacuole by a selective ion channel in the tonoplast membrane. As plants enter the light period, stomata close gradually and fixation of external CO_2 ceases. During phase II (not shown), an initial burst of CO_2 fixation generally occurs because both PEPC and Rubisco are active. In phase III (B), malate is released from the vacuole by passive efflux and decarboxylated by ME, leading to the release of CO_2 and the formation of pyruvate, which is converted to PEP by PPDK. Decarboxylation of malate proceeds concurrently with an increase in internal CO_2 concentrations, suppressing photorespiration. The liberated CO_2 is reassimilated via Rubisco and the photosynthetic carbon reduction (PCR) cycle. Pyruvate and PEP fuel gluconeogenesis to regenerate storage carbohydrate pools. In phase IV (not shown), internal CO_2 concentrations decline and stomata may open, allowing fixation of exogenous CO_2 to occur via Rubisco. PEPC may also become active during this phase as malate concentrations in the cytoplasm decline. The contribution of mitochondria to malate formation and decarboxylation is also shown. MT, Mitochondria; CP, chloroplast; CA, carbonic anhydrase; PGM, phosphoglyceromutase (which catalyzes 3-phosphoglycerate into 2-phosphoglycerate); PHI, phosphohexose isomerase (which catalyzes Fru-6-P into Glc-6-P); MAL, malate; PYR, pyruvate; FUM, fumarate; TCA, tricarboxylic acid. PEPC catalyzes PEP and HCO_3^- into oxaloacetate; MDH catalyzes oxaloacetate and NADH into malate and NAD^+; enolase catalyzes 2-phosphoglycerate into PEP.

olism enzymes increase up to 40-fold during the induction of the pathway by environmental stress due at least in part to de novo enzyme synthesis (see Cushman and Bohnert, 1996). Explanations for the basis of CAM shifting range from strictly ontogenetic to solely dependent on environmental conditions (Edwards et al., 1996). In *Peperomia*, an ontogenetic development of CAM that can be enhanced by water stress has been well documented (Ting et al., 1996). In others species, such as *Mesembryanthemum crystallinum*, the development of CAM in well-watered plants is likely to result from transient declines in leaf turgor pressure and water content during the day (Winter and Gademann, 1991) rather than from a developmental program. Stress-mediated CAM induction is reversible in several different facultative CAM species, supporting the exclusive involvement of environmental factors.

CAM-SPECIFIC GENES

The enzymatic machinery required to perform CAM is present in all higher plants. In CAM and C_4 plants, however, the activities of key enzymes such as PEPC, PPDK, NAD(P)-ME, and PEPCK are much higher than in C_3 plants. Unlike C_4 plants, however, in which the expression

patterns of these key photosynthetic enzymes differ on the basis of intercellular compartmentation, CAM plants have evolved diurnal patterns of expression and regulation to accommodate the enhanced carbon flux through glycolysis and gluconeogenesis required to satisfy the demand for PEP for nighttime CO_2 fixation and daytime decarboxylation of C_4 acids (Paul et al., 1993). Characterization of CAM-specific genes and their expression patterns in facultative CAM plants (summarized in Table I) has begun to shed light on the molecular mechanisms underlying the evolution and expression of these enzymes.

In CAM plants such as *M. crystallinum* and *Kalanchoë blossfeldiana*, the PEPC gene family is composed of up to four members, with one or two isoforms specific to CAM (Cushman et al., 1989; Gehrig et al., 1995). In *M. crystallinum*, the CAM-specific gene becomes highly expressed during CAM induction brought about by environmental stress (Cushman et al., 1989). Ancient gene duplication events followed by alterations in the promoters of these genes may have given rise to either the C_3 or CAM isoforms, each with distinct expression patterns. Multigene families have also been reported for ME, MDH, enolase, and phosphoglyceromutase in *M. crystallinum* (Cushman, 1992, 1993; Forsthoefel et al., 1995a, 1995b) and the H^+-ATPase c subunit in *M. crystallinum* (Tsiantis et al., 1996) and *Kalanchoë daigremontiana* (Bartholomew et al., 1996). Gene duplication, however, is not essential; products of a single gene can apparently play roles in both C_3 photosynthesis and CAM, as is the case for NAD-GAPDH (Ostrem et al., 1990) and PPDK (Fisslthaler et al., 1995). An important challenge for the future will be to determine the relative contribution of specific members of multigene families and the interplay of their gene products, particularly when they occupy different subcellular compartments. For example, both mitochondrial NAD-ME and cytosolic NADP-ME are coordinately induced to support daytime malate decarboxylation (Cushman, 1992; Saitou et al., 1994). Similarly, the combined activities of cytosolic NAD-MDH and chloroplastic NADP-MDH (Cushman, 1993) may be necessary for nocturnal malic acid formation. Furthermore, it will be important to understand if CAM-specific isozymes have undergone amino acid changes that result in alterations in regulatory and kinetic properties better suited for CAM. The characterization of more CAM-specific genes and genes from other CAM species should provide greater insight into the genetic basis of such alterations.

REGULATION OF CAM-SPECIFIC GENE EXPRESSION

CAM induction involves the regulation of an assemblage of enzymes and metabolite transporters, making it one of the more complex metabolic adaptations to environmental stress known. Studies concerning the regulation of CAM-specific gene expression have been confined largely to facultative CAM, wherein the shift from C_3 photosynthesis to CAM can be induced by a number of environmental

Table I. *Genes and transcripts involved in CAM*

Gene	Enzyme	Organism	Subcellular Location	Inducer	Reference
Ppc1	PEPC	*M. crystallinum*	Cytosol	NaCl, ABA, drought, 6-benzylaminopurine	Cushman et al., 1989; Thomas et al., 1992
Ppc	PEPC	*K. blossfeldiana*	Cytosol	ABA, drought, short-day length	Gehrig et al., 1995; Taybi et al., 1995
Mdh1	NADP-MDH	*M. crystallinum*	Chloroplast	NaCl	Cushman, 1993
Mod1	NADP-ME	*M. crystallinum*	Cytosol	NaCl	Cushman, 1992
Ppdk1	PPDK	*M. crystallinum*	Chloroplast	NaCl	Fisslthaler et al., 1995
Pgh1	Enolase	*M. crystallinum*	Cytosol	NaCl, drought, cold, hypoxia, ABA, 6-benzylaminopurine	Forsthoefel et al., 1995a
Pgm1	Phosphoglyceromutase	*M. crystallinum*	Cytosol	NaCl, drought, ABA, 6-benzylaminopurine	Forsthoefel et al., 1995b
Pgk1	Phosphoglyceratekinase	*M. crystallinum*	Cytosol	NaCl	N. Forsthoefel, personal communication
GapC1	GAPDH	*M. crystallinum*	Cytosol	NaCl	Ostrem et al., 1990
AtpvA	H^+-ATPase, A subunit[a]	*M. crystallinum*	Vacuole	NaCl	Löw et al., 1996
AtpvB	H^+-ATPase, B subunit[a]	*M. crystallinum*	Vacuole	NaCl	Löw et al., 1996
AtpvE	H^+-ATPase, E subunit	*M. crystallinum*	Vacuole	NaCl	Dietz and Arbinger, 1996
Atpvc	H^+-ATPase, c subunit	*M. crystallinum, K. daigremontiana*	Vacuole	NaCl, ABA, light	Bartholomew et al., 1996; Löw et al., 1996; Tsiantis et al., 1996
Sep1	Sulfhydryl endopeptidase[b]	*M. crystallinum*	Vacuole?	NaCl, drought, ABA, 6-benzylaminopurine, methyl jasmonate	N. Forsthoefel and J. Cushman, unpublished data
MK9	Ser/Thr kinase[c]	*M. crystallinum*	Cytosol	NaCl	Baur et al., 1994
MK6	Ser/Thr kinase[d]	*M. crystallinum*	Cytosol	NaCl, diurnal rhythm	B. Baur, personal communication

[a] Induced only in roots and young leaves. [b] Expressed only in leaves, possibly involved in remobilization of amino acids during CAM induction. [c] Possibly involved in the signal transduction of metabolic signals associated with CAM induction. [d] Possibly involved in regulation of diurnal rhythm of the CAM cycle.

factors such as photoperiod, drought, or high salinity (see Table I). Although transcriptional and posttranscriptional regulatory mechanisms are clearly involved, the exact regulatory mechanisms important for transcriptional activation and transcript stability and recruitment remain largely unknown.

Transcriptional Control

The expression of CAM-specific genes is regulated primarily at the level of transcription. Run-off transcription assays with nuclei isolated from leaves of *M. crystallinum* show that transcription of CAM-specific genes increases from 2- to 6-fold when plants are exposed to high salinity (Cushman et al., 1989; Forsthoefel et al., 1995a, 1995b). One intriguing question is how CAM-specific isogenes in a gene family exhibit expression patterns that are much higher than C_3-specific isogenes. Comparison of the promoter sequences of a CAM-specific and a C_3 "housekeeping" isogene of PEPC, *Ppc1* and *Ppc2*, respectively, indicates that these promoters are quite dissimilar. The *Ppc1* promoter contains consensus TATA and CAAT box motifs, whereas *Ppc2* does not (Cushman et al., 1993). The absence of such consensus motifs is often indicative of housekeeping genes. In contrast, the stress-inducible gene encoding NAD-GAPDH (*GapC1*) also lacks these consensus motifs, reinforcing the notion that *GapC1* functions in both a housekeeping and a CAM-specific capacity (Ostrem et al., 1990). However, this gene also contains additional *cis*-acting elements that confer stress-inducible expression patterns during CAM (see below). The promoter regions of the CAM-specific PEPC, NAD-GAPDH, and PPDK genes contain GT motifs (box II) that may function in light-responsive gene expression and consensus ABA response elements that may function in ABA-mediated gene expression events (Cushman et al., 1993; Schmitt et al., 1996). In contrast, these elements are conspicuously absent from the *Ppc2* promoter. The absence of nucleotide sequence similarities between these promoters argues against duplications of entire genes, but suggests that promoter elements may have evolved by genomic rearrangements such as transposon-induced translocation events (Kloeckener-Gruissem and Freeling, 1995).

Studying the stress-inducible expression of CAM-specific genes in facultative CAM species such as *M. crystallinum* may provide valuable insights into the nature of the regulatory *cis* elements governing their expression patterns. The coordinate expression of these genes in this species suggests they may share common *cis*-acting regulatory elements to mediate stress-inducible expression patterns. To investigate this hypothesis, Schaeffer et al. (1995) developed a transient expression system based on microprojectile bombardment of detached *M. crystallinum* leaves that faithfully reproduces CAM-specific patterns of gene expression. The stress-inducible *Ppc1* promoter contains a complex set of elements, including several redundant activator domains and one silencer region. Distal regions upstream of −721 from the transcriptional start site are important in modulating the amplitude of salt-inducible expression, and a region between −977 and −721 is sufficient to confer salt-responsive gene expression. More proximal regions also contribute to salt-inducible expression; however, the magnitude of induction, as well as the overall level of expression in both stress and unstressed plants, is reduced. The region between −487 and −320 appears to suppress salt-inducibility, suggesting that a silencer element is present within this region. In contrast, the *GapC1* promoter is less complex, with the elements responsible for salt-inducible expression contained within a 186-bp region located between −735 and −549 (Schaeffer et al., 1995). The elements located between −675 and −549 seem to be responsible for salt-inducible gene expression, whereas sequences from −735 to −675 appear to control the amplitude of expression in both unstressed and stressed plants. Comparison of the *Ppc1* and *GapC1* promoters in the distal regions sufficient for salt-inducible gene expression share multiple *myb/myc* consensus binding sites, suggesting that MYB/MYC-related transcription factors may be involved in controlling transcriptional activation events during CAM induction.

The exact nature of *trans*-acting factors involved in controlling CAM-specific transcription patterns remains largely unknown. Regions of the *Ppc1* and *GapC1* promoters sufficient to confer salt inducibility form slow-mobility DNA-protein complexes that are more abundant following CAM induction (Schaeffer et al., 1995) may be the result of de novo protein synthesis following salt stress. Alternatively, the factor(s) within this complex have undergone a modification to cause greater DNA-binding affinity under stress conditions. Further characterization of these DNA-binding proteins is required to elucidate the mechanisms by which they activate transcription. Studies with the 5′ flanking region of *Ppc1* show that multiple DNA-binding protein complexes interact with this promoter in vitro over a region of more than 600 nucleotides (Cushman and Bohnert, 1992) to mediate tissue-specific, diurnal, light, developmental, environmental stress, and hormone-responsive gene expression. One DNA complex, designated PCAT-1, interacts with AT-rich sequences between −205 and −128 of the *Ppc1* promoter and displays increased abundance or DNA-binding affinity in CAM-induced, salt-stressed plants (Cushman and Bohnert, 1992). PCAT-1 shares characteristics of high mobility group-like proteins, suggesting that it may play an architectural role in the assembly of active transcription complexes during CAM induction.

Posttranscriptional and Posttranslational Regulation

Apart from transcriptional regulation, posttranscriptional mechanisms such as increased mRNA stability may facilitate long-term CAM buildup during salt stress (Cushman et al., 1990), whereas mRNA destabilization may be the cause for a rapid decrease in mRNA levels during relief from salt stress. However, direct evidence for these control mechanisms is lacking. Increases in chloroplast RNA-binding proteins accompanying CAM induction have been observed and the distribution of transcripts in polysomes changes: *RbcS* transcripts decline, whereas transcripts for the CAM-specific PEPC increase (Bohnert et al., 1996). In-

direct evidence for translational regulation of enolase in *M. crystallinum* has also been presented (Forsthoefel et al., 1995a); however, further studies are required to confirm the importance of posttranscriptional mechanisms to CAM gene expression.

Posttranslational mechanisms govern short-term responses such as modulating translation, protein degradation, and enzyme activities in synchrony with the diurnal pattern of CAM. PEPC activity is allosterically controlled by both positive (Glc-6-P, triose phosphate) and negative (L-malate) effectors (Chollet et al., 1996). More importantly, covalent modification in the form of reversible phosphorylation/dephosphorylation cycles connects the activity of PEPC to the diurnal regulation of CAM, avoiding futile cycles of carboxylation and decarboxylation (Chollet et al., 1996). Regulation of PEPC activity by reversible phosphorylation was first described in *Bryophyllum fedschenkoi*, a constitutive CAM species (see Carter et al., 1996), and has now been demonstrated in *M. crystallinum* (Li and Chollet, 1994) and in C_3 and C_4 species.

The phosphorylation state of PEPC is controlled by PEPC kinase. In C_3 and C_4 plants, light activates and darkness inactivates this kinase. In contrast, the PEPC kinase activity of CAM plants is not directly related to changes in illumination, but is controlled by a circadian rhythm. In *M. crystallinum*, PEPC kinase is induced coincidentally with its target protein by salinity stress and is activated at night (Li and Chollet, 1994). Partial purification of this Ca^{2+}-independent kinase identified two polypeptides (39 and 32 kD) capable of phosphorylating purified PEPC from C_3, C_4, and CAM species. Whether these two proteins represent two isoforms or proteolytic forms of one protein remains unclear (Li and Chollet, 1994). Inhibitors of protein and RNA synthesis block the nocturnal appearance of kinase activity, suggesting that the protein is synthesized and degraded on a circadian basis (see Carter et al., 1996). Extremes in light intensity and temperature are known to perturb circadian rhythms of CO_2 fixation through modifications of PEPC kinase activity (Carter et al., 1996; Grams et al., 1996). In contrast, dephosphorylation of PEPC by a protein phosphatase 2A is not regulated diurnally.

Little is known about the regulation of CAM enzymes other than PEPC. Posttranscriptional control of enolase activity, possibly by reversible phosphorylation, has been suggested in studies using *M. crystallinum* (Forsthoefel et al., 1995a). Also, the activities of many chloroplastic and possibly cytosolic enzymes such as NAD-GAPDH, NADP-MDH, and Fru-1,6-bisphosphatase are light regulated by a covalent redox-modification cycle of the Fd-thioredoxin system, which acts at redox-sensitive Cys residues that participate in disulfide bridges (Anderson et al., 1995). Understanding the contribution of such posttranslational regulatory events to the control of CAM enzyme activity represents an important area for future investigations.

Tissue-Specific Regulation

Traditionally, CAM was thought to be performed by all chloroplast-containing cells in the plant, but recent studies indicate a tissue-specific division of labor. In *Peperomia*, the leaf is divided into four layers: an upper multiple epidermis, a middle one- to two-layered palisade mesophyll, a lower spongy mesophyll, and an abaxial epidermis. CAM enzyme expression and activity occur predominantly in the spongy mesophyll and to a lesser extent in the upper epidermis, whereas enzymes associated with C_3 photosynthesis occur predominantly in the palisade mesophyll. Tissue printing of *Peperomia* leaves confirms that PEPC protein and transcript expression is localized specifically to the multiple epidermis and spongy mesophyll (Ting et al., 1994). In contrast, Rubisco protein and mRNA are distributed throughout the leaf. Electron transport activity and chlorophyll-protein analysis indicate that the spongy mesophyll displays higher PSI:PSII ratios, which may be related to the increased ATP requirements of C_4 metabolism in this tissue (Nishio and Ting, 1993). Although it may be argued that *Peperomia* species are unique in their unequal distribution of CAM and C_3 enzymes, tissue-specific expression of CAM enzymes may be common, considering the morphological complexity of leaves in many CAM plants.

SIGNAL TRANSDUCTION

The mechanisms underlying the conversion of developmental and environmental signals into changes in CAM gene expression remain largely unexplored. A multitude of factors, such as intracellular Ca^{2+}, CO_2, malate, and carbohydrate concentrations, redox state, or cytosolic pH, may participate in signaling during CAM induction. In guard cells, high ABA or intracellular CO_2 concentrations are known to lead to elevated Ca^{2+} amounts, resulting in stomatal closure (Webb et al., 1996). Therefore, it is expected that changes in Ca^{2+} concentrations act to mediate stomatal closure during the daytime phases of the diurnal CAM cycle. Depletion of malate reserves lowers internal CO_2 and Ca^{2+} concentrations, leading to stomatal reopening toward the end of the day. Plants performing CAM, however, appear to undergo an inactivation of the blue- and red-light photoreceptors that normally mediate daytime stomatal opening, presumably so that stomatal movements can be modulated by other factors such as ABA and CO_2 (Lee and Assmann, 1992; Mawson and Zaugg, 1994). Whether the photoreceptors themselves or some other component of the signal transduction pathway(s) are rendered inoperative remains unclear.

Based on current evidence, CAM is controlled by a complex network of independent yet interrelated signaling pathways. For example, the physiological and gene-expression responses elicited by ABA persist in salt-stressed *M. crystallinum*, even when endogenous ABA increases are suppressed using lovastatin (Thomas et al., 1992), an inhibitor of isoprenoid biosynthesis, suggesting that ABA may act as a response modulator in conjunction with other stimuli, but not as a component of the primary signaling chain. This notion is reinforced by the observation that ABA requires high light intensity to induce PEPC expression (see Cushman and Bohnert, 1996). Recent evidence suggests that specific sets of protein kinases and phosphatases catalyze reversible phosphorylation steps

and modulate downstream events such as stomatal movement and changes in gene expression. Treatment of ice plant leaves with okadaic acid, a specific inhibitor of type 1 and 2A protein phosphatases, inhibits *Ppc1* transcript accumulation induced by dehydration, salinity stress, ABA, and methyl jasmonate treatments, suggesting that these target phosphatases participate in the signal transduction pathway leading to CAM induction. In contrast, treatment of detached leaves with W-7, a calmodulin antagonist, specifically inhibits *Ppc1* transcript accumulation in response to osmotic but not to dehydration stress (T. Taybi and J. Cushman, personal communication). These results suggest that independent signaling pathways operate in response to distinct environmental stresses and that at least one pathway involves changes in cytoplasmic Ca^{2+} concentrations. Treatment of *M. crystallinum* leaves with thapsigargin, a specific inhibitor of intracellular Ca^{2+}-ATPases and a stimulator of intracellular Ca^{2+} release, showed that leaves display increased *Ppc1* mRNA accumulation (M.S. Tsiantis, W. Deng, and J.A.C. Smith, personal communication), confirming the involvement of changes in cytoplasmic Ca^{2+} in CAM signaling pathways. Dissecting the specificity and possible cross-talk mechanisms for these pathways, however, will require characterization of the components of the signal transduction pathways themselves. MK9, a Ser/Thr protein kinase cDNA isolated from *M. crystallinum* (Baur et al., 1994) that shares similarity to an ABA-, dehydration-, cold-stress-, and osmotic-stress-inducible protein kinase from wheat, may be one such component.

LIFE AT THE TONOPLAST

Nocturnal CO_2 fixation results in the formation of large amounts of malate or other C_4 acids that must be sequestered in the vacuole to prevent acidification of the cytoplasm and feedback inhibition of PEPC. To accommodate the increased demands of malate uptake and storage into the vacuole during CAM, transport capacity must be increased. This transport is driven by the maintenance of an inside-positive electrochemical membrane potential gradient across the tonoplast membrane energized by tonoplast V_0V_1-H^+-ATPase and H^+-inorganic orthophosphatase activities (for review, see Lüttge et al., 1995). Malate is translocated across the tonoplast membrane in the form of a divalent anion (Fig. 2) via an ion channel (Iwasaki et al., 1992) or a malate transporter into the vacuole, where it becomes protonated. Malate transport into and out of the vacuole must be tightly regulated for precise fluctuations in C_4 acids to occur on a diurnal basis. Inward-rectifying, malate-selective ion channels favor malate influx at night, whereas during the day malate influx presumably becomes inactivated, leading to a net efflux of malate via a second channel (Iwasaki et al., 1992) or by passive diffusion through the tonoplast membrane. Although the kinetic characteristics of vacuolar malate transport are well known, the identity of the vacuolar ion channels or malate transporters remain unknown. However, a putative malate transporter has been partially purified and reconstituted in *Kalanchoë* (see Lüttge et al., 1995). Polyclonal antibodies raised against a protein fraction enriched in this putative transporter inhibit an ATP-dependent, malate-stimulated proton transport activity of isolated tonoplast vesicles and cross-react with a 32-kD protein (T. Pfeifer and R. Ratajczak, personal communication).

In salt-stressed *M. crystallinum*, vacuolar H^+-ATPase activity increases markedly to accommodate the increased rate of malate and Na^+ transport across the tonoplast, whereas H^+-inorganic orthophosphatase activities decline (Barkla et al., 1995). Salinity stress leads to a 2.5-fold increase in tonoplast H^+-ATPase protein in leaves. This salt-inducible increase is completely reversible within 2 d, indicating that considerable turnover of the complex occurs in *M. crystallinum*. Biochemical and electron microscopic studies seem to indicate that the tonoplast H^+-ATPase undergoes changes in composition and structure during the transition from C_3 to CAM (Lüttge et al., 1995); however, whether these structural changes reflect alterations in function or activity remains unclear at present. More recently, cDNAs for subunits A, B, E, and c of the leaf vacuolar H^+-ATPase from *M. crystallinum* (Dietz and Arbinger, 1996; Löw et al., 1996; Tsiantis et al., 1996) and the c subunit from *K. daigremontiana* (Bartholomew et al., 1996) have been reported. In *M. crystallinum*, transcript levels for these subunits increased about 2-fold in leaves following salt stress. A transcript of subunit c has also been reported, which has an expression that is increased following ABA and ionic (NaCl) but not osmotic (mannitol) stress (Tsiantis et al., 1996). Future studies are needed to clearly differentiate between the changes in tonoplast function that occur due to salt stress per se for energizing tonoplast Na^+/H^+ antiport activities (Barkla et al., 1995) and those changes that are required specifically for the energization of malate transport required during the transition from C_3 photosynthesis to CAM.

MOLECULAR EVOLUTION OF CAM

The widespread occurrence of CAM plants in arid environments, including epiphytic sites where water is often growth-limiting, and aquatic environments that experience daytime CO_2 depletion, strongly suggests that, like C_4 photosynthesis, CAM evolved primarily as a CO_2-concentrating mechanism (Ehleringer and Monson, 1993). The ecological and taxonomic diversity of CAM plants, which are distributed over 33 evolutionarily diverse plant families (in more than 328 genera), including monocots and dicots (Smith and Winter, 1996), has led to the speculation that CAM may have evolved in C_3 ancestors multiple times during evolution via a progression from intermediate CAM cycling to facultative or obligate CAM species (Ehleringer and Monson, 1993). Since the biochemical machinery of extant CAM is present in all plants, what molecular mechanisms can account for CAM evolution? One possibility is that CAM arose from mesophyll cells appropriating the gene expression patterns for elevated rates of malate synthesis and decarboxylation found in guard cells (Cockburn, 1983). However, the enzymatic machinery for malate synthesis and decarboxylation is present in all higher plant cells and is involved in a number of other aspects of me-

tabolism. Therefore, variations in photosynthetic carbon metabolism could just as easily have evolved directly from other cell types. Support for the molecular ontogeny of CAM from stomatal guard cell metabolism will require more detailed analysis of the genetic changes brought about by gene duplication or rearrangement events that led to elevated enzyme expression patterns in all photosynthetic cells. Additional evolutionary changes in signal transduction pathways targeting diurnal regulation of enzyme activities would also be necessary. Lacking comprehensive nucleotide sequence data sets for CAM genes from diverse species, reliable molecular phylogenies based on CAM-specific genes cannot yet be constructed. The limited data available on PEPC, the most extensively studied CAM-specific gene, however, indicate that these sequences cluster closer to C_3 than to C_4 forms (Cushman and Bohnert, 1996). Sequence information from genes involved in malate metabolism expressed in guard cells from C_3 and C_4 plants and in mesophyll cells of CAM plants should permit the identification of genetic modifications important to CAM and perhaps uncover clues to the processes driving CAM evolution in response to environmental stress.

FUTURE PERSPECTIVES

CAM is an extremely successful adaptation that permits plants to inhabit or successfully compete in environments with limited water or CO_2 availability. Ecophysiological, anatomical, taxonomic, evolutionary, and biochemical aspects of this adaptation have been extensively studied. These studies affirm that CAM and its induction by environmental stress provides one of the most interesting examples of genetic regulation of a complex metabolic pathway. Although rudimentary insights into the molecular genetics of CAM plants are now available from the characterization of the structure and regulation of some CAM-specific genes (Table I), much more work is needed. A significant challenge for the future will be to define and characterize the components of regulatory and signal transducing pathways that are involved in the induction and diurnal oscillation of CAM gene expression and enzymatic activities. Also, relatively little is known about the structure and regulation of key components of transport systems involved in the intracellular compartmentation and translocation of metabolites, particularly malate, that occur during the diurnal CAM cycle. Identification and characterization of carboxylate transporters and other carrier proteins and channels are sorely needed. Another essential goal for the future will be to better understand the role of plant growth regulators and their interaction with environmental and developmental signals leading to CAM induction. Plant growth regulators such as ABA and cytokinins have long been recognized to participate in the regulation of CAM; however, their relative importance to CAM induction and their interaction with environmental stimuli, their development, and their mode of action remain unclear. Increases in endogenous ABA pools precede or coincide with CAM induction (Taybi et al., 1995), and exogenous application of ABA has been shown to induce CAM (Ting, 1985) and the activities and expression of key CAM enzymes (Chu et al., 1990; Taybi et al., 1995) (Table I). Other plant growth regulators such as cytokinin and methyl jasmonate modulate the expression of CAM enzymes (Dai et al., 1994; Schmitt et al., 1996) (Table I); however, it remains unclear whether they exert their action directly at the level of gene expression or indirectly through, for example, changes in stomatal behavior.

To better understand the structural components essential to CAM, the mechanisms involved in its temporal regulation, and the signal transduction and developmental events that lead to CAM in facultative species, it will be essential to develop and exploit an amenable genetic model plant for CAM. *M. crystallinum* may prove to be an excellent choice for such a model. This rapidly growing, self-fertile species, which possesses a relatively small genome (approximately 2.5 times that of Arabidopsis), has provided much of the information we now know about the expression patterns and structure of CAM-specific genes. The rapid induction of CAM in *M. crystallinum* makes it particularly well suited for signal transduction studies, and the availability of photoautotrophic cell suspension cultures from this species (Willenbrink and Huesemann, 1995) may simplify and enhance such analyses. *M. crystallinum* can be regenerated from callus tissue and is amenable to *Agrobacterium*-mediated transformation, opening up the possibility of transgenic approaches for studying the function and regulation of crucial aspects of CAM. Furthermore, because CAM is optional in this species, disruption of genes essential to the metabolic pathway will not be detrimental to the development or survival of the plant. Ultimately, a combination of genetic and molecular strategies in this species and other suitable CAM species should further our understanding of the molecular genetic basis of CAM.

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