Differential Regulation of Transcripts Encoding Cytosolic NADP-Malic Enzyme in C_3 and C_4 *Flaveria* Species[1]

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A cytosolic NADP-malic enzyme (CYTME) has been described previously in several plants, all C_3 species. CYTME is distinct from the chloroplastic NADP-malic enzyme (CHLME) that is highly active in C_4 species. We show that at least one *CytMe* gene is present in all *Flaveria* spp., including C_3, C_4, and C_3-C_4 intermediate types. Based on the *CytMe* expression patterns in *Flaveria pringlei* (C_3) and *Flaveria trinervia* (C_4), we suggest CYTME has several distinct roles, including the supplying of NADPH for cytosolic metabolism, the supporting of wound response or repair, and the balancing of cellular pH in illuminated leaves. These three roles are likely correlated with *CytMe* mRNAs of apparent sizes 2.0, 2.2, and 2.4 kb, respectively, which differ in the length of the 5′ untranslated regions. Various regulatory mechanisms involving RNA processing and translational efficiency are discussed.

Malic enzymes (MEs) play an important role in a number of metabolic processes that require the conversion of malate to NAD(P)H, pyruvate, and CO_2. In plants, these enzymes contribute to C_4 photosynthesis, pH balancing mechanisms, and fruit ripening processes. MEs also provide reducing agents and carbon skeleton to biosynthetic pathways, with the NAD-ME isoforms functioning predominantly in the mitochondria and the NADP-ME isoforms functioning in the cytoplasm and plastid (Wedding, 1989; Edwards and Andreo, 1992; Drincovich et al., 2001). With the exception of the isoforms acting in C_4 photosynthesis, the correspondence between the observed cellular ME isoforms and their metabolic roles remain unclear. The genus *Flaveria* presents an opportunity to determine this correspondence.

The genus *Flaveria* (Asteraceae) contains C_3 and C_4 species and C_3-C_4 intermediate species with C_4 photosynthetic capacity ranging from C_3- to C_4-like. These species can be grouped into two lines based on genetic hybridization experiments, the number of phyllaries (floral bracts), and molecular phylogenetic studies (Powell, 1978; Bayraktaroglu, 1993; Kopriva et al., 1996; Marshall et al., 1996). All C_3 and C_4 species, in addition to some C_3-C_4 intermediates, belong to the 3-4 phyllary line, and the remaining C_3-C_4 species make up the 5-6 phyllary line. These well-characterized relationships make it possible to compare ME isoform abundance and gene expression patterns among closely related species with distinct metabolic schemes that utilize ME activity.

In the genus *Flaveria*, NADP-ME is encoded by a small multigene family in each species. Only *ChlMe1*, which encodes the chloroplastic NADP-malic enzyme (CHLME) that acts in the C_4 cycle, is expressed at high level in leaves of C_4 species. *ChlMe1* was cloned as a cDNA from *Flaveria trinervia* (C_4; Börsch and Westhoff, 1990; Rajeevan et al., 1991) and as genomic clones from *Flaveria bidentis* (C_4; Marshall et al., 1996) and *F. trinervia* (L. Lai and T. Nelson, unpublished data).A second gene (*ChlMe2*) encoding a plastidic NADP-ME has a non-leaf-specific expression pattern (Lipka et al., 1994; Marshall et al., 1996). The *ChlMe1* and *ChlMe2* genes are present and expressed in all *Flaveria* spp. regardless of photosynthetic type (Marshall et al., 1996; L. Lai, L. Wang, and T. Nelson, unpublished data).

Cytosolic NADP-ME (CYTME) has been described in several C_3 plants (Walter et al., 1988, 1990; van Doorsselaere et al., 1991; Franke and Adams, 1995; Knee et al., 1996) but not in C_4 plants. Here, we report the cloning of the *CytMe* cDNAs from *Flaveria pringlei* (C_3) and show that similar genes are present in C_4 and C_3-C_4 species of the same genus. Northern-blot analysis demonstrates that *CytMe* expression patterns are more complex in the C_4 species *F. trinervia* than in the C_3 species *F. pringlei*. This finding suggests that *CytMe* gene expression is regulated to accommodate C_4 photosynthesis, possibly to assure apportionment of the malate pool.

RESULTS

Isolation and Characterization of *F. pringlei* CytMe cDNAs

We screened a *F. pringlei* leaf cDNA library with a probe designed to detect both *ChlMe* and *CytMe*

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cDNAs, as described in "Materials and Methods." Of 5×10^5 primary phagemids screened, six clones were isolated which correspond to two highly similar *CytMe* mRNAs. No *ChlMe* cDNAs were isolated, consistent with the low level of expression of *ChlMe* in *F. pringlei* fully expanded leaves (see below; L. Lai, L. Wang, and T. Nelson, unpublished data).

Complete sequences were obtained for *CytMeA* (2,044 bp; accession no. AF288920), represented by four clones and *CytMeB* (2,021 bp; accession no. AF288921), represented by two clones. *CytMeA* cDNA comprises an open reading frame of 1,769 bp, a 5′-untranslated region (UTR) of 82 bp, and a 3′-UTR of 156 bp; *CytMeB* cDNA comprises an open reading frame of 1,769 bp, a 5′-UTR of 41 bp, and a 3′-UTR of 191 bp. These two cDNAs are 97% identical at the nucleotide level and 98% identical at the amino acid level (data not shown). *CytMeB* contains two regions absent in *CytMeA*: (a) eight bp in the 5′-UTR and (b) 12 bp in the 3′-UTR (Fig. 1, A and B, respectively). These regions are part of short tandem repeats (half-arrows in Fig. 1), which might play a role in the regulation of mRNA processing, stability, or translatability. A common poly(A) site was found in most clones; however, one clone in each class was found to end at a position 18 bp further downstream. At least one poly(A) signal, highly similar to the consensus AATAAA motif, was found within 40 bp upstream of these poly(A) sites (Fig. 1B, bold, italicized regions). The near-duplicate genes represented by these cDNAs may be a consequence of the allotetraploid nature of *F. pringlei*.

CytMe Genes Are Highly Similar to *ChlMe* Genes

The *CytMe* cDNAs are 74% and 75% identical with the *F. trinervia ChlMe1* cDNA (Börsch and Westhoff, 1990) and the *F. pringlei ChlMe2* cDNA (Lipka et al., 1994), respectively, in the regions encoding the mature proteins (data not shown). They differ from *ChlMe* genes by the absence of regions encoding the plastidic transit peptide and by many differences in the 5′- and 3′-UTRs (Fig. 1). In the 3′-UTRs, they are only 29% to 36% conserved. Low conservation in the 3′-UTRs (39%) is also found between the two *ChlMe* cDNAs. At the deduced amino acid level, CYTME is 76% and 78% identical with the *F. trinervia* CHLME1 and *F. pringlei* CHLME2, respectively, in the mature protein regions (data not shown).

In comparison with other plant NADP-MEs (data not shown), sequence conservation was found in three regions that form the dinucleotide-binding folds of NAD- and NADP-linked oxidoreductases and in the five cysteines conserved in all plant NADP-MEs (e.g. see Lipka et al., 1994).

CYTME Is Encoded by a Small Multigene Family in C_3 Species but by a Single Gene in C_3-C_4 and C_4 Species

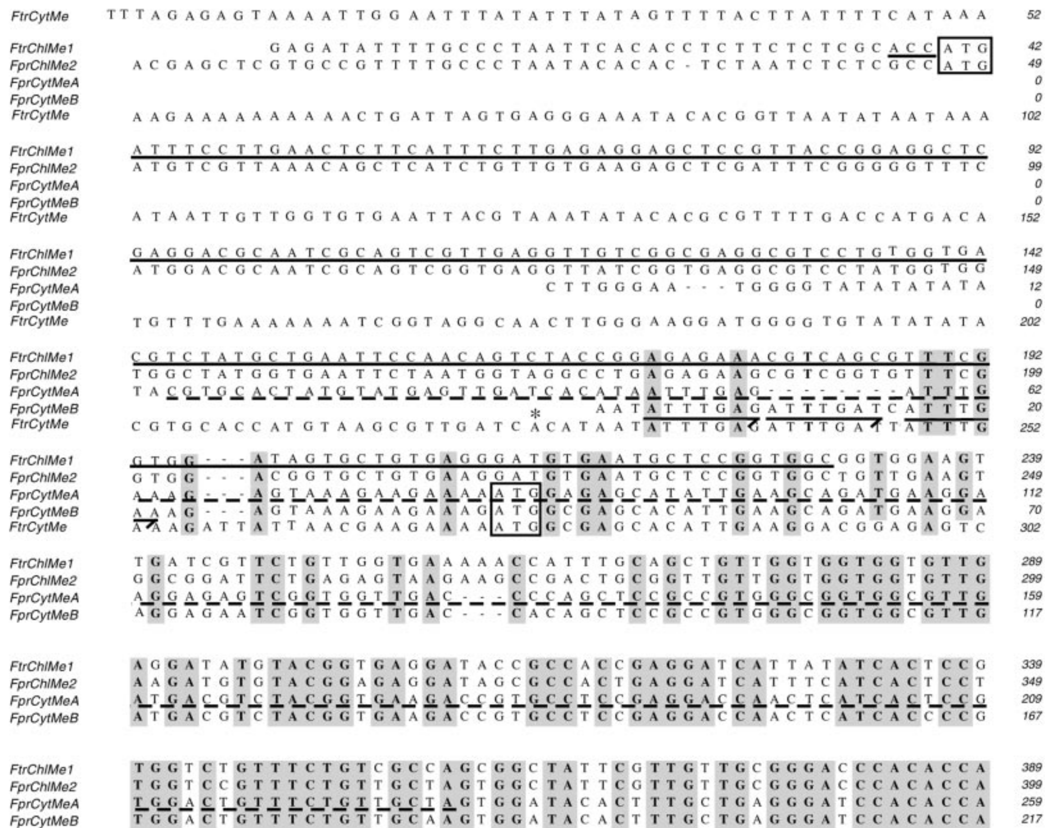
We found *CytMe* genes in all 10 *Flaveria* spp. examined. The species included were two C_3 species (*F. pringlei* and *Flaveria robusta*), six intermediate species (*Flaveria linearis*, *Flaveria floridana*, *Flaveria brownii*, *Flaveria angustifolia*, *Flaveria anomala*, and *Flaveria vaginata*), and two C_4 species (*F. trinervia* and *F. bidentis*). All species belong to the 3-4 phyllary line except for *F. linearis*, *F. floridana*, and *F. brownii*, which belong to the 5-6 phyllary line (Powell, 1978). For most of the species, the *CytMe*-specific probe (Fig. 1A) detected a single, strongly hybridizing fragment, suggesting they contain a single-copy gene (Fig. 2A). Multiple bands were detected in *F. anomala*, *F. pringlei*, and *F. robusta*, suggesting the presence of a small multigene (two–three copies) family in these species. To test whether the *CytMe* probe is specific for *CytMe* genes in all species, the DNA blots were reprobed with a *ChlMe* probe (Fig. 1A) that detects both *ChlMe1* and *ChlMe2* genes (Fig. 2B). The specificity of the probes is supported by the observation that they detect sets of bands with little or no overlap. The few apparently overlapping bands might be coincidental or attributable to the low-stringency hybridization conditions employed. However, the pattern obtained with each probe is unique.

CytMe Is Expressed in All Mature Organs of *F. pringlei* (C_3) and *F. trinervia* (C_4)

To determine whether the expression pattern of *CytMe* differs in leaves of C_3 and C_4 species, we compared *CytMe* mRNA levels in *F. pringlei* (C_3) and *F. trinervia* (C_4) and also examined the levels of both *ChlMe1* and *ChlMe2* mRNAs in toto (referred hereafter collectively as *ChlMe* mRNAs). Total RNA was isolated from leaves (fully expanded), stems, and roots of *F. pringlei* and *F. trinervia* and hybridized with the *CytMe*- and *ChlMe*-specific probes (Fig. 3). In *F. pringlei*, the patterns of *CytMe* and *ChlMe* mRNA accumulation are similar. Both mRNAs are found at comparable levels in stems and roots but accumulate to severalfold lower levels in leaves. In *F. trinervia*, *CytMe* mRNA was relatively uniform in abundance in all three organs, whereas *ChlMe* mRNA levels were extremely high in leaves compared with stems and roots. Based on the overall uniform expression of *CytMe* in all vegetative organs of both species, it is likely that *CytMe* plays a non-photosynthetic role in plant metabolism.

Alternative CytMe Transcripts in *F. trinervia* Leaves Vary in Proportion during Leaf Development

Because CYTME and CHLME roles may change with the onset of photosynthetic competence, we compared *CytMe* and *ChlMe* gene expression patterns throughout leaf development in both the C_3 and C_4



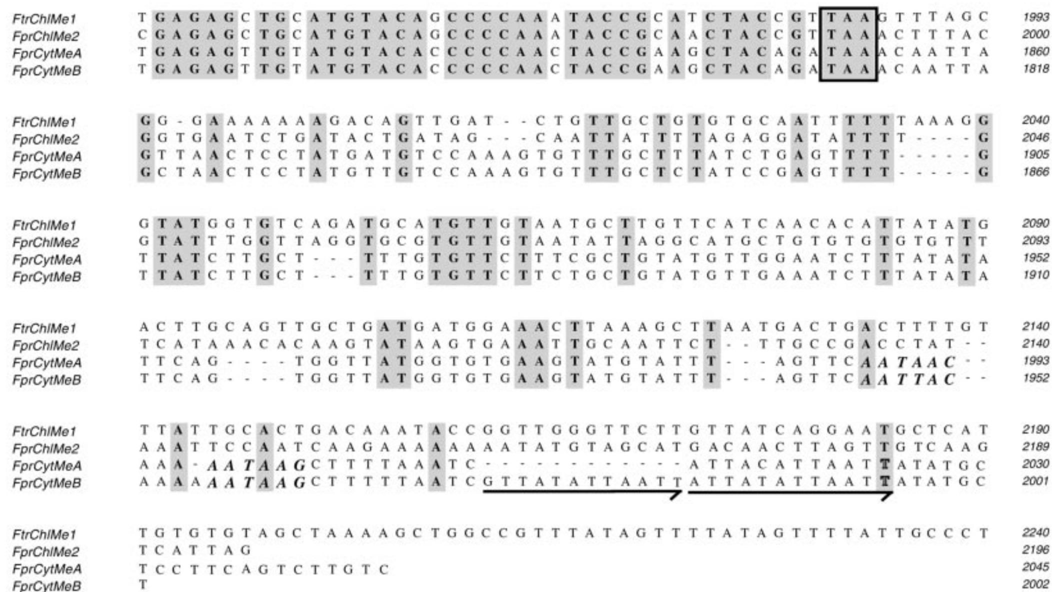
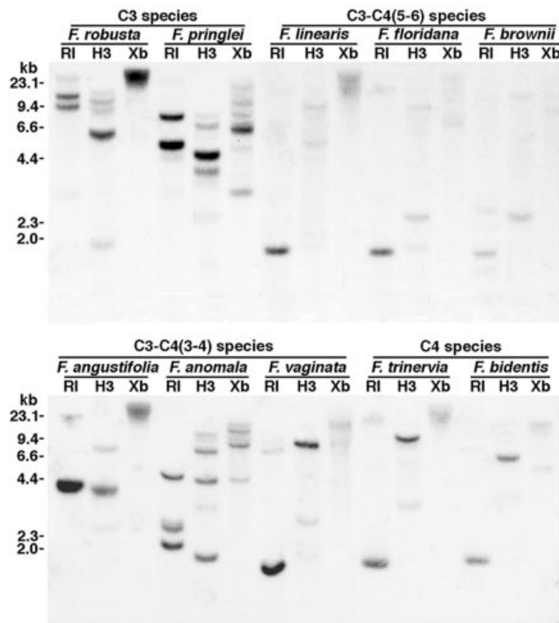


Figure 1. Alignment of the *Flaveria* spp. *ChlMe* and *CytMe* cDNA sequences. A, Sequences in the 5′ regions. The solid underline indicates the *ChlMe1* fragment used as the *ChlMe*-specific probe; the dashed underline indicates the *CytMeA* fragment used as the *CytMe*-specific probe. The 5′ end of the short *F. trinervia* mRNA is indicated by an asterisk. B, Sequences in the 3′ regions. The bold, italicized regions are the putative polyadenylation signals and the outlined nucleotide T indicates the upstream poly(A) addition site in the *CytMe* cDNAs. Identical nucleotides are highlighted with a gray background. Half-arrows indicate direct repeat sequences in the *CytMe* cDNAs. Boxes show the start codons in A and the stop codons in B. Ftr, *F. trinervia*; Fpr, *F. pringlei*.



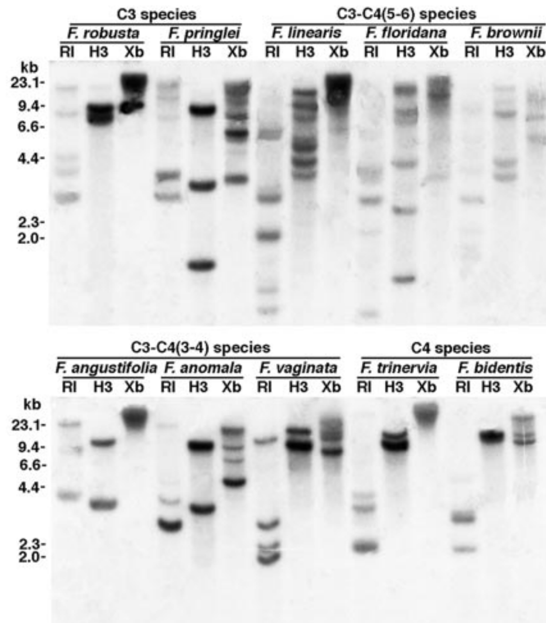


Figure 2. Southern analysis of genomic DNAs from *Flaveria* spp. using a *CytMe*-specific probe (A) and a *ChlMe*-specific probe (B). Both *ChlMe1* and *ChlMe2* are detected by the *ChlMe*-specific probe, resulting in multiple hybridizing bands in B. The species are grouped according to types of photosynthesis. Parentheses in each C_3-C_4 group indicate phyllary line. Within each of the C_3-C_4 groups, the species are placed in order of increasing C_4 capabilities (Ku et al., 1991). Note that *F. brownii* and *F. vaginata* have been characterized as C_4-like. RI, *Eco*RI; H3, *Hin*dIII; Xb, *Xba*I. Numbers at left indicate sizes in kilobase pairs.

Flaveria spp. In *F. pringlei* leaves, the level of *CytMe* mRNA increased slightly as the leaves matured (Fig. 4A). The deviations observed at 27% and 60% full-length stages might result from inadvertent stress and/or wounding in these leaf samples, because *CytMe* is responsive to these factors (Schaaf et al., 1995). *ChlMe* mRNA accumulation changed dramatically with leaf development, peaking early and then declining to a low and steady level.

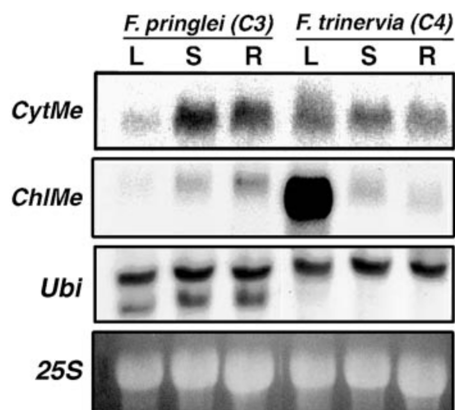
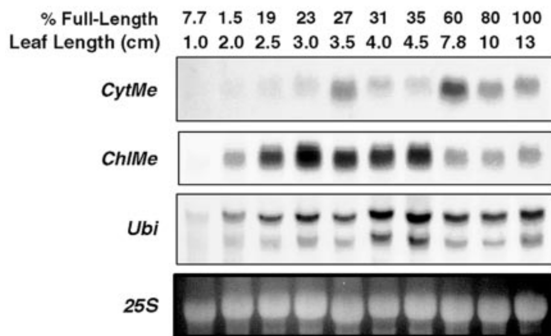


Figure 3. Organ-specific accumulation of *CytMe* and *ChlMe* mRNAs in *F. pringlei* and *F. trinervia* grown in a growth room. To normalize RNA loading, the blot was stripped and rehybridized with a ubiquitin probe (*Ubi*). A photograph of the 25S rRNA bands from the ethidium bromide-stained gel is also shown. L, Fully expanded leaves; S, stems; R, roots. Approximately 20 μg of total RNA was loaded per lane.

During leaf development in *F. trinervia*, distinct *CytMe* transcripts of apparent sizes 2.0 and 2.4 kb were present, depending on developmental state (Fig. 4B). In young leaves, the 2.4-kb transcript was predominant. From the 63% full-length stage onward, levels of the 2.0-kb transcripts increased. To determine whether these size variants were due to alternative splicing or to alternative 5′ or 3′ ends, we analyzed the *CytMe* mRNAs by reverse transcription (RT)-PCR and RACE, as described in "Materials and Methods." RT-PCR with a variety of primers revealed no size variation within the coding portions of the 2.0- and 2.4-kb mRNAs, suggesting they do not differ by alternative splices. By means of 3′ RACE assays, we found small variations in the poly(A) site that were insufficient to account for the size difference (Fig. 1B). However, by 5′ RACE assays, we found two fragments of different lengths, which when sequenced proved to result from differences in the length of 5′-UTRs of otherwise identical messages (Fig. 1A). When the sequences of these 5′-UTR variants were compared with the *CytMe* genomic sequence (GenBank accession no. AY033591), we found that they result from alternative transcription start sites and not from alternative 5′ leader exons. The longer transcript begins 225 bases upstream of the shorter counterpart. The size



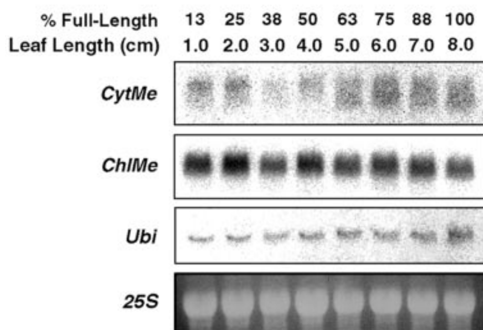


Figure 4. Changes in the levels of *CytMe* and *ChlMe* mRNAs during leaf development in *F. pringlei* (A) and *F. trinervia* (B). Leaves of various lengths representing different stages of maturity were collected from plants grown in a growth room. To normalize RNA loading, the blots were stripped and rehybridized with a ubiquitin probe (*Ubi*). Photographs of the 25S rRNA bands from the ethidium bromide-stained gels are also shown. Approximately 20 μg of total RNA was loaded per lane.

of the longer message is apparently exaggerated to 2.4 kb in gel electrophoresis because of the extensive secondary structures in its 5′ leader (see below). For simplicity we continue to refer to this mRNA by its size (2.4 kb) relative to size markers in electrophoresis. Of 20 independently cloned and sequenced mRNAs from *F. trinervia*, all were identical in sequence except for the length of 5′ leader, consistent with the presence of only a single *CytMe* gene. The 5′-UTRs of *F. trinervia CytMe* transcripts include the eight bases that distinguish the *F. pringlei CytMeB* from the *CytMeA* transcripts, and they include an additional three bases not observed in the UTRs of other *Me* mRNAs.

The m-fold algorithm (version 3.1; Mathews et al., 1999; Zuker et al., 1999) predicts four different stem-loop structures made possible by the additional 225 bases in the 5′ leader of the longer (2.4-kb) *CytMe* transcript. These structures include the entire 5′-UTR from eight bases upstream of the AUG to seven bases from the 5′ end. It is possible that these stable secondary structures might influence mRNA stability, processing or translation efficiency (see "Discussion").

The Expression of *CytMe* Is Affected by Light Only in Leaves

To compare the influence of light on the expression of *CytMe* and *ChlMe* genes in C_3 and C_4 species, mature greenhouse-grown plants were dark-adapted for 5 d and then re-illuminated. In *F. pringlei*, a single 2.0-kb *CytMe* transcript accumulated in light-grown leaves, the level of which remained unchanged during dark treatment and re-illumination (Fig. 5A). However, dark adaptation produced an additional 2.4-kb *CytMe* transcript, the levels of which peaked at 8 h after re-illumination and then decreased. *ChlMe* transcripts could not be detected in these leaves.

In *F. trinervia* leaves, two *CytMe* transcripts were present before the plants were shifted to darkness

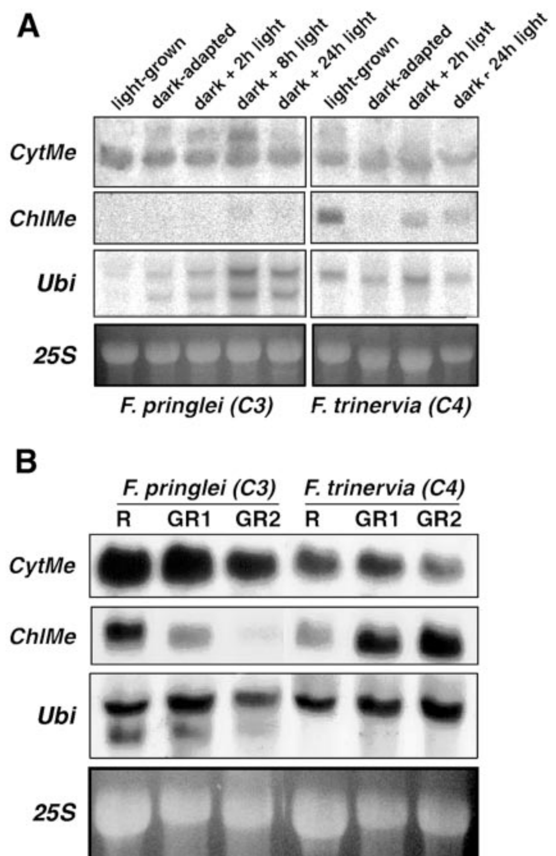


Figure 5. Effect of illumination on *CytMe* and *ChlMe* mRNA levels *in F. pringlei* and *F. trinervia*. A, Total RNA isolated from fully expanded leaves of plants grown in a greenhouse, dark-adapted for 5 d, and then returned to the greenhouse for 2 to 24 h. B, Total RNA isolated from roots of soil-grown plants (R) and roots of medium-grown plants (GR, green roots). GR1 and GR2 represent two different preparations. To normalize RNA loading, the blot was stripped and rehybridized with a ubiquitin probe (*Ubi*). Photographs of the 25S rRNA bands from the ethidium bromide-stained gels are also shown. Approximately 20 μg of total RNA was loaded per lane.

(Fig. 5A). The longer 2.4-kb transcript disappeared during dark adaptation and subsequent re-illumination, a pattern opposite to that observed in *F. pringlei* leaves. However, the level of the 2.0-kb transcript remained unaltered throughout the procedure. As expected for a C_4 species, *ChlMe* expression was positively regulated by light.

The effect of direct illumination on the accumulation of *CytMe* mRNA was also examined in roots of *F. pringlei* and *F. trinervia*, which green when exposed to direct light (Fig. 5B). Exposure to light had no effect on the expression of *CytMe* in roots of either species. In contrast, direct light exposure repressed the expression in roots of *ChlMe* genes in *F. pringlei* and induced expression in *F. trinervia*. Because illuminated roots were grown in medium with Murashige and Skoog salts and Suc rather than soil, it is possible that these patterns were influenced by Suc or other components of the medium.

CytMe Expression Is Influenced by Mechanical Injury

Because *CytMe* expression patterns have previously been shown to respond to wounding, possibly as a component of defense responses (Schaaf et al., 1995; Casati et al., 1999a; Drincovich et al., 2001), we compared *CytMe* and *ChlMe* mRNA levels in fully expanded leaves of the C_4 species *F. trinervia* after wounding treatments (Fig. 6). Fully expanded leaves of *F. trinervia* accumulate two *CytMe* transcripts of approximately 2.0 and 2.4 kb. Within 3 h of wounding, however, a third *CytMe* transcript of approximately 2.2 kb can be detected. The level of the 2.2-kb transcript increased with time after wounding for at least 7 h, whereas the level of the 2.4-kb transcripts decreased. This suggests that wounding induced a change in the start sites or processing of *CytMe* messages. In the same leaves, the level of *ChlMe* mRNA was unaffected by wounding.

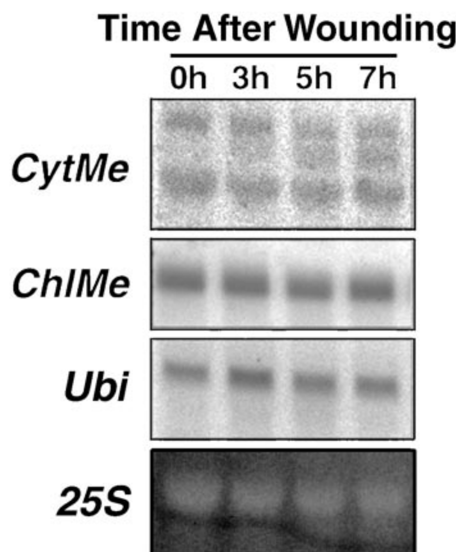


Figure 6. Effect of wounding on *CytMe* and *ChlMe* mRNA levels in fully expanded *F. trinervia* leaves. Leaves from the same node on a greenhouse-grown plant was mechanically wounded before samples were collected at the indicated times (see "Materials and Methods" for details). The experiment was repeated twice and similar results were obtained each time. To normalize RNA loading, the blot was stripped and rehybridized with a ubiquitin probe (*Ubi*). A photograph of the 25S rRNA bands from the ethidium bromide-stained gel is also shown. Approximately 20 μg of total RNA was loaded per lane.

DISCUSSION

Potential Roles of *CytMe* in C_3 and C_4 Species

CytMe mRNA species of 2.0, 2.2, and 2.4 kb are differentially present in C_3 and C_4 *Flaveria* spp. in patterns that suggests they correspond to roles for cytosolic ME in general metabolism, in wound responses, and in the control of cytoplasmic pH. In stems and roots of *F. trinervia* and in all organs of *F. pringlei*, only the 2.0-kb transcript accumulates. In leaves of both *F. pringlei* and *F. trinervia*, the 2.0-kb transcript increases with the extent of leaf maturation but is not influenced by light or wounding. The ubiquitous presence of the 2.0-kb transcript suggests that it is responsible for the majority of CYTME activity in both C_3 and C_4 plants. The 2.2- and 2.4-kb transcripts appear under more specific conditions.

Mechanical wounding in fully expanded *F. trinervia* leaves leads to accumulation of the 2.2-kb *CytMe* transcript at the expense of the 2.4-kb transcript. During injury, significant levels of reducing agents and carbon metabolites are needed to support cell wall building. Accumulation of the 2.2-kb *CytMe* transcript suggests that CYTME is involved in the supply of NADPH and pyruvate for this process. It is noteworthy that wound-induction of a cytosolic NADP-ME has been reported in bean (*Phaseolus vulgaris*), a C_3 plant (Schaaf et al., 1995).

The expression pattern of the 2.4-kb *CytMe* transcript indicates a correlation between this transcript and the ME required for controlling cytoplasmic pH in leaves. The phenomenon of illumination-induced cytoplasmic alkalization has been compared in the C_3 plant broad bean (*Vicia faba*) and the C_4 plant *Amaranthus caudatus* (Raghavendra et al., 1993; Yin et al., 1993). Whereas darkening led to rapid acidification in the C_3 leaf, alkalization continued in the darkened C_4 leaf. The observation that cytoplasmic pH can be controlled by balancing malate synthesis and degradation (Davies, 1973; Raven and Smith, 1974; Smith and Raven, 1979; Martinoia and Rentsch, 1994) provides a rationale for the opposite effects of illumination on the accumulation of the 2.4-kb *CytMe* transcript in the leaves of C_3 and C_4 *Flaveria* spp. (Fig. 5A). The appearance of the 2.4-kb *CytMe* transcript in *F. pringlei* (C_3) upon dark-adaptation suggests that CYTME is induced for malate degradation to offset acidification. By the same rationale, continued alkalization in the dark in *F. trinervia* (C_4) might repress CYTME, as evidenced by the decrease in the 2.4-kb

transcript (Fig. 5A), and thereby regulate the pathway in the opposite direction.

Although this study provides no direct experimental evidence regarding why *CytMe* mRNA species of different lengths (2.0, 2.2, and 2.4 kb) are synthesized to execute roles in general metabolism, wounding responses, and control of cytoplasmic pH, it is tempting to speculate on the bases for this finding. It is evident that depletion of the malate pool will result if dark-adaptation-induced acidification is to be offset or if NADPH levels need to be increased subsequent to wounding. However, the essential requirement of a large malate pool for photosynthesis in the C_4 species *F. trinervia* likely imposes an acute need for regulatory mechanisms that will precisely control the flux of malate for other cellular requirements. Although the levels of ME need to be rapidly increased if malate is to be degraded, the ME levels needs to be decreased immediately if the malate levels reach a lower threshold. It is in this dual context of rapid inducibility and repression that the different transcripts produced from the single *CytMe* gene in *Flaveria* spp. might play a role. First, because the 2.0- and 2.4-kb mRNAs (and perhaps the 2.2-kb mRNA) appear to vary only in the length of 5′-UTR, it is plausible that these mRNA species possess sequences/structures that render them suitable for acting as molecular switches that respond to physiological stimuli. The four alternative stem-loop structures predicted in the 5′UTR of the 2.4-kb mRNA all have a free energy of approximately −50 kcal/mol. Although such stable structures are usually associated with decreased translational efficiency, it has recently become evident that such structures can serve as regulatory elements. "Sensory" mechanisms controlling translation have been reported (Cereghino et al., 1995; Nielsen et al., 1995; Luo and Sachs, 1996; Wells et al., 1998; Morita et al., 1999; Diwa et al., 2000; Ravnum and Andersson, 2001). The introduction of secondary structures in the 5′-UTRs raises the possibility of translational control through trans-acting factors, a feature already documented in mammalian cells and plant systems (Kozak, 1999; Morris and Geballe, 2000; Wang and Wessler, 2001). The differences in the length of the *CytMe* mRNA transcripts (i.e. 2.2 versus 2.4 kb) subsequent to wounding and dark adaptation might reflect differences in the trans-acting factors that influence the expression of CYTME under these conditions. Second, the presence of the 5′-UTRs might enhance mRNA stability (Cereghino et al., 1995; Arnold et al., 1998) and thereby assist in fulfilling the need for higher CYTME activity during sustained increases in levels of cytosolic malate or acidity. Lastly, the significant accumulation of the longer transcripts might indicate that the mature mRNA transcript is generated by processing of a longer precursor transcript. This processing event might be conditionally regulated and leads to an mRNA that is translated with a higher efficiency and thereby fulfill the requirement for higher CYTME levels during the induction phase.

It is striking that in *Flaveria* spp., the multiple copies of the *CytMe* genes found in C_3 species are reduced in C_3-C_4 and C_4 species to a single copy. This may reflect the need in these latter species for rapid cellular responses to changes in the pool of C_4 metabolites to prevent futile cycling. Such rapid changes might be difficult to achieve by shifts in transcription to up- and down-regulate distinct ME genes and more easily achieved by transcriptional, post-transcriptional, and translational controls on a single gene, in addition to regulation of the enzymatic activity.

Correspondence between NADP-ME Genes and Protein Isoforms in *Flaveria* Species

Three NADP-ME isoforms, of molecular masses 62, 64, and 72 kD, have been detected immunologically in all *Flaveria* spp. (Drincovich et al., 1998; Casati et al., 1999b). The abundance of the 62-kD isoform is correlated with C_4 photosynthetic capacity, suggesting that the 62-kD isoform is the C_4-specific CHLME encoded by *ChlMe1*. Consistent with this, *ChlMe1* is highly transcribed specifically in the leaves of the C_4 species *F. trinervia* (Marshall et al., 1996; L. Lai, L. Wang, and T. Nelson, unpublished data). In the leaves of C_3 *Flaveria* spp. and non-photosynthetic organs of all *Flaveria* spp., the pool of NADP-ME was found almost exclusively to consist of the 72-kD isoform, similar to our finding with the *CytMe* mRNAs. Therefore, we suggest that the ubiquitous 72-kD isoform is encoded by the constitutively transcribed *CytMe* gene, because transcript and protein localization patterns show no correlation with photosynthesis. In contrast, the 64-kD isoform is found at higher levels in C_4-like and C_3-C_4 species (Drincovich et al., 1998) and is therefore likely to be encoded by *ChlMe2*; further biochemical studies are required to verify this premise. The levels and cellular localization of the 62- and 64-kD isoforms are similar in C_3-C_4 species (Drincovich et al., 1998; Casati et al., 1999b). It is likely that in the ancestral C_3 species, *ChlMe1* and *ChlMe2* were regulated in a similar fashion. When a chloroplastic NADP-ME activity was required early in C_4 evolution, as represented by *Flaveria* C_3-C_4 species, the expression of both genes might have been increased, followed by the divergence and distinct expression patterns of the genes exhibited in C_4 species.

MATERIALS AND METHODS

Plant Materials and Treatments

Multiple isolates of *Flaveria* spp. were provided by Dr. Scott Holaday (Texas Tech University, Lubbock), Dr. Harold Brown (University of Georgia, Athens), Drs. Maurice S. B. Ku and Gerald Edwards (Washington State Univer-

sity, Pullman), and Dr. Peter Westhoff (Heinrich-Heine-Universitat, Dusseldorf). *Flaveria trinervia* and *Flaveria bidentis* were grown from seeds, whereas all other *Flaveria* spp. were propagated from vegetative cuttings. Greenhouse-grown plants were used for light-shift and wounding experiments as well as for isolation of mRNA for the *Flaveria pringlei* leaf cDNA library. The greenhouse was maintained at an average temperature of 28°C during summer and 25°C during winter, with supplemental lighting to obtain a 16-h light/8-h dark cycle in the range of 700 to 1,000 μmol m^{-2} s^{-1}. For all other experiments, plants were maintained in a controlled growth room at 28°C with a 16-h light/8-h dark cycle at 700 to 900 μmol m^{-2} s^{-1} supplied by a combination of white fluorescent and incandescent lights.

For northern-blot analysis of *CytMe* expression during leaf development, leaf length was used as an indicator of expansion stage in plants grown in a growth chamber. Leaves ranging in size from 1 cm to the maximum lengths of 13 cm (for *F. pringlei*) and 8 cm (for *F. trinervia*) were collected. Senescing leaves were avoided by collecting leaves only from the top five nodes of plants with at least 10 nodes.

In re-illumination experiments, only fully expanded leaves from greenhouse-grown plants at the 6- to 7-node stage were used, to eliminate the effects of developmental regulation. After being dark-adapted for 5 d, plants were re-illuminated in the greenhouse for up to 24 h and leaves were collected at the indicated times.

In wounding experiments, the youngest pairs of fully expanded leaves from three different mature *F. trinervia* plants (greenhouse-grown) were treated. Each leaf was severed along both sides of the midrib, yielding two halves of midrib-less blades that were still attached to the plant by the petiole. Each plant therefore yielded four leaf-halves, each of which was collected at 0 (control), 3, 5, and 7 h after treatment.

Oligonucleotides

The following oligonucleotides were used in the various parts of this study: VN-1, 5′-ACCATGATTTCCTTGAACTC-3′; VN-2, 5′-ACCATGATTTCCTTGAACTC-3′; FtrMe-3, 5′-GCCACCGGAGCATTCACATCC-3′; CytMe-1, 5′-TTGCGTCCTCGAGCCTCCGG-3′; CytMe-1A, 5′-CCAAAGTGTTTGCTTTATC-3′; CytMe-2, 5′-AAGCTTATTTTTGTTATTGAACT-3′; CytMe-3, 5′-CGTGCACTATGTATGAGTTG-3′; CytMe-4, 5′-AATATTTTGCATCAATTTCTTCT-3′; CytMe-5, 5′-GTGGTATGATGGAACTCG-3′; CytMe-6, 5′-GAACTCTTCAAGTAAATCATA-3′; CytMe-7, 5′-GGTGAAAAGGTCCTCGTG-3′; CytMe-8, 5′-TTAGAACAGATGGTTTGATG-3′; CytMe-9, 5′-GAGGCATGGCAGCCTT-3′; CytMe-10, 5′-TCACCAGATCAGTAGGGC-3′; Cy5R1, 5′-CCTCGGAGGCRCGGTCMTC-3′; Cy5R2, 5-CGCCACCGCCCACGGC-3; Cy5R5, 5′-GTGGATCCCTCAGCAAAGTG-3′; Trcytlong, 5′-GAGAGTAAAATTGGAATTTATATT-3′; Tanchor, 5′-GGATCCGTCGAGT(15)-3′; and Tadaptor, 5-GGATCCGTCGAGTTT-3′.

Isolation of *CytMe* cDNAs

Poly(A) RNA was isolated from fully expanded leaves of greenhouse-grown *F. pringlei* and was used in constructing a cDNA library with the ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA). The library was screened with a 322-bp fragment encoding the chloroplast transit peptide and the first 45 amino acids of CHLME1. The latter region is very similar in CHLME and CYTME proteins. The 322-bp probe was generated by RT-PCR using the primers VN-1 and VN-2 and *F. trinervia* leaf RNA (B. McGonigle and T. Nelson, unpublished data). Successive rounds of screening were carried out by hybridization in 5× SSC, 5× Denhardt's, 0.1% (w/v) SDS, 0.1% (w/v) tetrasodium pyrophosphate (Na_4PPi), and 200 μg mL^{-1} salmon sperm DNA at 65°C. Subsequently, two 30-min washes at 65°C were performed using 1× SSC, 1% (w/v) SDS, and 0.1% (w/v) Na_4PPi as the first wash and 0.1× SSC, 1% (w/v) SDS, and 0.1% (w/v) Na_4PPi as the second wash. Sequence assembly and analysis were performed using GeneWorks (version 2.5.1, Intelligenetics, Inc., Mountain View, CA).

Labeling of Gene-Specific Fragments for DNA and RNA Gel-Blot Analyses

PCR was used to radiolabel fragments (Mertz and Rashtchian, 1994) with 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. The *CytMe*-specific fragment was obtained using the *F. pringlei CytMeA* cDNA as template and the primers CytMe-3 and VN-2. This probe comprises the 5′ portion of *CytMeA* cDNA, which has low homology to the corresponding regions in the *ChlMe* cDNAs (Fig. 1A). The *ChlMe*-specific fragment was obtained using the 322-bp fragment described above as template and the primers VN-1 and FtrMe-3. This probe contained the complete coding region for the plastidic transit peptide which is unique to *ChlMe* genes (Fig. 1A).

Estimation of Gene Copy Number

Ten to 15 μg of genomic DNA was digested with restriction enzymes, separated by electrophoresis in a 1% (w/v) agarose gel, and then transferred to Nytran Plus membrane (Schleicher & Schuell, Keene, NH). To ensure hybridization to genomic DNAs from all species used, low-stringency hybridization was carried out in 25% (w/v) formamide, 5× SSC, 5× Denhardt's, 1% (w/v) SDS, 0.05% (w/v) Na_4PPi, 250 μg mL^{-1} salmon sperm DNA, and 5% (w/v) dextran sulfate at 42°C. Subsequently, two 20-min washes at 42°C were performed using 2× SSC, 0.1% (w/v) SDS and 1× SSC, 0.1% (w/v) SDS as the first and second wash solutions, respectively.

Measurement of CytMe and ChlMe mRNA Levels

Total RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Approximately 20 μg of total RNA was separated by formaldehyde agarose gel electrophoresis and transferred to Nytran Plus membrane (Schleicher &

Schuell, Keene, NH). High-stringency hybridization was carried out in 50% (w/v) formamide, 5× SSPE, 5× Denhardt's, 0.5% (w/v) SDS, 25 mM EDTA, 200 μg mL^{-1} salmon sperm DNA, and 5% (w/v) dextran sulfate at 42°C. Subsequently, two 20-min washes were carried out at 42°C with 2× SSC, 0.1% (w/v) SDS. Blots were analyzed using the Fujix BAS-2000 phosphorimager (Fuji, Tokyo).

Ubiquitin mRNA levels were analyzed to normalize for differences in RNA loading. Two ubiquitin probes were used. The first probe, the insert of pSKUB1 (Christensen and Quail, 1989), was randomly labeled and used with the high-stringency hybridization method. The second ubiquitin probe was a 5′ end-labeled oligonucleotide UBQRep (5′-CTCCTTCTGGATGTTGTAGT-3′), which is complementary to the repeat sequences highly conserved among all known plant ubiquitin mRNAs (Xia and Mahon, 1998). The oligonucleotide probe was hybridized in 0.6 M NaCl, 0.18 M Na_2HPO_4, 6 nM EDTA, pH 6.8, 0.75% (w/v) *N*-lauroyl-sarcosine, 10% (w/v) dextran sulfate, and 0.5 mg mL^{-1} heparin (Dellaporta and Moreno, 1994) at 42°C and washed twice in 6× SSC, 0.1% (w/v) SDS for 10 min each at 37°C. Both ubiquitin probes detected three mRNA species of sizes 0.8, 1.6, and 2.1 kb in *F. pringlei* RNA samples, as reported for maize (Christensen and Quail, 1989). In *F. trinervia* RNA samples, only the 0.8- and 2.1-kb mRNAs were detected.

Determination of mRNA Size Variants

Total RNA from young *F. trinervia* leaves (up to 4 cm long) was used for all of the following experiments. For detection of alternative splice events, the first-strand cDNA was obtained using the RETROscript kit (Ambion, Austin, TX) and the oligo(dT) primer. Regions of the cDNA suspected to contain introns were amplified using PCR for 35 cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min. Positive and negative controls were included in each run and each region was tested at least twice. The primers used were CytMe-2 through CytMe-10.

3′ RACE was carried out using the primer Tanchor and Superscript II reverse transcriptase (Life Technologies). Glass Max spin columns (Life Technologies) were used to rid the product of extra primers. PCR reactions included the *CytMe*-specific primer CytMe-1 (194 bp upstream of the major poly[A] site) and the primer Tadaptor for the first round and CytMe-1A and Tadaptor for the second round.

5′ RACE was carried out using the 5′ RACE kit Version 2.0 (Life Technologies) and the *CytMe*-specific primer CytMe-4 (291 bp downstream of the start codon), followed by two rounds of PCR amplification with the nested primers Cy5R5 and Cy5R1. Potential RNA secondary structure within the 5′-UTR was identified using the RNA m-fold algorithm (version 3.1) at http://bioinfo.math.rpi.edu/~mfold/, maintained by M. Zuker (Rensselaer Polytechnic Institute). To determine the origin of the 5′-UTR extension in the longer *CytMe* transcript, its sequence was compared with the genomic sequence. The genomic *F. trinervia* DNA was amplified first using the primers Cy5R1 and Trcytlong. The products from this reaction were re-amplified using Trcytlong and Cy5R2 as the primers.

PCR products from the 5′ RACE reactions, genomic amplification, and some of the reactions designed to detect alternate splicing were cloned (TOPO TA kit, Invitrogen, Carlsbad, CA) and sequenced (Keck Sequencing Facility, Yale University).

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