Final Technical Report for DOE grant DE-FG02-91ER20038

PI: Timothy Nelson
Yale University
"Spatial Regulation of C4 Genes in C3, C4 and C3/C4 Intermediate Flaveria Species"

AIMS
The long-range goal of this project was to understand the control of plant cell differentiation in spatial patterns, using the differentiation of BS and M cells surrounding veins of C4 leaves as a model. Based on our prior studies on the structure and regulation of BS- and M-specific C4 genes and on cellular patterns of their expression in several C4 species, we proposed and later refined a model that invoked the developing veins as sources of light-dependent positional information that guides the differentiation of BS and M cells at near or far distances, respectively. In the absence of this information (too distant or in darkness), cells take on a default state, with a distinct C3 pattern of photosynthetic gene expression. The existence of species within the genus *Flaveria* with differing leaf cellular arrangements and photosynthetic schemes (C4, C3, & C3-C4) provided an opportunity to test this model and to identify DNA elements involved in the spatial regulation of leaf cell differentiation.

In 1991, I proposed the following specific aims:

1. To compare C3 and C4 versions of a representative bundle sheath (BS) cell-specific gene and a representative mesophyll (M) cell-specific gene. We compared the BS-specific genes *rbcS* and NADP-malic enzyme (*me*), and the M-specific gene NADP-malate dehydrogenase (*mdh*), in *F. pringlei* (C3) and *F. trinervia* from the genus *Flaveria*.

2. To introduce reporter gene constructs with BS- or M-cell specific promoters and other elements into the different anatomical contexts provided by C3, C4, and C3/C4 intermediate species of *Flaveria*. We proposed transient expression experiments to test the function of M- and BS-specific promoters and elements in species that express their endogenous versions of the corresponding genes in different spatial and cellular patterns. We developed a microbombardment in situ transient assay for seven species of *Flaveria*, as well as stable transgenic assays for a C4 and a C3 species.

Based on results from the first funding period, we added the following aims (1995):

3. To determine the extent and distribution of BS vs. M posttranscriptional differences in C4 and C3/C4 species. We performed a combination of nuclear run-off, RNA quantitation, transient assays, and transgenic assays to make this determination.

4. To determine the mechanism of cell-specific posttranscriptional control in *Flaveria* species. We performed transient and transgenic assays of various reporter constructs with 5' and 3' flanking sequences from C4 genes.

RESULTS
Our findings are listed below, followed by a brief description of the data. Details of results are or will be in the public domain as peer-reviewed journal publications, which are listed at the end of this report.
DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.
DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.
1. *rbcS* promoters and 3' UTRs are highly similar in C4 and C3 species of *Flaveria*. Using a cDNA for *F. trinervia* *rbcS* (gift of Dr. S. Sun, U. Hawaii), we cloned and sequenced several *rbcS* cDNAs from the C3 species *F. pringlei* and from the C4 species *F. trinervia*, then used these to isolate genomic clones from our λGEM-11 libraries of each species. Although we characterized many different cDNAs, we concentrated on one highly expressed *rbcS* gene from each species. A comparison of the 5' regions of each revealed that the two are highly similar throughout the first 500 bp. In contrast, sequence comparison of the unusually long 3' transcribed untranslated regions (UTRs) of C3 and C4 *rbcS* cDNAs (300-400 nt, depending on which cDNA) revealed blocks of similarity interrupted by dissimilar regions. This is significant because of the possibility of C3 vs. C4 differences in posttranscriptional processes, perhaps mRNA stability (see below).

2. *rbcS* genes are transcribed in both BS and M cells in *F. trinervia* (C4). In the course of characterization of the *rbcS* cDNAs from *F. trinervia* and subsequent functional promoter testing in leaves (see below), we performed transcription run-off experiments with nuclei from separated BS and M cells of *F. trinervia* leaves. This required the design of an efficient Percoll step-gradient method for cell separations in this species, which should be of general use for C4 dicots. The surprising result was that *rbcS* genes are transcribed at significant levels in both BS and M cells, although measurably higher in BS cells (Table 1). This was consistent with results of the transient expression experiments described below. It is notable that *rbcS* genes in some C3 dicots and monocots are subject to posttranscriptional control as part of their organ- or light-specificity. In addition, *rbcS* genes in the C4 plants maize and amaranth exhibit apparent posttranscriptional control as a function of cell type or illumination. Since *rbcS* genes in C4 plants are developmentally regulated in a manner distinct from other C4 genes, perhaps because of their important role under C3 conditions, we decided to also test another BS-specific gene, that encoding NADP-malic enzyme.

3. *Flaveria* *me* gene families include C4 and non-C4 members, both plastidic and non-plastidic. To characterize the promoters and 3'UTRs of BS-specific *me* from *F. trinervia*, we obtained *me* genomic clones of *F. trinervia* and *F. pringlei*, using the *F. trinervia* *me* cDNA as probe. In each species, two genes encode plastidic ME with a transit peptide, one a C4 enzyme (ChlMe1) and the other with an undetermined role (ChlMe2). Additional genes encode cytosolic ME (CytMe), lacking a transit peptide, but with high similarity to the plastid isoform in the mature protein portion. We subsequently isolated cDNAs for ChlMe and CytMe for additional C4, C3, and C3-C4 *Flaveria* species by RT-PCR. This pattern of gene family complexity extends to all *Flaveria* species examined, supporting the view that C4 pathway recruited existing metabolic genes during evolution. Comparisons of corresponding genes in different species was informative. *F. trinervia* and *F. bidentis* 5' regions of ChlMe1 are nearly identical, except *bidentis* includes a 500bp AT-rich block related to the Tourist & Stowaway putative retrotransposons described in Sue Wessler's lab. The promoters in all 3 species include specialized regulatory elements associated with light-regulated inducible genes, such as ASF-1, GT-1, and opaque-2 sites. We also identified several putative ABA-response

### Table 1. Summary of nuclear run-off experiments in *F. trinervia*. Results expressed as % ubiquitin transcripts from image analysis of dot-blot signals

<table>
<thead>
<tr>
<th>Probe</th>
<th>M Transcripts</th>
<th>BS Transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td>ubiquitin (contr.)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>rbcS</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>me</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>mdh</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>ppk</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>ppc</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>
elements in the *F. trinervia* and *bidentis* leaf ME genes. CytME promoters include elements associated with general metabolic enzymes such as NIT-2 sites and motifs found in Adh1 and PAL genes.

4. **The promoters for ChlMe1 and ChlMe2 have distinct cell specificity and developmental patterns.** The C4 ChlMe1 mRNA accumulates in BS-specific fashion in mature leaves of *F. trinervia*. Nuclear run-off experiments demonstrated transcription of *me* at high levels in both M and BS nuclei (see Table 1), suggesting that posttranscriptional processes must be involved in conferring the BS-specific accumulation of *me* mRNA. However, our transgenic experiments using the ChlMe1 promoter to drive GUS expression suggest that 5' sequences alone are sufficient to achieve BS-specificity of GUS expression. The inclusion of 3'UTR sequences was not required. ChlMe2 and CytMe are transcribed in non-cell specific fashion throughout the leaf. Based on transgenic studies, the ChlMe1 promoter is active in both M and BS cells early in leaf development or in dark-grown cotyledons, and BS specificity depends on developmental age and illumination.

5. **MDH (NADP-dependent malate dehydrogenase) is encoded by a single gene, in C4, C3, and C3/C4 species.** To clone the C4-MDH from *F. trinervia*, we first used NADP-MDH amino acid sequence consensus information to clone and sequence a *F. trinervia* RT-PCR product from leaf RNA, and used this to screen a *F. trinervia* cDNA library (our maize *mdh* probes were not similar enough). The 26 cDNAs isolated were all derived from the same gene, with three alternate polyadenylation sites. cDNA probes were used to demonstrate that each of the *Flaveria* species used in our experiments contained a single MDH gene, instead of paired C4 and housekeeping genes, as in the monocots maize and sorghum. *mdh* mRNA and protein levels in each species were in proportion to the photosynthetic scheme: C3 species = low; C3/C4 species intermediate = medium; C4 species = high. We used the *mdh* cDNA as probe against our *F. trinervia* genomic library and isolated several positives that included a substantial amount of 5' and 3' regions flanking the *mdh* gene.

6. **Positional response of C4 genes: Regulation of C3 and C4 rbcS, me, and mdh promoters in C4, C3 and C3-C4 cellular contexts.** Our original plan was to use transient assays in separated BS and M protoplasts along with some transgenic plants. When the plant groups at Yale jointly obtained a DuPont Biolistic device, a more effective method for *in situ* expression analysis was possible—one that avoided possible programming of transgenes thru development (e.g., methylation) as can occur in transgenics and that avoided the tissue damage of protoplast preparation. BS and M cells can easily be visualized by DIC optics in intact leaf tissue following bombardment, GUS expression, staining, and tissue clearing. Extensive optimization was necessary for use of the biolistic method in *Flaveria* species. Each species (e.g., *F. trinervia* (C4) *F. pringlei* (C3), *F. chloraeofolia* (C3/C4), *F. linearis* (C3/C4)) exhibited different He pressure and projectile distance optima for bombardment. In addition, *Flaveria* experiments required that GUS be assayed within 8 hours, at which time its accumulation peaked and begins its decline. This relatively rapid turnover of GUS has not been observed in other plant species, including maize. This optimized assay is now efficient and reproducible in 7 species of *Flaveria*. 
7. Leaves of *Flaveria* C3, C4, and C3/C4 species express control promoters with differing efficiency. A strong constitutive promoter, the double-35S, was used to drive GUS expression as a non-cell-specific control. All leaf cells in each of the *Flaveria* species are capable of expressing this construct, with the frequency of each cell type approximately in proportion to its relative target area. The number of GUS-expressing cells per bombardment varied more than ten-fold among the *Flaveria* species, although each species was internally consistent (Table 2). This was in proportion to the total GUS activity recovered in leaf extracts from each species following bombardment, based on quantitative fluorometric GUS assays. The relative activity of the double-35S control promoter has proved to be a good predictor of the efficiency of C4 promoters in each *Flaveria* species. After extensive testing of various reporters (e.g., luciferase) to serve an internal (co-bombarded) control for test promoter constructs, we found that the use of an external control (35S-GUS) was as reliable and reproducible, and did not require a separate assay procedure. The successful development of these *Flaveria in situ* assays permitted us to perform the interspecific C3 vs. C4 promoter tests we proposed, which identified the importance of posttranscriptional controls, consistent with results from our nuclear run-off experiments.

8. *mdh* promoter from *F. trinervia* exhibits a 5-fold preference for M cells in C4 species and is non-specific in intermediate and C3 species. 5' sequences from the *F. trinervia* *mdh* gene were translationally fused to a GUS reporter and nos 3' terminator, and confirmed by sequencing. In microbombardment experiments, constructs containing 1.7 kb of *mdh* 5' sequence exhibited M-specificity (5-fold, from 3 individual experiments; example in Fig. 1 & Table 2), but far from enough to account for the absolute M-specificity of mRNA accumulation (at least 50x by RNA blots, absolute by *in situ* hybridization). The same constructs were active but not M-specific in C3 and intermediate species. In C3 species, expression was observed in all cells (including epidermal); in C3/C4 species, expression was limited to photosynthetic cells (BS, M, and stomatal). Both the pattern of expression and the high level of expression in non-C4 species is significant, because they suggest that the endogenous C4 (M-specific) pattern of *mdh* mRNA accumulation evolved from C3 (non-specific) and C3/C4 (gradient accumulation) pattern by the superimposing of cell-specific posttranscriptional controls upon the products of genes with strong non-specific promoters.

9. The *rbcS* promoter from *F. trinervia* (C4) exhibits a 3-5-fold preference for BS cells; *F. pringlei* (C3) *rbcS* promoter exhibits no preference for BS or M cells in C4 or C3 species. We proposed to test the activity of a C3 *rbcS* promoter in C4 and intermediate contexts, to see if cell-specific transcription was the consequence of the host cellular distribution of factors or of sequence differences in C3 vs C4 promoters. The latter seemed unlikely, based on the above 5' sequence similarity, although the 3' differences or farther upstream 5' differences may be significant. Bombardment of C3 *rbcS* promoter constructs into C3/C4 intermediate or C4 species exhibited no BS vs. M cell-specificity, although the constructs were highly active in all species tested (higher than that conferred by the double 35S promoter). Example experiments are shown in Table 3. Bombardment of C4 *rbcS* promoter constructs into the same species revealed BS-preferred (3-5-fold) GUS expression in C4 and intermediate species, and high levels of
activity in C3 M cells (no BS cells in these species). The fact that both \( rbcS \) and \( rndh \) C4 promoters are highly active in the C3 context, and the C3 versions are active in the C4 context, all without strong cell-specificity, suggests that much of the specificity arises from suppression of mRNA accumulation in the incorrect cells rather than from cell-specific enhancement of its accumulation.

10. The \( F. \) pringlei \( rbcS-1019 \) promoter is uniformly active in photosynthetic cells, and is 2 to 4-fold stronger than the double-35S promoter in all species of \( Flaveria \) tested. We performed quantitative measurements of the strengths of all promoters used for the GUS experiments in two ways: (1) quantitative fluorometric assays of promoter-GUS fusions in extracts from bombarded leaves and (2) luminometer assays of promoter-luciferase fusions in similar extracts.

From the in situ GUS assays described above, we know this expression is limited to photosynthetic cells but is otherwise non-specific. This very high level of activity was useful for experiments in C3/C4 intermediate species in which overall leaf transcriptional activities were low with other promoters.

11. Significance of in situ expression experiments. Taken together, the partial cell-specificity of \( Flaveria \) C4 \( rbcS \) and \( mdh \) promoters in microbombardment experiments and the results of nuclear run-off experiments lead to the conclusion that BS- and M-specific C4 genes are transcribed in both BS and M cells, but that the transcripts are subject to different posttranscriptional controls in the two cell types. This form of regulation in C4 species may be an evolutionary "add-on" to the non-cell-specific expression of these genes in their C3 ancestor plants. In the case of \( rbcS \), this form of regulation may reflect the metabolic requirement that it be expressed in both cell types, even in C4 plants, under some environmental conditions, possibly more rapidly than turnover of transcription factors could permit. It is particularly intriguing that \( rbcS \) mRNA accumulation in light vs. dark appears to be posttranscriptionally regulated in some C3 plants. It is also noteworthy that cDNAs for \( Flaveria \) C4 genes (\( rbcS \), me, mdh) all contain 3' untranslated regions (UTRs) of between 160-400 bp, although our studies of the me genes suggest that most or all controls are in 5' regions.

12. Molecular phylogeny of genus \( Flaveria \). The interpretation of gene expression experiments including several species—even from the same genus—requires a detailed knowledge of the relationships of the species at a molecular genetic level. We therefore completed a molecular phylogeny of the genus \( Flaveria \), in collaboration with molecular systematics experts Drs. Doug and Pamela Soltis (Wash. State Univ.). We compiled a collection of molecular and morphological data, including chloroplast RFLPs (using spinach probes provided by Dr. Robert Janssen, Univ. TX, Austin), nuclear rDNA spacer sequences (obtained by PCR methods), and leaf vascular patterns (with the help of Dr. Leo Hickey, Yale). A large enough dataset was obtained that parsimony "tree-making" programs (PAUP and others) generated a simple relationship among the 15 species we analyzed. This molecular-based tree is very similar to the morphology-based relationship proposed by Powell, although there are significant differences. The most
important observation is that all Flaveria C4 species (F. trinervia, F. bidentis, F. australasica) are clustered as a single group, linked to the C3 species (F. pringlei, F. robusta) though several intermediate species (F. ramosissima, F. linearis). An independent "branch" of intermediates (F. floridana, F. chloraeofolia) link the C4-like species F. brownii to C3 ancestors, which are in turn closest related to the (C3) Composite outgroups (Coreopsis, Helianthus). The bottom line is that all the species within the genus are indeed closely related at a DNA level, thus validating promoter swapping experiments, and that a second phyllary branch does not quite achieve the C4 strategy. The regions we sequenced in rbcS and mdh genes are almost sequence-identical in C3 and C4 species.

13. Effect of calcium sulfate on growth of Flaveria. In the course of measuring the accumulation of mRNAs for ME, MDH, and RbcS in various Flaveria species, we found that Flaveria species cannot be dark-adapted for dark-light or light-dark shifts if grown on normal soil mixtures. Seed germinated in the dark develop no further than cotyledon stage, and plantlets shifted from light growth into darkness cease growth and die rapidly. We reasoned that since Flaveria grows normally on gypseous soils, the vigor of plants growing under suboptimal conditions might be improved by supplementation with calcium sulfate. We supplemented Flaveria species growing in 0%, 5% and 50% sunlight with from 1 to 100 ppm CaSO4. All supplemented plants exhibited increased vigor; dark-grown plants in 1 ppm initiated 3-4 leaves on elongated internodes before losing vigor and dying. However, the 5% and 50% sunlight/1 ppm CaSO4 plants were healthy enough to use for studies of light intensity effects on C4 genes.

14. Stable transgenics of Flaveria C4 & C3 species. At the time of the original proposal (1991) we were excited about preliminary results in regenerating plantlets from leaf disks of F. trinervia, F. linearis, and F. pringlei, and proposed to generate a number of transgenics to confirm and extend the observations from transient leaf assays. Despite considerable effort, we were unable to regenerate transformed plants from the first two of these species. Apparently, the procedures for selection of transformed tissue were incompatible with regeneration. Subsequently, however, the Taylor laboratory (CSIRO) defined reproducible and efficient methods for regeneration of transformed F. bidentis (C4) and F. pringlei (C3) from hypocotyl segments, which we successfully adopted. Key features of the protocol are the gradual reduction in concentration of the IAA/kinetin hormone environment, inclusion of GA3, and use of Phytogel, although considerable tissue culture craft is still required. We utilized the transgenic approach for all of our later studies, because we encountered difficulty in publishing our biolistic transient expression studies of C4 gene promoters and 3'UTRs. The method has an inherent variability that we addressed with controls, but which some reviewers found unacceptable.

The transgenic studies largely just confirmed our earlier transient expression studies. However, they also enabled us to observe several unexpected features. First, the ChlMe1 promoters are very strong and BS-specific in leaves—making them of potential use for engineering purposes. Second, the same constructs are moderately expressed in phloem cells of primary and lateral roots. The degree of its cell-specificity may have prevented previous detection of substantial ME activity in roots. Third, the CytME promoters appear to function throughout the leaf early in development, and are shut off in a pattern suggesting a correspondence to the sink-source transition in maturing leaves. There is no apparent cell-specificity in leaves. Expression appears to be concentrated in vascular tissues of the stem. The 3'UTRs do not change these patterns. Fourth, histochemical GUS assays permitted us to detect responses by not previous observed from Northern blot analysis.
15. Function of ME gene family members in C3 and C4 species. The role of the two ME forms in C3 and C4 species is unknown, although several roles have been proposed. We need to understand these roles in order to understand the origin of the C4 pathway and the effects of future efforts to engineer the pathway into non-C4 species. Using GUS reporter constructs, we found that the ChlME1 genes are strongly repressed by sucrose. 3% sucrose inhibits germination and growth of F. bidentis and F. trinervia, although this was included in early transformation protocols. At 0.1% sucrose, germination and growth are enhanced, and ME gene expression is boosted. We have also determined that Flaveria species can be dark-grown for studies requiring sugar feeding. This requires a light pretreatment of 24h to permit seed germination. This enabled us to test the effects of inhibitors of the C4 pathway, wounding, and redox state on reporter gene expression.

We largely achieved the aims of the initial and renewal project proposals. The next logical steps would have been to characterize in further detail the regulatory elements and factors that govern the C4 pathway expression of these three gene families at transcriptional and posttranscriptional levels. However, the requirement imposed by peer review that we perform all of these studies by means of transgenic plants made progress slow (approx. 6 months to create and analyze Flaveria transgenics), and we decided that it was unwise to continue with the Flaveria system.

Publications supported by this project

Ph.D. dissertations