Untranslated Regions of \textit{FbRbcS1} mRNA Mediate Bundle Sheath Cell-specific Gene Expression in Leaves of a C\textsubscript{4} Plant*§

Minesh Patel\textsuperscript{1}, Alan J. Siegel, and James O. Berry\textsuperscript{2}

From the Department of Biological Sciences, State University of New York, Buffalo, New York 14260

C\textsubscript{4} photosynthesis typically requires two specialized leaf cell types, bundle sheath (bs) and mesophyll (mp), which provide the foundation for this highly efficient carbon assimilation pathway. In leaves of \textit{Flaveria bidentis}, a dicotyledonous C\textsubscript{4} plant, ribulose 1,5-bisphosphate carboxylase (rubisco) accumulates only in bs cells surrounding the vascular centers and not in mp cells. This is in contrast to the more common C\textsubscript{3} plants, which accumulate rubisco in all photosynthetic cells. Many previous studies have focused on transcriptional control of C\textsubscript{4} cell type-specificity; however, post-transcriptional regulation has also been implicated in the bs-specific expression of genes encoding the rubisco subunits. In this current study, a biolistic leaf transformation assay has provided direct evidence that the 5' and 3'-untranslated regions (UTRs) of \textit{F. bidentis FbRbcS1} mRNA (from a nuclear gene encoding the rubisco small subunit), in themselves, confer strong bs cell-specific expression to \textit{gfpA} reporter gene transcripts when transcribed from a constitutive CaMV promoter. In transformed leaf regions, strong bs cell-specific GFP expression was accompanied by corresponding bs cell-specific accumulation of the constitutively transcribed \textit{FbRbcS1} 5'-UTR-gfpA-3'-UTR mRNAs. Control constructs lacking any \textit{RbcS} mRNA sequences were expressed in all leaf cell types. These findings demonstrate that characteristic cell type-specific \textit{FbRbcS1} expression patterns in C\textsubscript{4} leaves can be established entirely by sequences contained within the transcribed UTRs of \textit{FbRbcS1} mRNAs. We conclude that selective transcript stabilization (in bs cells) or degradation (in mp cells) plays a key role in determining bs cell-specific localization of the rubisco enzyme.

Plants that utilize the highly efficient C\textsubscript{4} pathway of carbon fixation typically possess a Kranz-type leaf anatomy that consists of two distinct photosynthetic cell types (1–4). Bundle sheath (bs)\textsuperscript{3} cells occur as a layer around each leaf vein, while mesophyll (mp) cells occur in one or more layers surrounding the vascular associated rings of bs cells. This specialized leaf anatomy compartmentalizes two sets of photosynthetic reactions that make up the C\textsubscript{4} pathway. In the C\textsubscript{4} dicot \textit{Flaveria bidentis}, atmospheric CO\textsubscript{2} entering via the leaf stomata is initially incorporated into four carbon acids by phosphoenolpyruvate carboxylase, an enzyme found only in the leaf mp cells. The C\textsubscript{4} acids diffuse from mp cells to bs cells, where they are decarboxylated by a photosynthetic malic enzyme. Within bs chloroplasts, the released CO\textsubscript{2} is incorporated into the Calvin cycle by ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco), the primary enzyme of photosynthetic carbon fixation.

The specialized anatomical framework and compartmentalized reactions of the C\textsubscript{4} pathway function as a "CO\textsubscript{2} pump" that concentrates CO\textsubscript{2} in bs cells, where rubisco is specifically localized (1, 2, 5, 6). As a result, the carboxylase activity of this enzyme is increased, while its oxygenase activity, which can decrease photosynthetic productivity, is essentially eliminated. This two-cell compartmentalization of carboxylation/decarboxylation reactions does not occur in leaves of the more common C\textsubscript{3} plants, which possess only one photosynthetic cell type and use rubisco as the initial enzyme of biological carboxylation. The result is that C\textsubscript{4} plants are much more efficient at using biological energy (derived from photosynthetic electron transport) for CO\textsubscript{2} fixation, particularly in warm and arid environments where carbon assimilation in C\textsubscript{3} plants can be severely limited (1–3, 6, 7).

In mature C\textsubscript{4} leaves, rubisco and other photosynthetic proteins (such as the mp cell-specific phosphoenolpyruvate carboxylase) are confined to only one of the two photosynthetic cell types, and transcripts encoding these enzymes are correspondingly bs or mp cell-specific (8, 9). For this reason, many previous studies have focused on regulation of transcription as the primary determinant of bs and mp cell specificity. In some cases transcriptional control has been demonstrated, and transcriptional regulatory elements involved in determining cell type specificity have been identified (8–15).

Genes encoding both small and large subunits of the rubisco enzyme (\textit{RbcS} in the nucleus and \textit{rbcL} in the plastid) often show patterns of expression that are distinct from other \textit{C\textsubscript{4}} enzymes. For example, in many \textit{C\textsubscript{4}} species \textit{RbcS} and \textit{rbcL} genes are initially expressed in all leaf cell types, similar to their expression in \textit{C\textsubscript{3}} plant species (8, 9, 16, 17). To achieve cell type-specific localization of rubisco, which is essential for the function of the C\textsubscript{4} pathway, \textit{RbcS} and \textit{rbcL} genes become selectively down-regulated in mp leaf cells while continuing to be expressed abundantly in bs cells. This C\textsubscript{3}-C\textsubscript{4} transition in rubisco gene expression occurs during leaf development and is influenced by

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\textsuperscript{1} Current address: USDA ARS Cereal Crops Research Unit, Barley and Malt Laboratory, 501 Walnut St., Madison, WI 53726.

\textsuperscript{2} To whom correspondence should be addressed: Dept. of Biological Sciences, State University of New York, Buffalo, NY 14260. Tel.: 716-645-2363 (ext. 145); Fax: 716-645-3369; E-mail: camjob@buffalo.edu.

\textsuperscript{3} The abbreviations used are: bs, bundle sheath; mp, mesophyll; rubisco, ribulose 1,5-bisphosphate carboxylase; UTR, untranslated region; GFP, green fluorescent protein; RACE, rapid amplification of cDNA ends.
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light and/or photosynthetic metabolism (8, 9, 17–19). Some studies have indicated that unlike other C₄ enzymes, the initiation of bs cell-specific rubisco gene expression during leaf development and its maintenance in mature leaves of C₄ plants is due, at least in part, to post-transcriptional regulation; control of translation and mRNA stability have both been implicated (8, 9, 19, 20). Of particular significance, transcriptional run-on studies have shown that, while the rubisco mRNAs accumulate only in bs cells of mature C₄ leaves, their corresponding genes are transcribed in the nuclei and plastids of both bs and mp cells, suggesting cell type-specific control of transcript stability (9, 21). A recent study showed that untranslabeled regions (UTRs) from a heterologous rubisco transcript (AhRbcS1 from amaranth, another C₄ dicot), in themselves, conferred partial bs cell-specific expression to a gusA reporter gene at the level of mRNA accumulation when transcribed from a constitutive promoter in transgenic F. bidentis plants (22). This provided further evidence that post-transcriptional regulation contributes to bs cell-specific gene expression.

To better understand the role of post-transcriptional gene expression in determining the bs cell-specific localization of rubisco in C₄ leaves, we made use of a rapid and efficient biolistic transient expression assay. For this assay, transcripts with 5′- and 3′-untranslated regions (UTRs) from an F. bidentis RbcS gene (FbRbcS1) were linked in frame with a gfpA reporter gene and placed under the control of a constitutive promoter. When introduced into intact F. bidentis leaves, these constructs produced striking patterns of bs cell-specific GFP fluorescence and transcript localization within the transformed leaf regions. In contrast, gfpA transcripts lacking the FbRbcS1 UTRs produced no cell-type specificity in fluorescence or mRNA localization. Because GFP transcript accumulation, as well as protein accumulation, was found to be highly specific to bs cells in the intact transformed leaves, we conclude that this strong cell type-specific expression occurred at the level of mRNA stability and was mediated by the FbRbcS1 UTRs.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—F. bidentis seedlings were germinated and grown under sterile conditions on Murashige and Skoog media (Sigma) in PlantCon (ICN) growth containers maintained in a growth chamber with 14 h/day illumination at 170–200 mmol photons m⁻² s⁻¹.

FbRbcS cDNAs—cDNAs of F. bidentis FbRbcS transcripts were prepared by 5′- and 3′-RACE PCR (FirstChoice RLM-RACE Kit, Ambion) according to manufacturers protocols, using primers provided with the kit together with gene-specific primers corresponding to sequences within the FbRbcS coding regions (see supplemental material). 5′-Amplifications (performed under conditions that required the presence of a cap to ensure authentic 5′ ends) revealed the presence of two classes of cDNA, designated FbRbcS1 and FbRbcS2 (accession numbers AY267350 and AY267351, respectively), based on the length and sequence of the 5′-UTRs. Full-length cDNAs were isolated using 5′ end sense primers specific to each class of FbRbcS cDNA, together with a 3′ primer provided with the kit.

Primer extension analysis was performed (Promega AMV Reverse Transcriptase-Primer Extension System and manufacturers protocols) using total RNA isolated from F. bidentis leaves and cotyledons, together with a ³²P-end-labeled primer corresponding to a conserved region within the FbRbcS1 and FbRbcS2 coding regions. Primer extension products were analyzed using a 6% acrylamide-urea sequencing gel.

Post-transcriptional Expression Vectors—The experimental and control expression constructs were carried in the transient expression vector pBl221 (Clontech), downstream of the CaMV promoter, and upstream of the nopaline synthase transcription terminator. The control transient expression vector CaMV-gfpA was based on the pBl221XS expression cassette described by Patel et al. (22) and was created by replacing the β-glucuronidase (gusA) gene located within the Smal and Xhol restriction sites of that vector, with the gfpA gene. To prepare the FbRbcS1 5′-UTR-gfpA-3′-UTR expression vector, two chimeric gene fragments were initially amplified. The 5′-fragment corresponded to the FbRbcS1 5′-UTR plus the 5′ portion of the gfpA open reading frame and was amplified from a gfpA DNA template (using primers 5GFPFor and GFPRev; supplemental material). The forward primer incorporated a Smal restriction site at start of the FbRbcS1 5′-UTR. The 3′-fragment was amplified from the FbRbcS1 cDNA template (primers GFP49_3FbFor and G3FbRev; supplemental material) and contained 49 bases from the 3′ end of the gfpA coding sequence, plus the FbRbcS1 3′-UTR and an Xhol site at the 3′ end. These 5′-UTR-gfpA and gfpA 3′-UTR fragments overlapped by 49 bases within the gfpA open reading frame. The full-length FbRbcS1 5′-UTR-gfpA-3′-UTR expression cassette was prepared by denaturing and annealing these overlapping fragments and then extending the overlapping DNA strands with Klenow DNA polymerase (New England Biolabs). The transient expression vector FbRbcS1 5′-UTR-gfpA-3′-UTR was created by replacing the gusA fragment of pBl221XS (22) with this expression cassette, using the restriction sites Smal and Xhol.

Biolistic Transformation—Leaves (2–3 cm in length) from 6-week-old F. bidentis plants were detached and placed into a Petri dish lined with H₂O-moistened filter paper (Whatman 3MM). Four to five leaves were used per dish. For particle bombardment, gold particles (1.0 μm in size) were washed, prepared, and coated with DNA (either FbRbcS1 5′-UTR-gfpA-3′-UTR or CaMV-gfpA) according to manufacturer’s protocols (Biolistic PDS-1000/He Particle Delivery System, Bio-Rad). Leaves were bombarded once at 1100 p.s.i. using a PDS-1000/He Particle Delivery System (Bio-Rad). Each bombardment was performed using 10 μl gold/plasmid DNA suspension, using macrocarriers and stopping plates (Bio-Rad) placed ~6 cm from the leaves. After bombardment, the leaves were incubated in the dishes with illumination at 25 °C for 16 h and analyzed by microscopy.

Microscopy and Tissue Preparation—Following incubation, the bombarded leaves were initially placed on microscope slides over a thin layer of H₂O and visually scanned under 4× and 10× objectives of an Axiosvert 10 microscope (Zeiss, Oberkochen, Germany), using a 450–490 FT510, LP520 filter system. Localized regions of leaf transformation (foci) showing GFP fluorescence were carefully excised on the microscope stage using a scalpel, and these were used immediately for capturing leaf surface images of the foci and subsequent tissue...
processing. All of the images shown were captured using a DM IRE2 inverted compound microscope (Leica Microsystems, Wetzlar, Germany) equipped with fluorescent and brightfield imaging systems and a Retiga Exi cooled CCD camera (QImaging, Burnaby, British Columbia, Canada). Immediately after leaf surface images were taken, the foci were placed either in optimal cutting temperature compound (O.C.T., TissueTek) for cryosectioning or processed for paraffin embedding/sectioning (13, 16, 22). 10-μm cryosections were prepared using a Reichert-Jung Cryocut 1800 (Leica Microsystems) at 18 °C. 15-μm-thick paraffin-embedded sections were prepared using a rotary microtome and fixed to glass slides treated with Sta-On (Surgipath, Richmond, IL).

In Situ Hybridization and Immunolocalization—In situ hybridization analysis of paraffin-embedded sections from GFP-expressing foci was performed as described previously (13, 16, 22). Sections were hybridized to a gfpA antisense RNA probe that was transcribed in vitro using biotin-16 UTP (Roche Applied Science). Hybridized transcripts were detected using a streptavidin-alkaline phosphatase conjugate (NeutrAvidin; Pierce Chemical) with an enzymatic color reaction (SigmaFast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets).

Immunolocalization analysis on serial paraffin-embedded sections of GFP-expressing foci was performed as described (13, 16, 22), using GFP antiserum (Assay Designs, Inc.). Fluorescent imaging of the antibody reactions was performed using biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) together with an R-phycoerythrin/streptavidin conjugate (Molecular Probes).

RESULTS
The goal of this investigation was to determine the role of 5′- and 3′-UTRs of a F. bidentis RbcS mRNA in mediating bs cell-specific gene expression in the leaves of a C4 plant. Therefore, to ensure that FbRbcS cDNA used for vector construction contained a full-length 5′-UTR, we used a RACE PCR protocol that required the presence of a 5′-cap. Approximately 100 clones were characterized, some of which contained small variations within their coding regions that identified them as being derived from individual members of an FbRbcS gene family. These comprised two distinct classes, based on the length and sequence of their 5′-UTRs, and by some differences in their 3′-UTRs. FbRbcS1 and FbRbcS2 (supplemental Fig. S1, A and C) are representative of these two classes. Primer extension analysis confirmed that two main classes of FbRbcS mRNA accumulate in F. bidentis in vivo, with 5′ termini corresponding to the 5′ ends of these two classes of cDNA (supplemental Fig. S1B).

The post-transcriptional expression construct derived from FbRbcS1 (supplemental Fig. S2) was prepared by linking the full-length 5′- and 3′-UTRs of this transcript (nucleotides 1–26 and 552–735, respectively) in frame with a gfpA
reporter gene. To ensure that expression patterns were due entirely to post-transcriptional control, transcription of experimental, as well as control mRNAs (which lacked the FbRbcS1 UTRs, supplemental Fig. S2), was placed under the control of the constitutive CaMV promoter. These were inserted into the plasmid pBI221 for biolistic transformation.

Following bombardment, images taken from the leaf surface clearly show bs cell-specific GFP fluorescence within transformed regions (foci) expressing the FbRbcS1 5'UTR-gfpA-3'UTR construct (Fig. 1, A–C). These three images show GFP expression that is highly specific to bs cells that surround the leaf veins, with no GFP fluorescence observable outside of a single layer of cells surrounding the leaf vascular centers or within the veins themselves. In contrast, leaves bombarded with a CaMV-gfpA control construct, which lacked the FbRbcS1 UTRs, showed GFP fluorescence distributed throughout all of the cell types observable from the leaf surface (Fig. 1, D–F). Note that for A and D, images taken with a GFP imaging filter system were merged with the corresponding bright-field images, for clear visualization of transformed and untransformed leaf structures (leaf veins and bs cells). Images in other panels were captured using the GFP imaging filters alone and clearly show the differences in fluorescence between GFP-expressing and non-expressing cells.

To observe patterns of expression for the FbRbcS1-UTR and control constructs in more detail, cryogenic cross-sections were prepared from GFP-expressing foci that were excised from the intact leaves. Due to higher levels of GFP expression from FbRbcS1-UTR constructs relative to control constructs (similar findings were reported for AhRbcS1-UTR versus control constructs in ref. 22), exposures were adjusted for equal visualization of GFP fluorescence. In cross-sections, the FbRbcS1 5'-UTR-gfpA-3'-UTR construct clearly shows strong bs cell-specific expression (Fig. 2, A–C), whereas the control construct showed expression in all of the leaf cell types (D–F). With both the experimental and control constructs, transformed as well as non-transformed cells were observed within the foci. For example, both GFP-expressing (gbs) and non-expressing (bs) bundle sheath cells are observable in Fig. 2, A–C; expressing and non-expressing examples of all leaf cell types are observable in Fig. 2, D–F. Such cell to cell variations in transformation within a foci were most likely due to the nature of the biolistic protocol, which normally results in random clusters of cell transformations by the DNA-coated biolistic beads.

The images in Figs. 1 and 2 are representative of more than 60 independent biolistic transformation events. In all cases, the FbRbcS1 5'-UTR-gfpA-3'-UTR construct produced transfor-
Information foci showing strong GFP fluorescence only in bs cells, whereas transformation foci produced by the control construct showed GFP fluorescence in all cell types. Because both experimental and control constructs were transcribed from the same constitutive promoter, we conclude that bs cell-specific GFP expression mediated by the \textit{FbRbcS1-UTRs} was due to post-transcriptional regulation.

In situ hybridizations were performed to determine whether the strong bs cell specificity mediated by the \textit{FbRbcS1-UTRs} was due to regulation at the level of translation (\textit{gfpA} mRNA would be present in all transformed cell types) or mRNA stability (\textit{gfpA} mRNA would be present only in transformed bs cells). It was necessary to use paraffin-embedded sections for \textit{in situ} localization of GFP mRNAs, since it was not possible to maintain the integrity of frozen sections from biolistically transformed \textit{F. bidentis} leaf foci through the \textit{in situ} hybridization procedure. These sections were hybridized to a biotin-labeled GFP antisense RNA probe, and hybridization was detected using streptavidin-alkaline phosphatase and an enzymatic color reaction.

Fig. 3, \textit{A–C}, show representative cross-sections of foci from \textit{F. bidentis} leaves bombarded with constructs containing the \textit{FbRbcS1 5′-UTR-gfpA-3′-UTR} construct (all of which showed strong bs cell-specific GFP expression when viewed from the leaf surface). In these images (and in more than 15 additional sections), hybridization to the GFP antisense probe occurred primarily in leaf bs cells; only minimal signal was observed in mp cells of the biolistically transformed leaves. The very low levels of hybridization signal observed in some leaf mp cells was most likely due to low levels of mRNA accumulation from the \textit{FbRbcS1 5′-UTR-gfpA-3′-UTR} construct, since the GFP antisense probe did not normally produce detectable background signal in non-expressing leaf cells (for example, see Fig. 3, right portion of section shown in \textit{F}). In contrast, in sections from foci of leaves bombarded with the control construct (all of which showed GFP expression in all cell types when viewed from the leaf surface), hybridization to the GFP antisense probe occurred in all transformed leaf cell types, including bs and mp cells (Fig. 3, \textit{E–G}). Note that Fig. 3\textit{F} shows hybridization to a section from near the edge of an excised foci that contains both transformed cells (which showed hybridization to the \textit{gfpA} probe) and non-transformed cells (which showed no hybridization). This observation is consistent with control hybridization reactions (data not shown) indicating that the \textit{gfpA} antisense probe did not

\textbf{FIGURE 3.} Bs cell-specific GFP mRNA in cross-sections prepared from paraffin-embedded GFP foci excised from biolistically transformed \textit{F. bidentis} leaves. For \textit{in situ} hybridizations, leaf sections from GFP-expressing leaf regions were prepared and hybridized with biotin-labeled GFP antisense RNA probe (see “Experimental Procedures”). \textit{A–C}, \textit{in situ} hybridization of sections prepared from regions transformed with \textit{FbRbcS1-UTR/GFP} constructs. \textit{E–G}, \textit{in situ} hybridization of sections prepared from regions transformed with the control (no \textit{RbcS UTR}) constructs. Note that \textit{F} shows both biolistically transformed (GFP-expressing) and non-transformed (no GFP expression) regions. \textit{D} and \textit{H}, adjacent serial sections corresponding to \textit{C} and \textit{G}, respectively, reacted with GFP antisera and visualized using an R-phycoerythrin fluorescent detection system. \textit{Bar} = 100 μm.
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hybridize to sections from non-transformed *F. bidentis* leaves under conditions used here.

To co-localize GFP mRNA and protein accumulation within the same paraffin-embedded transformed leaf foci, serial sections were reacted with GFP antisera; imaging of the antibody reactions was performed using an R-phycoerythrin/streptavidin detection system. Fig. 3D (serial section corresponding to 3C) shows the same highly specific pattern of GFP protein accumulation in bs cells that was observed for GFP transcripts. Similarly, GFP protein accumulation in leaf regions bombarded with the control construct (Fig. 3H, serial section corresponding to Fig. 3G) shows the same nonspecific accumulation patterns as the control GFP mRNAs.

It should be noted that differences between *in situ* hybridization versus immunolocalization protocols can lead to slight variations in tissue morphology, accounting for the apparent incomplete overlap between serial sections shown in Fig. 3, D and C and H and G).

**DISCUSSION**

This current study provides strong evidence for post-transcriptional control of bs cell-specific *RbcS* gene expression in the leaves of a *C₄* dicot. Taken together, the data shown in Figs. 1–3 show that constitutively transcribed reporter gene transcripts containing both 5’- and 3’-UTRs from endogenous *FbRbcS1* mRNAs display strong bs cell-specific patterns of expression in biolistically transformed *F. bidentis* leaves. Because gfp/A transcript accumulation as well as expression of the GFP protein were both highly specific to bs cells, we conclude that this regulation occurred at the level of mRNA stability. This conclusion is supported by initial observations of regenerating *F. bidentis* T₀ plants stably transformed with the *FbRbcS1* 5’-UTR-gfpA-3’-UTR construct, which also show GFP fluorescence that is highly specific to leaf bs cells in a pattern very similar to the foci shown here.⁴

Findings presented here differ from those of our previous study (22), in which 5’- and 3’-UTRs from heterologous amaranth *AhRbcS1* mRNAs conferred only partial bs cell specificity to a *gusA* reporter gene, a pattern that was also likely mediated by post-transcriptional regulation of mRNA accumulation. The difference in expression patterns between these two systems is likely due to the heterologous origin of the *AhRbcS1* 5’- and 3’-UTRs sequences (these share 65 and 69% similarity, respectively, with the endogenous *FbRbcS1* UTRs, see supplemental Fig. S1, A and C), which might not have been fully recognized by the *F. bidentis* post-transcriptional regulatory processes, resulting in “leaky” bs cell specificity. In consideration of our current findings, we conclude that 5’- and 3’-UTRs of endogenous *F. bidentis* RbcS transcripts are capable of mediating strong bs cell-specific protein as well as mRNA accumulation in *C₄* leaves and thus contain the information needed to generate the bs cell-specific expression patterns that characterize these plants. In initial studies, we have found that neither UTR by itself is capable of mediating bs-specific gfp expression, suggesting that interactions between the 5’- and 3’-UTRs may be required for this process.

Rubisco genes are known to be highly regulated; in many plants their expression is modulated by light (18, 23, 24), development (20, 25, 26), cell type (6, 9, 16), photosynthetic metabolism (17, 19, 27–29), and even pathogen infection (30). While regulation of transcription has been implicated in some of these processes, post-transcriptional control is often a primary regulatory determinant (21, 23, 26, 29, 31, 32). This current study indicates that post-transcriptional control was utilized as a primary mechanism when *RbcS* gene expression was adapted for the specialized cell-type specific expression that provides the foundation for the *C₄* pathway in *F. bidentis* leaves.

Post-transcriptional regulation of *FbRbcS1* transcript accumulation would appear to be fully sufficient for mediating bs cell-specific gene expression. However, additional regulatory mechanisms might also contribute to this process in *F. bidentis* and other *C₄* systems. Separate studies from our laboratory and others have also implicated regulation of transcription and translation in determining bs cell-specificity (8, 9, 19, 20). For example, bs cell-specific localization of the rubisco proteins is separable from the specific localization of their corresponding transcripts in amaranth leaves during specific stages of leaf or photosynthetic development, implicating regulation at the level of translation (19, 20). Therefore, the establishment of full bs cell-specific *RbcS* gene expression in *C₄* leaves might in fact result from the integration of multiple levels of regulation. Each mechanism could act independently during different developmental stages (as in Refs. 19 and 20) or perhaps at some times in synergy, ultimately leading to full bs cell-specific localization of the rubisco proteins. Emerging evidence suggests that close interactions between different phases of transcriptional and post-transcriptional regulation may be a common occurrence in eukaryotic gene expression (33). Such redundancy and overlap for mechanisms determining bs cell specificity would ensure that full *C₄* photosynthetic capacity occurs in the leaves of these plants, at all stages of development and under diverse environmental conditions.

A less likely alternative explanation for bs cell-specific localization of *FbRbcS1* mRNA is that sequences within the UTRs have an effect on transcriptional elongation. Inhibition or termination of transcription by downstream control elements is known to occur in eukaryotic viruses and more rarely in cellular genes (in both plants and animals; Refs. 34–37). Measurements of gfp/A mRNA transcription and turnover in stable transgenic *F. bidentis* lines will provide mechanistic details about UTR-mediated control of bs cell-specific *FbRbcS1* mRNA accumulation.

Direct evidence that bs cell specificity is controlled by sequences within the *FbRbcS1* UTRs will focus future *C₄* studies (38) on an analysis of post-transcriptional mechanisms involved in this process. Control of gene expression through differential transcript stabilization occurs in all organisms (39–46); transcripts targeted for regulated turnover often contain specific sequences within their 5’- and 3’-UTRs that interact with cytoplasmic RNA-binding proteins to mediate their specific decay (40, 41, 44, 46–48). In many cases, these mRNAs contain AU-rich elements within their 3’ UTRs that mediate turnover (41, 42, 44, 46–48). *RbcS* mRNAs from the *C₄* dicots

⁴ M. Patel and J. O. Berry, unpublished data.
F. bidentis and amaranth contain multiple AU-rich elements within their 3′-UTR (supplemental Fig. S1C); interestingly, one of these repeats, UUUAAU, is identical to a repeat in human mRNAs that become destabilized when this sequence interacts with a specific class of RNA binding protein (46). Identification of regulatory sequences within the RbcS 5′- and 3′-UTRs, as well as the RNA-binding proteins they interact with, will aid in the functional analysis of specific molecular recognition processes responsible for post-transcriptional, cell-specific transcript accumulation in C₄ leaves.

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REFERENCES

1. Hatch, M. D. (1987) Biochim. Biophys. Acta 895, 81–106
2. Kanai, R., and Edwards, G. E. (1999) in C₄ Plant Biology (Sage, R. F., and Monson, R. K., eds) pp. 49–88, Academic Press, San Diego, CA
3. Furbank, R. T., Hatch, M. D., and Jenkins, C. L. D. (2000) in Advances in Photosynthesis. Photosynthesis: Physiology and Metabolism (Leegood, R. C., Sharkey, T. D., von Caemmerer, S., and Kennedy, R., eds) pp. 459–469, Kluwer Academic Publishers, Dorecht, the Netherlands
4. Edwards, G. E., Franceschi, V. R., Ku, M. S. B., Voznesenskaya, E. V., Pyankov, V. I., and Andreo, C. S. (2001) J. Exp. Bot. 52, 577–590
5. von Caemmerer, S., and Furbank, R. T. (2003) Photosynth. Res. 77, 191–207
6. Sage, R. F. (2004) New Phytol. 161, 341–370
7. Ehleringer, J. R., Cerling, T. E., and Helliker, B. R. (1997) Oecologia 112, 285–299
8. Furbank, R. T., and Taylor, W. C. (1995) Plant Cell 7, 797–807
9. Sheen, J. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 187–217
10. Langdale, J. A., Taylor, W. C., and Nelson, T. (1991) Mol. Gen. Genet. 225, 49–55
11. Bansal, K. C., Virett, J.-F., Haley, J., Khan, B. M., Schantz, R., and Bogorad, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 89, 3654–3658
12. Viret, J.-F., Mabrouk, Y., and Bogorad, L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8577–8581
13. Long, J. J., and Berry, J. O. (1996) Plant Physiol. 112, 473–482
14. Nomura, M., Sentoku, N., Nishimura, A., Lin, J.-H., Honda, C., Taniguchi, M., Ishida, Y., Ohta, S., Komari, T., Miyao-Tokutomi, M., Kano-Murakami, Y., Tajima, S., Ku, M. S., and Matsuoka, M. (2000) Plant J. 22, 211–221
15. Gowik, U., Burscheidt, J., Akylidiz, M., Schläue, U., Koczor, M., Steubel, M., and Westhoff, P. (2004) Plant Cell 16, 1077–1090
16. Wang, J.-L., Klessig, D. F., and Berry, J. O. (1992) Plant Cell 4, 173–184
17. Wang, J.-L., Turgeon, R., Carr, J. P., and Berry, J. O. (1993) Plant Cell 5, 289–296
18. Wang, J.-L., Long, J. J., Hotchkiss, T., and Berry, J. O. (1993) Plant Physiol. 102, 1085–1093
19. McCormac, D. J., Boinski, J. I., Ramsperger, V. C., and Berry, J. O. (1997) Plant Physiol. 114, 801–815
20. Ramsperger, V. C., Summers, R. G., and Berry, J. O. (1996) Plant Physiol. 111, 999–1010
21. Boinski, J. I., Wang, J.-L., Xu, P., Hotchkiss, T., and Berry, J. O. (1993) Plant Mol. Biol. 22, 397–410
22. Patel, M., Corey, A. C., Yin, L.-P., Ali, S., Taylor, W. C., and Berry, J. O. (2004) Plant Physiol. 136, 3550–3561
23. Berry, J. O., Breiding, D. E., and Klessig, D. F. (1990) Plant Cell 2, 795–803
24. Zhou, J., Ma, L., Zhang, S., Zhu, Y., and Sun, D. (2001) Plant Cell Physiol. 42, 1049–1055
25. Wanner, L. A., and Gruissem, W. (1991) Plant Cell 3, 1289–1303
26. Hensel, L. L., Grbic, V., Baumgarten, D. A., and Bleecker, A. B. (1993) Plant Cell 3, 553–564
27. Jiang, C. Z., Rodermel, S. R., and Shibles, R. M. (1993) Plant Physiol. 101, 105–112
28. Cheng, S.-H., Moore, B. D., and Seemann, J. R. (1998) Plant Physiol. 116, 715–723
29. Sinha, A. K., Hofmann, M. G., Römer, Ü., Köckenberger, W., Elling, L., and Roitsch, T. (2002) Plant Physiol. 128, 1480–1489
30. Berger, S., Papadopoulos, M., Schreiber, U., Kaiser, W., and Roitsch, T. (2004) Physiol. Plant. 122, 419–428
31. Thompson, D. M., and Meagher, R. B. (1990) Nucleic Acids Res. 18, 3621–3629
32. Rodermel, S., Haley, J., Jiang, C.-Z., Tsai, C.-H., and Bogorad, L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3881–3885
33. Orphanides, G., and Reinberg, D. (2002) Cell 108, 439–451
34. Wright, S. (1993) Mol. Biol. Cell. 4, 661–668
35. Curie, C., and McCormick, S. (1997) Plant Cell 9, 2025–2036
36. He, X., Fütterer, J., and Hohn, T. (2002) Nucleic Acids Res. 30, 497–506
37. Hu, W.-W., Gong, H., and Pua, E. C. (2005) Plant Physiol. 138, 276–286
38. Brown, N. J., Parsley, D. K., and Hibberd, J. M. (2005) Trends Plant Sci. 10, 215–221
39. Shirley, B. W., and Meagher, R. B. (1990) Nucleic Acids Res. 18, 3377–3385
40. Seeley, K. A., Byrne, D. H., and Colbert, J. T. (1992) Plant Cell 4, 29–38
41. Green, P. J. (1993) Plant Physiol. 102, 1065–1070
42. Zubiaga, A. M., Belasco, J. G., and Greenberg, M. E. (1995) Mol. Cell. Biol. 15, 2219–2230
43. Staton, J. M., Thomson, A. M., and Leedman, P. J. (2000) J. Mol. Endocri- nol. 25, 17–34
44. Fedoroff, N. V. (2002) Curr. Opin. Plant Biol. 5, 452–459
45. Gutierrez, R. A., Ewing, R. M., Cherry, J. M., and Green, P. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11513–11518
46. Blackshear, P. J., Lai, W. S., Kennington, E. A., Brewer, G., Wilson, G. M., Guan, X., and Zhou, P. (2003) J. Biol. Chem. 278, 19947–19955
47. Laroia, G., Cuesta, R., Brewer, G., and Schneider, R. J. (1999) Science 284, 499–502
48. Cheng, Y., and Chen, X. (2004) Curr. Opin. Plant Biol. 7, 20–25