Untranslated Regions from C₄ Amaranth AhRbcS1 mRNAs Confer Translational Enhancement and Preferential Bundle Sheath Cell Expression in Transgenic C₄ Flaveria bidentis

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Many aspects of photosynthetic gene expression are posttranscriptionally regulated in C₄ plants. To determine if RbcS mRNA untranslated regions (UTRs) in themselves could confer any characteristic C₄ expression patterns, 5′- and 3′-UTRs of AhRbcS1 mRNA from the C₄ dicot amaranth were linked to a gusA reporter gene. These were constitutively transcribed from a cauliflower mosaic virus promoter and assayed for posttranscriptional expression patterns in transgenic lines of the C₄ dicot Flaveria bidentis. Three characteristic C₄ expression patterns were conferred by heterologous AhRbcS1 UTRs in transgenic F. bidentis. First, the AhRbcS1 UTRs conferred strong translational enhancement of gusA expression, relative to control constructs lacking these UTRs. Second, while the UTRs did not appear to confer tissue-specific expression when analyzed by β-glucuronidase activity assays, differences in gusA mRNA accumulation were observed in leaves, stems, and roots. Third, the AhRbcS1 UTRs conferred preferential gusA expression (enzyme activity and gusA mRNA accumulation) in leaf bundle sheath cells. AhRbcS1 UTR-mediated translational enhancement was also observed in transgenic C₃ plants (tobacco [Nicotiana tabacum]) and in in vitro translation extracts. These mRNAs appear to be translated with different efficiencies in C₄ versus C₃ plants, indicating that processes determining overall translational efficiency may vary between these two categories of higher plants. Our findings suggest that the AhRbcS1 5′-UTR functions as a strong translational enhancer in leaves and other tissues, and may work synergistically with the 3′-UTR to modulate overall levels of Rubisco gene expression in different tissues and cell types of C₄ plants.

Flaveria bidentis and amaranth (Amaranthus hypochondriacus) are both dicotyledonous plants that utilize the C₄ pathway of photosynthesis. This specialized CO₂ fixation pathway requires the abundant expression of genes encoding the various C₄ enzymes in one of two leaf cell types. One set of CO₂ fixation enzymes, including Rubisco, accumulates only in leaf bundle sheath (bs) cells, while others, such as phosphoenolpyruvate carboxylase, are produced only in leaf mesophyll (mp) cells. In all C₄ species, the various photosynthetic enzymes are regulated independently (Furbank and Taylor, 1995; Berry et al., 1997; Sheen, 1999) yet share many characteristic gene expression patterns required for their function in CO₂ fixation. These include very high levels of expression, localization to specific cell types within leaves or other photosynthetic tissues, and regulation by light. For many C₄ genes, including those encoding the Rubisco small subunit (SSU; encoded by nuclear RbcS genes) and large subunit (encoded by plastid rbcL genes), posttranscriptional control processes have been implicated in one or more aspects of their regulation (Furbank and Taylor, 1995; Berry et al., 1997; Sheen, 1999).

In all plants, the Rubisco SSU is encoded by a multi-gene family (Dean et al., 1989; Galili et al., 1998, 2000). Within a given RbcS family, the transit and mature SSU polypeptides encoded by different RbcS genes are usually highly conserved, while the promoter and mRNA untranslated regions (UTRs) can be conserved or may vary in sequence (Dean et al., 1989; Corey et al., 1999). For some plants, the expression of individual RbcS genes can vary significantly within a given tissue (Dean et al., 1989; Wanner and Gruissem, 1991). Quantitative differences in expression between individual RbcS genes may be due to differences in transcription (Poulson et al., 1986; Poulson and Chua, 1988; Wanner...
and Gruissem, 1991) or, in some cases, mRNA decay rates (Manzara and Gruissem, 1988; Dean et al., 1989).

Many aspects of Rubisco gene expression are common to both C_{3} and C_{4} plants, the most notable of these being expression primarily in photosynthetic leaves and cotyledons, and regulation by light within these tissues (Gilmartin et al., 1990; Cory, 1994; Viret et al., 1994; Berry et al., 1997). In addition, overall levels of RbcS and rbcL gene expression are often controlled by development (Wanner and Gruissem, 1991; Hensel et al., 1993; Ramsperger et al., 1996), cell and tissue type (Furbank and Taylor, 1995; Sheen, 1999), and photosynthetic metabolism (Sheen, 1990; Krapp et al., 1993; Wang et al., 1993a; McCormac et al., 1997). For RbcS genes, control of expression at the level of transcription has been implicated in all these processes (Fluur et al., 1986; Poulsone et al., 1986; Poulsone and Chua, 1988; Sheen, 1990; Wanner and Gruissem, 1991; Dedonder et al., 1993; Furbank and Taylor, 1995). In addition, many developmentally and environmentally induced changes in RbcS and rbcL gene expression occur at posttranscriptional levels, such as mRNA processing (selective degradation or stabilization of transcripts) and regulation of translation (Berry et al., 1988, 1990, 1997; Thompson and Meagher, 1990; Gallie, 1993; Furbank and Taylor, 1995; Sheen, 1999).

The aspect of Rubisco regulation that is most characteristic of C_{4} plants, including *F. bidentis* and amaranth, is that in mature leaves and cotyledons genes encoding both Rubisco subunits are selectively down-regulated in mesophyll cells, while continuing high levels of expression in bundle sheath cells. The end result is that Rubisco, an abundant enzyme in all photosynthetic cells of C_{3} plants, accumulates only in the bundle sheath cells of C_{4} plants, with little or no accumulation of Rubisco transcripts or subunit proteins in mesophyll cells. In the *F. bidentis* dicot amaranth, posttranscriptional regulation contributes substantially to the establishment and maintenance of bundle sheath-specific *RbcS* and *rbcL* gene expression during C_{4} leaf development (Boinski et al., 1993; Ramsperger et al., 1996) and in response to changes in photosynthetic activity (McCormac et al., 1997). Posttranscriptional control may be a property of Rubisco expression in many C_{4} plants; such regulation has been shown to be involved with determination of bundle sheath specificity in C_{4} monocots as well as C_{4} dicots (Schaffner and Sheen, 1991; Bansal et al., 1992; Kubicki et al., 1994; Viret et al., 1994; Roth et al., 1996; Berry et al., 1997; Sheen, 1999).

To better understand the role of posttranscriptional regulation in determining highly abundant cell type and tissue-specific Rubisco gene expression in C_{4} plants, and the possible conservation of regulatory mechanisms among C_{4} dicots, we have prepared posttranscriptional gene expression constructs derived from the amaranth *AhRbcS1* gene (Corey et al., 1999) for expression in transgenic lines of *F. bidentis*. The goal of this study was to determine what properties of C_{4} gene expression, if any, could be conferred to a *gusA* reporter gene by the UTRs of *AhRbcS1* mRNA, a transcript known to be highly regulated at posttranscriptional levels in amaranth (Berry et al., 1988, 1990, 1997; Ramsperger et al., 1996; McCormac et al., 1997). Since the 5’- and 3’-UTRs of many mRNAs are involved in posttranscriptional regulation, full-length 5’- and 3’-UTRs of *AhRbcS1* mRNAs were linked to a *gusA* reporter gene. To ensure that any observed gene expression patterns were due entirely to regulation at posttranscriptional levels in transgenic plants, these *AhRbcS1* 5’-UTR-*gusA*-3’-UTR fusions were transcribed from the constitutive cauliflower mosaic virus (CaMV) 35S promoter instead of from an *RbcS* promoter. For analysis of translation in vitro, these mRNAs were also transcribed in vitro from a T7 promoter. Our results show that RbcS 5’-UTRs conferred greatly enhanced levels of translation to *gusA* transcripts in leaves and other tissues of C_{4} plants and to a lesser extent in C_{3} plants or plant-derived translation extracts. *AhRbcS1*-derived UTRs in themselves could mediate tissue-specific accumulation of *gusA* mRNAs in transgenic *F. bidentis* plants, although transcript accumulation levels were clearly separable from levels of β-glucuronidase (GUS) activity in the different plant tissues. In themselves, the heterologous *AhRbcS1* UTRs were sufficient to confer partial C_{4}-like bundle sheath-specific *gusA* expression patterns in leaves of transgenic *F. bidentis* plants.

**RESULTS**

**Posttranscriptional Gene Expression Constructs Derived from *AhRbcS1* mRNA**

To investigate the roles of 5’- and 3’-UTRs in the posttranscriptional regulation of C_{4} RbcS gene expression, tagged RNA expression constructs were prepared from *AhRbcS1* mRNA sequences (Corey et al., 1999). The purpose of these constructs was to investigate aspects of posttranscriptional C_{4}-specific RbcS gene expression that are conferred by regions of the mRNA molecules themselves, in the absence of any contribution from the RbcS promoter. Thus, while these lack an *AhRbcS* promoter, they contain 5’- and/or 3’-UTRs from the *AhRbcS* mRNAs, linked (in frame) with a *gusA* reporter gene, and were transcribed in transgenic plants from the constitutive CaMV promoter. The *AhRbcS1*-derived constructs used in this study were initially prepared using the transient expression vector pBI221 (Stratagene, La Jolla, CA) and contained the expression cassettes shown in Figure 1. The expression construct designated pGG53 contained the 5’- and 3’-UTRs of *AhRbcS1*; pBI221XS contained *AhRbcS* UTRs and was used as a control. Constructs containing the *AhRbcS1* 5’-UTR alone (pGG5) or the 3’-UTR alone (pGG3) were also prepared.

**35S-UTR- *gusA*-3’-UTR Expression Levels in Leaves of Transgenic C_{4} Plants**

For production of transgenic plants, the *AhRbcS1* UTR-derived expression constructs and the control...
construct described above were inserted into the binary vector pGA482 (An, 1987) and used for transformation of F. bidentis hypocotyls (Chitty et al. 1994). Although all three of the AhRbcS1 UTR-derived expression constructs described above were used for transformation of F. bidentis hypocotyls, only the binary vector containing the CaMV/AhRbcS1-5′-UTR-gusA-3′-UTR expression cassette (from pGG53) led to the recovery of regenerated C4 plants. F. bidentis lines containing the AhRbcS1 5′-UTR alone (from pGG5) or 3′-UTR alone (from pGG3) expression cassettes have not been recovered and could not be included in this study.

Overall levels of GUS activity were significantly higher in leaves of F. bidentis plants transformed with the CaMV/AhRbcS1-5′-UTR-gusA-3′-UTR expression construct (lines designated FbA53), relative to control plants (lines designated CaMV-GUS) expressing the gusA gene alone (lacking the AhRbcS UTRs) from the CaMV promoter. As shown in Figure 2A, GUS activity levels in leaves ranged from 18- to 250-fold higher in the FbA53 lines, relative to the control CaMV-GUS lines. Despite the great differences in gusA expression levels observed between the experimental and control plants, accumulation levels of gusA mRNA did not differ significantly between the two plant groups. Although there were some differences in mRNA accumulation between individual transgenic plants (Fig. 2B), when standardized to 18S rRNA levels the amount of gusA mRNA in the leaves of FbA53 and CaMV-GUS plants were approximately equal. These results clearly show that the enhanced levels of gusA expression in the FbA53 leaves, relative to the CaMV-GUS control leaves, was not due to increased levels of gusA mRNA accumulation.

When GUS activity levels in leaves of transgenic plants were standardized to levels of gusA mRNA accumulation and averaged for six FbA53 and three control CaMV-GUS lines (from lines shown in Fig. 2, A and B, and in Fig. 3), there was an average 48-fold enhancement of translation from the transcripts containing the AhRbcS1 UTRs, relative to control gusA transcripts lacking these UTRs (Fig. 2C). To alleviate concerns about potential background in reporter gene assays or hybridization analysis, wild-type (nontransformed) plants showed no discernable GUS activity in fluorescent 4-methyl umbelliferyl β-D-glucuronide (MUG) assays or any hybridization to the gusA DNA.
Expression in Transgenic C_4 Plants

analyzed by northern analysis, using $^{32}$P-labeled DNA fragment probes 2.65 and CaMV-GUS10. RNA samples prepared from each tissue were visualized and quantified using a phosphorimager. Hybridizations were reduced greater than 6-fold in stems and greater than 20-fold in roots. In contrast, gusA mRNA levels in the CaMV-GUS control plants were present at similar levels in all three tissues. Taken together, it appears that some degree of tissue-specific regulation of gusA mRNA accumulation was conferred by the AhRbcS1 UTRs in the transgenic F. bidentis plants. Since the gusA gene was transcribed from a constitutive CaMV promoter, it is likely that the tissue-specific differences in gusA mRNA accumulation were regulated posttranscriptionally, through differential stabilization of the hybrid AhRbcS1 5' -UTR-gusA-3' -UTR mRNAs. In addition, it is clear that synthesis of GUS from these mRNAs in FbA53 transgenic plants was enhanced to similar high levels in leaves, stems, and roots, independent of levels of gusA transcripts that accumulated in these tissues. This suggests that rates of GUS protein synthesis and levels of mRNA accumulation are controlled by separate determinants in this transgenic C_4 plant system.

Visual examination of various tissues isolated and stained for GUS activity from several transgenic plants indicated that, while overall levels of GUS activity were much higher in FbA53 than in control plants, both constructs expressed the gusA transgene throughout the plants. The quantitative data shown in Figure 3 are representative of these plants. Quantitative MUG activity assays revealed that enhanced levels of GUS activity from the CaMV/AhRbcS1 5' -UTR-gusA-3' -UTR expression cassette occurred in all tissues of the FbA53 plants examined, including photosynthetic leaves as well as nonphotosynthetic roots (Fig. 3A). It is especially interesting to note that, while GUS activity levels were enhanced to similar levels in the leaves, stems, and roots of FbA53 transgenic plants, gusA mRNA levels produced from the CaMV/AhRbcS1 5' -UTR-gusA-3' -UTR expression cassette were significantly lower in the nonleaf tissues of these plants (when standardized to levels of 18S rRNA; Fig. 3B). Relative to levels in leaves, gusA-containing transcripts were reduced greater than 6-fold in stems and greater than 20-fold in roots. In contrast, gusA mRNA levels in the CaMV-GUS control plants were present at similar levels in all three tissues. Taken together, it appears that some degree of tissue-specific regulation of gusA mRNA accumulation was conferred by the AhRbcS1 UTRs in the transgenic F. bidentis plants. Since the gusA gene was transcribed from a constitutive CaMV promoter, it is likely that the tissue-specific differences in gusA mRNA accumulation were regulated posttranscriptionally, through differential stabilization of the hybrid AhRbcS1 5' -UTR-gusA-3' -UTR mRNAs. In addition, it is clear that synthesis of GUS from these mRNAs in FbA53 transgenic plants was enhanced to similar high levels in leaves, stems, and roots, independent of levels of gusA transcripts that accumulated in these tissues. This suggests that rates of GUS protein synthesis and levels of mRNA accumulation are controlled by separate determinants in this transgenic C_4 plant system.

Visual observation of GUS-staining patterns within leaf cross-sections from transgenic FbA53 plants showed higher GUS activity in bs cells than in mp cells (Fig. 4A). In contrast, cross-sections from CaMV-GUS control plants showed homogenous (although much lower overall) levels of GUS staining in all leaf cell types (Fig. 4B). In situ hybridization using an antisense RNA probe of the gusA-coding region indicated patterns of gusA mRNA accumulation for the transgene mRNAs that were reflective of the GUS activity staining patterns. Higher levels of gusA mRNA accumulation were observed in leaf bs cell than in mp cells of the FbA53 lines (Fig. 4D), while similar amounts of gusA mRNA were present in both leaf cell types of CaMV-GUS lines (Fig. 4E). In control hybridizations with adjacent serial sections, FbRbcS1 mRNAs (detected using an FbRbcS1 antisense probe) were fully bs specific (Fig. 4F). Based on these findings, we conclude that gusA mRNAs containing the AhRbcS1-derived UTRs were preferentially expressed in bs cells, primarily at the level of mRNA accumulation, in leaves of the heterologous F. bidentis plants.

Localization of AhRbcS1 5' -UTR-gusA-3' -UTR Expression in Transgenic C_4 Plants

Visual observation of GUS-staining patterns within leaf cross-sections from transgenic FbA53 plants showed higher GUS activity in bs cells than in mp cells (Fig. 4A). In contrast, cross-sections from CaMV-GUS control plants showed homogenous (although much lower overall) levels of GUS staining in all leaf cell types (Fig. 4B). In situ hybridization using an antisense RNA probe of the gusA-coding region indicated patterns of gusA mRNA accumulation for the transgene mRNAs that were reflective of the GUS activity staining patterns. Higher levels of gusA mRNA accumulation were observed in leaf bs cell than in mp cells of the FbA53 lines (Fig. 4D), while similar amounts of gusA mRNA were present in both leaf cell types of CaMV-GUS lines (Fig. 4E). In control hybridizations with adjacent serial sections, FbRbcS1 mRNAs (detected using an FbRbcS1 antisense probe) were fully bs specific (Fig. 4F). Based on these findings, we conclude that gusA mRNAs containing the AhRbcS1-derived UTRs were preferentially expressed in bs cells, primarily at the level of mRNA accumulation, in leaves of the heterologous F. bidentis plants.

AhRbcS1 UTRs Confer Translational Enhancement in Other Plant Systems

The AhRbcS1 UTR sequences conferred enhanced rates of translation in the transgenic F. bidentis plants, relative to plants lacking these UTRs. While this is not surprising, considering that these mRNAs encode

![Image](https://example.com/image.png)

**Figure 3.** GUS activity and mRNA accumulation in three tissues of transgenic F. bidentis FbA53 and CaMV-GUS plants. A, GUS activity. Fluorescent MUG assays were performed using total protein extracts from leaf, root, or stems of representative AhRbcS1 5'-gusA-3' (FbA53-2.65) and CaMV-GUS control (CaMV-GUS10) transgenic C_4 plants. B, gusA mRNA and 18S rRNA levels in leaves, roots, or stems of FbA53-2.65 and CaMV-GUS10. RNA samples prepared from each tissue were analyzed by northern analysis, using $^{32}$P-labeled DNA fragment probes for each gene. Hybridizations were visualized and quantified using a phosphorimager.
photosynthetic proteins that accumulate to very high levels in plant cells, enhanced translational ability represents a very interesting and significant property of the C₄ AhRbcS₁ transcripts. To determine if the enhancement of gene expression conferred by the AhRbcS₁ UTRs was specific to expression in the C₄ dicot F. bidentis or if increased translation could occur in a C₃ plant as well, binary vectors containing AhRbcS₁ UTR-derived expression cassettes and the CaMV-GUS control cassette (Fig. 1) were transformed into the C₃ dicot tobacco (Nicotiana tabacum). In addition to the pGG53 cassette, transgenic plants, stable lines transformed with expression constructs containing the AhRbcS₁ 5' -UTR alone (pGG5 cassette) or the 3' -UTR alone (pGG3 cassette), were also recovered.

Transgenic lines of tobacco expressing all three AhRbcS₁-derived cassettes and the control cassette were analyzed for GUS activity, gusA, and 18S rRNA levels, as described above. As in the transgenic F. bidentis plants, GUS activity from the CaMV/AhRbcS₁-5' -UTR-gusA-3' -UTR construct, as well as the other constructs tested, was observed in all tissues of the plants, including leaves, stems, and roots. To quantify levels of translation from each construct, GUS activity in leaves was standardized to levels of gusA mRNA and averaged over at least five independent T₀ lines transformed with each of the constructs. Figure 5 shows that there was an enhancement of GUS activity from a construct containing the AhRbcS₁ 5' - and 3' -UTRs (tobacco lines 5'3'GUSAh), and from a

Figure 4. Bs-preferential GUS activity and gusA mRNA accumulation in transgenic FbA53 and CaMV-GUS plants. For GUS activity staining, leaves excised from transgenic or wild-type plants were first incubated with GUS-staining solution and then paraffin embedded and sectioned as described in “Materials and Methods.” For in situ hybridizations, leaf sections from transgenic C₄ plants were prepared and hybridized with biotin-labeled antisense RNA probes, as described. A, Leaf cross-section from FbA53-2.67 transgenic plant, GUS staining. B, Section from CAMV-GUS9 plant, GUS staining. C, Section from leaf of wild-type (nontransformed) F. bidentis, GUS staining. D, Leaf section from FbA53-2.67, hybridized to gusA antisense RNA probe. E, Leaf section from CaMV-GUS9, hybridized to gusA antisense RNA probe. F, Leaf section from CAMV-GUS9 plant, hybridized to FhRbcS₁ antisense RNA probe.
construct containing the 5′-UTR alone (5′GUSAh), relative to the control CaMV-GUS tobacco plants. However, this enhancement was not as strong as that observed in F. bidentis leaves (approximately 3-fold in transgenic tobacco lines expressing the pGG53 or pGG5 cassette).

Interestingly, the AhRbcS1 3′-UTR (tobacco line 3′GUSAh) in itself appeared to repress expression in the C₃ tobacco plants. When compared to expression in lines expressing the AhRbcS1-5′-UTR-containing constructs (5′GUSAh and 5′3′GUSAh) and even to the tobacco CaMV-GUS control lines, GUS activity relative to gusA mRNA accumulation in the 3′-UTR alone lines was considerably reduced. In the presence of the 5′-UTR, translation from the gusA mRNAs remained high. Such findings suggest that the AhRbcS1 3′-UTR, in the absence of the 5′-UTR, may act as an inhibitor of translation.

The pGG53 and pBI221XS gusA expression cassettes shown in Figure 1 were inserted downstream of a T7 promoter to determine if AhRbcS1 UTR-mediated enhanced-translational ability could also occur in vivo in plant-derived cell-free translation extracts. Equal amounts of transcripts generated in vitro from each of these cassettes were translated in wheat germ cell-free translation extracts. Equal amounts of transcripts were translated in vitro using a wheat germ system (Promega) in the presence of 35S-Met. Reactions were separated by SDS-PAGE and visualized using a phosphorimagery. Note: Smaller band is likely degradation product of GUS protein. Lane 1: Control (no added RNA); lane 2: gusA control construct (pBI221XS) RNA, lacking RbcS UTRs; lane 3: AhRbcS1 5′ gusA3′ (pGG53) RNA. B, Northern blot showing relative amounts of gusA RNA present in each translation extract after termination of the reaction. RNA samples prepared from aliquots of each extract were separated on an agarose/formaldehyde gel, blotted, and hybridized to a gusA probe. Relative IVT/RNA (Av. of 2 rxns), relative rates of in vitro GUS protein synthesis standardized to gusA RNA levels.

Figure 5. Averaged GUS activity, standardized to gusA mRNA levels, in leaves of transgenic tobacco, a C₄ dicot. 5′ GUSAh, pGG53 (AhRbcS1 5′-UTR) expression cassette; 5′GUS3′Ah, pGG53 (AhRbcS1 5′- and 3′-UTR) cassette; and 3′GUSAh, pGG3 (AhRbcS1 3′-UTR) expression cassette. CaMV-GUS, No AhRbcS1 UTRs; N, nontransformed control plants. MUG values were standardized relative to gusA mRNA/18s RNA levels. For each construct, bars represent data from T₀ plants from at least five independent transformation events.

Figure 6A shows that in vitro transcribed RNAs containing 5′- and 3′-UTRs derived from amaranth AhRbcS1 mRNAs showed enhanced levels of synthesis in vitro. Phosphorimagery analysis revealed that there was a 7- to 8-fold increase in translation of the 68-kD GUS protein conferred by the AhRbcS1 UTRs (lane 3) compared to control RNA, which lacks RbcS UTRs (lane 2). In two independent experiments, when levels of GUS protein synthesized in vitro were standardized to amounts of RNA present, transcripts containing the AhRbcS1 UTRs were found to confer nearly identical increased levels of in vitro translation of the gusA coding region (7.52 and 7.48 in two experimental repeats), relative to control transcripts that lack the RbcS UTR (Fig. 6, bottom row).

Taken together, findings from transgenic C₄ and C₃ plants, and the in vitro translation studies, demonstrate that UTRs derived from C₃ RbcS mRNAs confer enhanced rates of translation to a gusA reporter gene. This enhancement can occur, with some variation in overall levels, in a wide range of experimental plant systems.
DISCUSSION

Although the C₄ pathway of photosynthesis has been known for nearly four decades (Hatch and Slack, 1966; Hatch, 1997), underlying mechanisms responsible for determining the unique and complex gene expression patterns characteristic of this diverse group of plant species are not well understood. To date, evidence indicates that, during the evolution of C₄ photosynthesis, multiple levels of regulation have been utilized and coordinated to achieve the cell-specific gene expression patterns required for the function of this specialized CO₂ fixation pathway. Control of C₄ gene expression at the level of transcription has been implicated in many studies (Nelson and Langdale, 1992; Furbank and Taylor, 1995; Berry et al., 1997; Sheen, 1999; Furbank et al., 2000; Matsuoka et al., 2001, Leegood, 2002; Shoichi and Burnell, 2003; Gowik et al., 2004). Photosynthetic promoters linked to reporter genes have reproduced some level of cell type-specific or tissue-specific expression patterns when these are expressed in C₄ plants, although in some cases only partial bs or mp cell specificity has been obtained. Promoter regions from a maize (Zea mays) bs-specific RbcS gene, or mp-specific phosphoenolpyruvate carboxylase or pyruvate orthophosphate dikinase genes, conferred reporter gene expression that was highly specific to their respective cell types in transgenic maize plants (Matsuoka et al., 1993; Nomura et al., 2000a, 2000b). However, contribution from posttranscriptional control cannot be ruled out, since these expression constructs also contained 5′-UTR and coding regions of the mRNAs. Some sequences controlling bs cell specificity and high-level expression of the F. bidentis gene encoding the C₄ NADP malic enzyme occur outside of the gene’s promoter, within regions corresponding to the 5′- and 3′-UTRs and N-terminal coding regions of the Mε1 transcript (Ali and Taylor, 2001a, 2001b). These findings suggest that regulation at transcriptional as well as posttranscriptional levels contribute to the C₄ expression patterns of this bs-specific gene in F. bidentis. In amaranth, observations of AhRbcS gene expression during early leaf development (Ramsperger et al., 1996) and in photosynthetically inactive leaf regions (McCormac et al., 1997) indicate that control of bs cell specificity is initially regulated at the level of transcription, followed by control at the level of transcription or transcript stability.

While previous investigations have focused on the role of promoters and transcriptional control in the regulation of bs- or mp-specific gene expression, the potential involvement of regulatory sequences within the photosynthetic transcripts themselves have been largely overlooked. In this study, we examined how the UTRs from bs-specific AhRbcS1 mRNAs affect overall levels as well as tissue and cell type expression patterns in transgenic C₄ plants and in other plant systems. Findings presented here indicate that UTRs of the amaranth AhRbcS1 mRNAs conferred significantly enhanced levels of expression to a gusA reporter gene, relative to mRNAs that lacked these UTRs, in all tissues of transgenic F. bidentis plants. Significant but less dramatic enhancement was also observed in transgenic tobacco or in in vitro wheat germ extracts. Considering that RbcS mRNAs encode photosynthetic proteins that accumulate to very high levels in the chloroplasts of C₄ as well as C₃ plants (Edwards and Huber, 1981; Mizioroko and Lorimer, 1983; Sage et al., 1987), it might be expected that these transcripts would contain sequences capable of acting as strong translational enhancers. Translational enhancement is primarily known from some plant and animal viral RNAs (Gallie, 1993, 2002; De La Luna et al., 1995; Chizhikov and Patton, 2000; Salvatore et al., 2002). There are also several examples of plant cellular transcripts showing enhanced translation capabilities (Pitto et al., 1992; Gallie and Young, 1994; Bate et al., 1996; Ling et al., 2000). Translational enhancement of cellular and viral mRNAs is often mediated through sequences located in their 5′- or 3′-UTRs and in some cases involves interactions with specific cellular proteins.

Translational enhancement from AhRbcS1 mRNAs occurred very strongly in F. bidentis leaves and, to a lesser degree, in tobacco and in the cell-free wheat germ extracts. Differences in translational abilities for some transcripts are known to occur between different groups of plants; for example, transcripts containing the tobacco mosaic virus Ω enhancer are translated more efficiently in dicots than in monocots (Gallie et al., 1989). Tissue-specific or developmentally related differences in translational efficiencies for some highly translated plant mRNAs are also known. UTRs from a barley (Hordeum vulgare) aleurome mRNA increased translational efficiency mostly in aleurome or endothelium protoplasts (Gallie and Young, 1994), while the lat52 5′-UTR conferred enhanced translation specifically in developing pollen grains during maturation stage of development (Bate et al., 1996). Heat shock protein HSP101-mediated translational enhancement of Fed-1 mRNA occurred in response to light and during specific stages of leaf development (Ling et al., 2000). It is therefore not surprising that levels of translational enhancement for C₄ RbcS mRNAs would be stronger in F. bidentis than in tobacco, or in translation extracts derived from the C₃ monocot wheat. This could be indicative of differences in the presence or levels of specific RbcS translational control factors between C₃ and C₄ dicots, or general differences in overall translational abilities for photosynthetic mRNAs between these two groups of plants.

At least some of the enhanced translational ability of the AhRbcS1-derived constructs may be due to the fact that their 5′-UTR has a better context for the initiator AUG than the CaMV-gusA control construct. Both constructs share identical gusA context downstream of the AUG codon (which is less optimal than Ah or Fb RbcS mRNAs; Corey et al., 1999; GenBank accession nos. AY267350 and AY267351; Sawant et al., 2001). However these differ in upstream context (Kozak, 1999). Most significantly, the AhRbcS1-derived UTR
UTRs of Ah tissue-specific regulation is mediated through the presence of the 3′-UTR. Furthermore, the 3′-UTR alone did not increase translation levels relative to the CaMV-GUS control. (In fact, the 3′-UTR alone lowered translation levels to less than the CaMV-GUS control in transgenic tobacco.) These observations imply that enhanced translation ability is a property of the 5′-UTR in itself. It is possible that the very high levels of translation mediated by the AhRbcS1-5′-UTR-gusA-3′-UTR mRNAs in the heterologous C₄ dicot may in fact not be reflective of the translation rates for endogenous intact FbRbcS mRNAs of F. bidentis. Transgenic mRNAs containing the heterologous AhRbcS1 5′-UTR may be capable of very high-level, unregulated translation, beyond that which would occur in intact FbRbcS mRNAs. However, it is clear that some posttranscriptional regulatory patterns were conferred by the AhRbcS1 UTRs in the heterologous C₄ plants, including tissue-specific mRNA accumulation and preferential expression in leaf bs cells.

The 5′- and 3′-UTRs of AhRbcS1 mRNA in themselves were sufficient to confer tissue-specific accumulation patterns to gusA mRNA in transgenic F. bidentis plants. Highest levels of gusA transcripts in FbA53 plants occurred in leaves, with less accumulation in stems and significantly reduced accumulation in roots. This is reflective of relative levels of overall RbcS mRNA accumulation that occur in these tissue in intact amaranth plants (Nikolaou and Klessig, 1987) and is typical of tissue-specific RbcS gene expression levels observed in other plant species (Silverthorne and Tobin, 1990; Wanner and Gruissem, 1991; Meier et al., 1995; Galili et al., 1998). Because transcription was from the constitutive CaMV promoter, differential accumulation of the AhRbcS1 UTR-containing mRNAs in different tissues of the FbA53 plants was likely due to differences in their stability. Since gusA transcripts expressed in transgenic CaMV-GUS control plants lacked the AhRbcS1 UTRs, these would not be subject to the same differential regulation of stability and thus accumulated to equal levels in the different tissues. Posttranscriptional regulation of RbcS mRNA accumulation in different tissues or under different growth conditions has been clearly documented in a variety of plant species (Shirley and Meagher, 1990; Silverthorne and Tobin, 1990; Wanner and Gruissem, 1991; Deidonner et al., 1993; Galili et al., 1998). Findings presented here indicate that a significant amount of tissue-specific regulation is mediated through the UTRs of AhRbcS1 mRNAs. Posttranscriptional regulatory mechanisms responsible for tissue-specific accumulation of RbcS mRNAs appear to be conserved in these two species of C₄ dicot, since the AhRbcS1 UTR sequences were recognizable and functional in the heterologous transgenic plants.

Normally, specific degradation resulting in reduced levels of an mRNA would be expected to also result in reduced levels of the encoded protein. In the case of the FbA53 plants, reduced levels of gusA mRNA in stems and roots did not result in a corresponding lower levels of GUS activity in these tissues, suggesting there are separate determinants of GUS protein synthesis and mRNA accumulation in these transgenic C₄ plants. The equal levels of GUS activity in these tissues did not result from saturation of the MUG assays, since increasing dilution of the extracts (up to 0.025-fold) produced the same relative levels of activity in the three tissues. Taking into consideration gusA transcript accumulation in the three tissues, together with the GUS activity assays, it appears that only a small amount of the more abundant gusA mRNAs in leaves of FbA53 plants were utilized for synthesis of the GUS protein, or else the less abundant transcripts accumulating in stems and roots were able to be utilized more efficiently. When the GUS activity data shown in Figure 3 were calculated relative to gusA mRNA levels, translation from AhRbcS1-5′-UTR-gusA-3′-UTR mRNA appeared to be at least 6- and 20-fold higher in stems and roots, respectively, relative to its translation in leaves. The reason for the disparity between mRNA accumulation and GUS activity in different tissues of FbA53 plants is not clear. Because of the highly enhanced protein synthesis ability of transcripts expressed from this construct, it is conceivable that levels of these hybrid mRNAs accumulating within the cells greatly exceeded the cells’ ability to regulate their translation. This could result in the synthesis of equal amounts of the GUS enzyme from different levels of gusA transcript in all tissues of the FbA53 plants. An important consideration from these findings is that, while in the majority of situations measured levels of GUS enzymatic activity accurately reflect of levels of gusA mRNA accumulation and therefore gene expression (Jefferson, 1987), it is clearly possible for exceptions to this precept to occur.

Preferential accumulation of the gusA mRNAs in bs cells was observed in cross-sections of mature leaves from FbA53 plants. GUS activity levels observed in these cross-sections were reflective of the amount of gusA transcript present in the two cell types, with higher levels present in bs cells than in other leaf cell types. Bs-preferential gusA mRNA accumulation or expression was not observed in leaf cross-sections from the CaMV-GUS control lines. Based on these analyses, we conclude that the AhRbcS1 UTRs were conserved enough and sufficient in themselves to confer at least partial C₄-like bs cell-specific expression to the reporter gene in leaves of transgenic F. bidentis plants. Since the transgenes were constitutively transcribed, preferential gusA mRNA accumulation was
regulated posttranscriptionally and is likely to be mediated by differential processing or stabilization of the AhRbcS1-derived transcripts in mature leaves of the FbA53 plants.

Previous studies have demonstrated that bs-specific accumulation of transcripts encoding both Rubisco subunits is posttranscriptionally regulated in mature leaves of C₄ plants. In maize, nuclear run-on experiments have shown that RbcS mRNAs are transcribed in nuclei isolated from both bs and mp cells, whereas these transcripts accumulate only in bs cells of mature leaves (Schaffner and Sheen, 1991). Experiments comparing run-on transcription and mRNA accumulation for the plastid rbcL gene of amaranth, maize, and sorghum (Sorghum bicolor) have provided similar results (Boinski et al., 1993; Kubicki et al., 1994). In each of these plant systems, rbcL mRNAs were transcribed at similar levels in plastids isolated from both cell types, whereas they accumulate only in bs chloroplasts, and not in mp chloroplasts, of mature leaves. Such findings indicate that both RbcS and rbcL transcripts are transcribed in all of the leaf photosynthetic cells but have different stabilities in bs versus mp cells.

The lack of full cell type-specific regulation for the CaMV/AhRbcS1-5' -UTR-gusA-3' -UTR construct in transgenic FbA53 leaves could be due to the heterologous UTR sequences present in the AhRbcS1 mRNA, which may not be fully recognized by the endogenous C₄ regulatory mechanisms of F. bidentis. In fact, initial evidence from the first regenerating leaves of new transgenic C₄ lines indicates that constructs containing UTRs of the endogenous FbRbcS1 mRNAs show much tighter bs-specific expression than constructs derived from the heterologous AhRbcS1 mRNAs (M. Patel and J.O. Berry, unpublished data). Even small variations between these two plant species in terms of their mRNA regulatory sequences and the factors that recognize them might be expected to result in alterations to normal expression patterns, such as the greatly enhanced translation of the heterologous AhRbcS1-based constructs observed in the FbA53 plants. A comparison of UTR sequences from AhRbcS1 and two endogenous F. bidentis RbcS mRNAs (Corey et al., 1999; and GenBank accession nos. AY267350 and AY267351) reveal some similarities. These 5'-UTRs are all relatively short for nuclear-encoded plant genes (29–53 nt, most plant genes possess leader regions of 40–80 nt long; Gallie, 1993, 1996). All three 5'-UTRs are very AU rich (66%–68%) and contain similar 17 to 18 nt AU-rich regions. The 3'-UTRs are 129, 184, and 212 nt long for AhRbcS1, FbRbcS1, and FbRbcS2, respectively. These are even more AU rich than the 5'-UTRs (73%–88%) and contain similar multiple UUAUU(C) repeats. Overall comparisons of three amaranth AhRbcS (Corey et al., 1999) and the two F. bidentis FbRbcS mRNAs indicated that among all of these transcripts, the 5'-UTRs share 63% to 75% similarity, while the 3'-UTRs share 64% to 67% similarity. The lineages of Caryophyllales (Amaranth) and Asterids (Flaveria) diverged between 83 and 151 million years ago (Schneider et al., 2004). The fact that RbcS mRNAs from two divergent C₄ plants have high overall similarities, with similar AU rich sequences in their 5'- and 3'-UTRs, suggests that these might serve a similar regulatory function (but perhaps still not identical) in both C₄ species. Additional comparative functional and phylogenetic analyses of conserved regulatory sequences in the UTRs of RbcS mRNAs may help to identify cis-acting elements that mediate translation or mRNA stability in different C₄ species.

Findings presented here confirm that some aspects of RbcS gene expression in C₄ plants are controlled posttranscriptionally and that regulatory processes are at least partially conserved between these two species of C₄ dicot. Constitutively transcribed gusA mRNAs containing the AhRbcS1 UTRs showed enhanced overall levels of expression, tissue-specific mRNA accumulation, and preferential expression in leaf bs cells. The fact that full tissue- or bs cell-specific expression was not achieved from the AhRbcS1-derived construct in F. bidentis could be due to the heterologous origin of the UTR sequences. An alternative explanation is that additional mRNA sequences occurring elsewhere on full-length RbcS mRNAs might also be involved in mediating posttranscriptional photosynthetic gene expression in C₄ plants. While cis-acting control regions often occur within the 5'- or 3'-UTRs of an mRNA, sequences within the coding region can also be involved (Gallie, 1993, 1996, 1998; Gillham et al., 1994; Viret et al., 1994; Mayfield et al., 1995; Stern et al., 1997; Ali and Taylor, 2001a, 2001b). Such regions could function independently or possibly in synergy with the UTRs, to achieve the full tissue- and bs-specific RbcS expression patterns characteristic of C₄ plants. Additional studies examining the expression of posttranscriptional expression constructs derived from the endogenous FbRbcS mRNAs (currently in regeneration) may allow us to resolve these questions.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All plants (transgenic and wild-type Flaveria bidentis, amaranth, and tobacco [Nicotiana tabacum]) were germinated and grown in growth chambers (14 h/d illumination at approximately 170–200 μmol photons m⁻² s⁻¹).

RNA Isolation, Northern Analysis, and Hybridization Probes

Total RNA was extracted from transgenic and wild-type plants, and northern- or slot-blot analysis was performed as previously described (Berry et al., 1985; Long and Berry, 1996). Transcripts of gusA were detected using a DNA probe fragment corresponding to the entire gusA open reading frame. Equalized loading of the mRNA samples was confirmed by stripping the blots and rehybridizing with an 18SrRNA probe (a 250-bp fragment that was PCR amplified from an F. bidentis 18S rRNA clone). The DNA probe fragments were [³²P]-labeled using a Megaprime kit (Amersham Biosciences, Piscataway, NJ). Hybridizations were visualized and quantified using a phosphorimagery equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).
**AhRbcS1-Derived Posttranscriptional Expression Vectors**

All of the AhRbcS1-derived constructs used for this study were prepared from a modified form of the transient expression vector pBl221 (CLONTECH Laboratories, Palo Alto, CA). This vector, designated pBl221XS (Fig. 1), was produced by inserting a short DNA fragment containing an Xhol site into pBl221 at the Smal site located just upstream of the nopaline synthase terminator.

To prepare pGG3 (the AhRbcS1 3′-UTR construct), the full-length 3′-UTR was PCR amplified from an AhRbcS1 cDNA clone. The forward primer contained an Xhol site followed by an SstI site, followed by (from 5′→3′) the first 20 bases of the 3′-UTR (from nt 600 through 619). The reverse primer contained (from 5′→3′) the same two restriction cut sites, followed by the 20 bases immediately upstream of the polyA tail (antisense; from nt 728 through 709). The amplified 3′-UTR (3E103′XS) was then digested with Xhol, gel purified, and ligated into dephosphorylated pBl221XS. Electroporation of XL1-MRP cells (Stratagene) yielded a clone containing the 3′-UTR in correct orientation downstream of the gusA open reading frame (confirmed by restriction mapping and dideoxynucleotide sequencing), designated pGG3.

To prepare pGG5 (the AhRbcS1 5′-UTR-gusA-3′-UTR construct) an oligonucleotide was synthesized that contained a BamHI site at its 5′ end, followed by the 47 base AhRbcS1 5′-UTR sequence attached in frame to the first 21 bases of the sense strand of the gusA reporter gene-coding region. PCR was performed using this as the forward primer, together with a reverse primer specific for the 3′ end of the AhRbcS1 3′-UTR, using the 3′-UTR-gusA construct pGG3 as the template. This amplified fragment was inserted into the BamHI/Xhol sites of pBl221XS from which the gusA gene had been removed. Following transformation, a clone containing the 5′- and 3′-UTRs in correct positions relative to the gusA open reading frame was identified by restriction mapping and dideoxynucleotide sequencing and designated pGG5 (Fig. 1).

For pGG3, the 3′-UTR region was removed from the amplified pGG5 fragment described above before insertion into pBl221XS. Following transformation, a clone containing the 5′-UTR in correct position upstream of the gusA open reading frame was identified by restriction mapping and dideoxynucleotide sequencing and designated pGG5.

**Production and Analysis of Transgenic F. bidentis and Tobacco**

The AhRbcS1-derived expression cassettes contained within the constructs described above and shown in Figure 1 were all inserted into the binary vector pGA482 (An, 1987) and transformed into Agrobacterium strains AGL1 or LBA4404 for production of transgenic F. bidentis or tobacco plants, respectively. Transgenic F. bidentis plants were prepared according to the methods of Chitty et al. (1994). Transgenic tobacco plants were prepared as described previously (Kinal et al., 1995; Park et al., 1996). Transgenic F. bidentis plants used for analysis were first (T1) or second (T2) generation progeny of self-pollinated primary transformants (T0). All transgenic tobacco plants were primary T0 transformants. Genomic DNA blots were used to determine copy number of inserted transgenes and determine independent transformations, as previously described (Kinal et al., 1995; Park et al., 1996).

**Analysis of Expression Levels for the gusA Transgene**

For analysis of transgene expression, samples were harvested from young leaves of 1- to 3-month-old plants. To prepare protein extracts for fluorometric MUG assays, six leaf discs (6 mm diameter) were removed from each plant and placed into 500 μL of protein extraction buffer (50 mM Tris, pH 7.2, 1 mM EDTA, 10 mM β-mercaptoethanol) with 1 μL of 1 mg/mL phenylmethylsulfonyl fluoride. Tissues were ground in a glass homogenizer, and the extracts were clarified in a microfuge. The supernatant was frozen in aliquots at −70°C. MUG assays were performed according to the assay described by Jefferson (1987). Due to the very high levels of Gus activity in some of the plants, the original protein extract was diluted as much as 0.025-fold before being added to the assay solution. For other samples with little or no Gus activity, up to 50 μL of extract was directly added to each 0.5-ml reaction. Aliquots from these enzymatic reactions were removed, at 2, 7, 12, and 17 min after initiation and the reactions terminated. MUG concentrations at each time point were determined with a spectrophotometer, using excitation at 365 nm and emission at 455 nm.

Since the focus of these analyses was posttranscriptional control, Gus activity levels were standardized to levels of gusA mRNA for each sample. Since loading can vary somewhat between gel lanes or loading slots, gusA mRNA levels in each sample were first standardized to levels of 18S rRNA.

**Analysis of gusA Transgene Expression in Leaf Bs and Mp Cells**

For localization of Gus enzyme activity, leaves (20–40 mm in length) of transgenic or wild-type F. bidentis were harvested from young (3–4-week-old) plants and stained with 5-bromo-4-chloro-3-indolyl β-D-glucuronide as follows. Leaves were cut into 2 to 4 mm strips and incubated with enough staining solution (5-bromo-4-chloro-3-indolyl β-D-glucuronide tablets, Sigma-Aldrich, St. Louis) to completely submerge the tissue. The tissues were then vacuum infiltrated three times for 5 min each, and incubated overnight at 37°C. The tissues were then fixed in acetic ethanol (2:3) overnight at room temperature. Samples were paraffin embedded and then sectioned to a thickness of 10 μm using a rotary microtome equipped with methods described previously (Wang et al., 1992; Long and Berry, 1996). Two 10-min incubations in xylenes were used to remove paraffin from the tissues, which were then mounted with a glass coverslip using Permount (Fisher Scientific, Pittsburgh, PA).

Sections from transgenic or wild-type F. bidentis leaves (20–40 mm in length) were prepared for in situ hybridization as described (Wang et al., 1992; Long and Berry, 1996). For detection of gusA or endogenous RbcS mRNAs, plasmids containing either gusA coding region or cDNA for a full-length FbRbcS gene (FbRbcS1) were linearized and used to generate antisense RNAs in vitro. These were transcribed using biotin-16 UTP (Roche Applied Science, Indianapolis), and hybridized transcripts were detected using a streptavidin-alkaline phosphatase conjugate (NeutrAvidin; Pierce Chemical, Rockford, IL) with an enzymatic color reaction (SigmaFast BCIP/NBT tablets).

All sections were visualized and images were captured using a Leica DM IRE HC inverted compound microscope equipped with a Retiga-cooled CCD camera (Leica Microsystems, Wetzlar, Germany).

**In Vitro Translation**

The pGG53 and pBl221XS gusA expression cassettes were inserted into pBlueScript II SK+ for generation of mRNA from the T7 promoter for use in in vitro translation assays. In vitro translation was performed in the presence of [35S]Met (PerkinElmer Life Sciences, Boston, MA) using wheat germ extracts (TNT system; Promega, Madison, WI). After the reactions were completed, aliquots were taken from each of the translation extracts for northern analysis of gusA transcripts present in the extracts. Translated proteins were run on 15% SDS-PAGE gel. RNAs were separated on an agarose-formaldehyde gel and analyzed by northern analysis using a gusA gene probe. Both gels were visualized and quantified using a phosphorimagert and ImageQuant software.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY267350 and AY267351.

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