Expression of the C₄ Me₁ Gene from Flaveria bidentis Requires an Interaction between 5′ and 3′ Sequences

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The efficient functioning of C₄ photosynthesis requires the strict compartmentation of a suite of enzymes in either mesophyll or bundle sheath cells. To determine the mechanism controlling bundle sheath cell-specific expression of the NADP-malic enzyme, we made a set of chimeric constructs using the 5′ and 3′ regions of the Flaveria bidentis Me₁ gene fused to the β-glucuronidase gusA reporter gene. The pattern of GUS activity in stably transformed F. bidentis plants was analyzed by histochemical and cell separation techniques. We conclude that the 5′ region of Me₁ determines bundle sheath specificity, whereas the 3′ region contains an apparent enhancer-like element that confers high-level expression in leaves. The interaction of 5′ and 3′ sequences was dependent on factors that are present in the C₄ plant but not found in tobacco.

INTRODUCTION

In the C₄ photosynthetic pathway, plants spatially separate the initial fixation and subsequent utilization of CO₂ to avoid the wasteful process of photorespiration. The efficient operation of C₄ photosynthesis requires the strict compartmentation of a number of key enzymes in either mesophyll or bundle sheath cells (Hatch, 1987). This compartmentation in turn relies on cell-specific expression of the genes encoding the enzymes (Furbank and Taylor, 1995). For this reason, C₄ plants provide an attractive system for studying the factors regulating cell-specific gene expression.

In several recent studies, attempts have been made to elucidate the molecular basis of cell-specific expression of various genes required in C₄ photosynthesis. However, because of the lack of a facile transformation system for any C₄ species, these studies have relied on more or less indirect approaches. Transient expression assays of promoter-reporter gene fusions introduced into isolated maize mesophyll protoplasts (Sheen, 1990, 1991) or maize leaf sections (Matsuoka and Numazawa, 1991; Bansal et al., 1992; Bansal and Bogorad, 1993; Viret et al., 1994; Purcell et al., 1995) are among the approaches that have been used. In addition, the expression of reporter genes driven by promoters from C₄ genes has been studied in stably transformed C₃ plants (Matsuoka and Sanada, 1991; Matsuoka et al., 1993, 1994; Stockhaus et al., 1994).

Although much information has been gained from the above-mentioned studies, each of the approaches used has some potential shortcomings. For example, the disturbance caused by isolation of protoplasts and by electroporation or particle bombardment may alter gene expression. In addition, changes in the physical characteristics of leaves during development may affect the penetration of DNA-coated particles into different cell types (Viret et al., 1994). Finally, the analysis of the expression of genes required for C₄ photosynthesis using C₃ plants has obvious limitations. For instance, C₃-specific regulatory factors are unlikely to be present in C₃ plants, thus requiring cautious interpretation of data obtained in such a heterologous system.

To avoid these limitations, we have developed an efficient method for Agrobacterium-mediated stable transformation of the C₄ dicot Flaveria bidentis (Chitty et al., 1994). The development of this method has opened the door for the study of factors regulating the cell-specific expression of genes required for C₄ photosynthesis in a homologous C₄ plant. In the work presented here, we used the F. bidentis Me₁ gene, which encodes the isoform of NADP-malic enzyme (NADP-ME) used in C₄ photosynthesis (Marshall et al., 1996), to study the cis regulatory elements that lead to high-level, bundle sheath cell-specific expression. We found that both upstream and downstream regions of the Me₁ gene are required to confer the expected expression pattern and that the effects of these
two regions are distinct. Elements located in the upstream region of Me7 are responsible for bundle sheath preferential expression, whereas elements located downstream of the Me7 stop codon are required for high-level expression in leaves.

RESULTS

Construction of Me7–gusA Chimeric Genes

To determine the role(s) of the regions flanking the F. bidentis Me7 gene in directing the high-level, bundle sheath–specific expression of Me7, we made a series of constructs containing the gusA reporter gene encoding β-glucuronidase (GUS; Jefferson, 1987). Two constructs contained 2121 bp of DNA upstream of the Me7 translation start codon plus exon 1 (30 bp), intron 1 (186 bp), and 24 bp of exon 2 fused in-frame to the 5' end of the gusA gene from pKIWI101 (Janssen and Gardner, 1989). In the first construct, termed ME20, the gusA gene was followed by the octopine synthase (ocs) 3' terminator, which was also derived from pKIWI101 (Figure 1A). In the second construct (ME29), the ocs 3' terminator was replaced by a 5.9-kb fragment containing the region immediately downstream of the Me7 translation stop codon (Figure 1B). Assuming the correct excision of intron 1 from Me7, both constructs were predicted to encode a chimeric protein containing the first 18 (of an estimated 60) amino acids of the NADP-ME chloroplast transit peptide followed by a single threonine and the entire GUS protein.

Because most plant genes are regulated primarily by sequences at their 5' ends, we also made a series of constructs containing progressive deletions of upstream sequences from Me7. As shown in Figure 1C, the three constructs included all of the Me7 5' untranslated region plus 311, 578, or 1023 bp of upstream DNA fused to gusA.

The 3' Region of Me7 Is Required for High-Level Expression in Leaves

Sections of leaves taken from greenhouse-grown F. bidentis plants transformed with ME20 or ME29 were incubated in a 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) solution to allow histochemical determination of the level and pattern of GUS expression conferred by these gene constructs. There were no differences in patterns or levels of staining between primary transformants and their progeny. Figure 2 shows a typical pattern of staining for ME20-transformed plants. Only very faint staining was observed in leaves, even after prolonged incubation. Because of this very low level of staining, it was not possible to determine histochemically the cell-type distribution of GUS expression in leaves of ME20-transformed plants. Similar results were obtained in plants transformed with ME25, ME26, and ME27 (Figure 1C). These constructs have shorter Me7 5' regions (data not shown). Thus, constructs with only 5' regions of the Me7 gene produced very faint or no detectable histochemical staining in leaves.

In contrast, intense blue staining was observed when leaves of ME29-transformed plants were incubated in X-gluc solution for relatively short times. Figure 3A shows a surface view of a typical leaf in which it was evident that the blue color was concentrated around the veins. To determine the cell-type distribution of GUS activity, leaves were sectioned after histochemical staining and examined at higher magnification by using both bright- and dark-field illumination. As shown in Figure 4A, the GUS reaction product was most concentrated in the bundle sheath cells of leaves of ME29-transformed plants. Bundle sheath cells are clearly visible as a tightly organized ring of cells around each vein. Thin sections examined under dark-field illumination provided higher resolution. Figures 4B and 4C also show that GUS was concentrated in bundle sheath cells. Some of the GUS product was observed in mesophyll cells, particularly those adjacent to bundle sheath cells. However, the level of GUS staining observed in mesophyll cells was always significantly lower than that seen in bundle sheath cells, unless X-gluc incubations were performed for extended periods of time. High levels of GUS were also detected in some parts of veins, as shown in Figure 4D, in which one vein was sectioned longitudinally.

Histochemical Determination of GUS Activity in Other Organs

Histochemical GUS staining also was observed in other regions of plants transformed with both ME20 and ME29. As shown in Figures 2 and 3, high levels of GUS were seen in stem sections and in the meristematic regions of shoot apices. The intensity and pattern of this non-leaf-associated staining were similar in plants transformed with either construct. GUS staining in stems and shoot apices was also detected in plants transformed with the 5' deletion constructs ME25, ME26, and ME27 at levels similar to ME20 and ME29 (data not shown). The dark staining in stems was found in xylem parenchyma cells, in cells of the cambium, and in a layer of parenchyma cells surrounding the outer side of the phloem (Figure 3B). All of these cells contain significant numbers of chloroplasts. Other parenchyma cells containing fewer chloroplasts also showed GUS staining; however, GUS accumulation was lower in these cells. Figure 3C shows a longitudinal section of a shoot tip in which GUS staining is evident in lateral meristematic regions, similar to that seen in Figure 2. (Apical staining was also observed in ME29-transformed material, but it is not visible in this glancing section, which does not pass through the shoot apex.) In Figure 2, additional staining is visible below the apex, but this is due to staining of vascular parenchyma cells as described for stems.
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Figure 1. Chimeric Me1–gus Constructs Used in Plant Transformation.
(A) The ME20 construct with 2.36 kb of ME1 5' fused to gusA with an ocs 3' end. Exon 2 from ME1 was fused in-frame to the gusA start codon.
(B) The ME29 construct with the same 5' ME1 sequences as ME20 but with 5.9 kb of the ME1 3' end replacing the ocs 3' fragment. The ME1 3' fragment begins at the translation stop codon.
(C) Constructs containing deletions of ME1 5'. Transcriptional fusions were made that included the 5' untranslated region of ME1 and progressively shorter portions of ME1 upstream DNA.
(D) Constructs used to determine whether ME1 3' functions with a heterologous promoter. pBS218 contains 539 bp of the component 4 promoter of subterranean clover stunt virus (SC4-5') fused to the same gus–ocs 3' fragment as that used in ME20. In pBS237, the ocs 3' fragment was replaced by 0.9 kb of ME1 3' extending downstream from the translation stop codon.

Boxes indicate transcribed regions of Me1; exons are shown in black, and introns are diagonally striped. Thick lines indicate non-transcribed regions of Me1. Thin lines and open boxes indicate gusA, ocs, or SC sequences. Numbers indicate positions relative to the ME1 start of transcription.
Figure 2. Histochemical Localization of GUS Activity in ME20-Transformed *F. bidentis*.

(Left) Shoot apex. (Middle) Leaf section. (Right) Transverse section of a stem. All sections were taken from the same T₁ plant and were incubated overnight in X-gluc solution. SA, shoot apex; MV, midvein.

Figure 3. Histochemical Localization of GUS Activity in ME29-Transformed *F. bidentis*.

(A) Leaf section. Bar = 100 μm. (B) Transverse section of a stem. Bar = 100 μm. (C) Longitudinal section of a shoot apex. Bar = 1 mm. All sections were taken from the same T₁ plant and were incubated for 4 hr in X-gluc solution. V+B, vein plus bundle sheath; C, cambium; XP, xylem parenchyma; LM, lateral meristem.

The 5’ and 3’ Regions of Me1 Do Not Confer Significant Expression in the C₃ Plant Tobacco

To test whether the regulatory elements present in the 5’ and 3’ regions of Me1 were active in C₃ plants, ME20 and ME29 were used to transform tobacco. This species was chosen because to date, no C₃ *Flaveria* species has been successfully transformed. At present, transformation systems are available only for C₄ (*F. bidentis*; Chitty et al., 1994) and C₃-C₄ (*F. pubescens*; Chu et al., 1997) *Flaveria* species.

Leaf, stem, and flower sections from six ME20- and 19 ME29-transformed tobacco plants were analyzed histochemically. In 21 of the plants, no blue color development was observed after overnight incubation. However, in two ME20-transformed plants and one ME29-transformed plant, some blue was detected in stem and leaf sections, but only in small areas.
patches. In addition, in one ME29-transformed plant, some blue color was observed in stigmas (data not shown). Biochemical assays of the selectable marker neomycin phosphotransferase were used to verify that all of the tobacco plants tested for GUS activity were transformed.

The 3' Region of Me1 Functions as an Efficient Terminator with a Heterologous Promoter

Having demonstrated the role of the 3' region in Me1 expression, we asked how the 3' region would function in combination with a heterologous promoter. We selected the promoter of the component 4 gene of subterranean clover stunt virus, a multicomponent, single-stranded DNA virus (Boevink, 1995). This promoter has been shown to provide constitutive gusA gene expression at levels similar to the cauliflower mosaic virus 35S promoter in a number of dicotyledonous plants (B. Surin and P.M. Waterhouse, unpublished results). A chimeric construct was made in which a 539-bp fragment of virus component 4 (SC4) was fused upstream of gusA, which was followed by a 0.9-kb EcoRI fragment representing the region immediately downstream of the Me1 stop codon (Figure 1D). For comparison, we also made a second construct in which the 0.9-kb Me1 3' fragment was replaced by the ocs 3' end, as was done in ME20 (Figure 1D). When introduced into Nicotiana plumbaginifolia protoplasts, similar levels of transient GUS expression were obtained with both constructs (Table 4). Stable transformation experiments

Table 1. GUS Activities in Extracts of Leaves from ME20- and ME29-Transformed Plants

<table>
<thead>
<tr>
<th>Plant</th>
<th>Construct Used</th>
<th>Net GUS Activity (FU μg^-1 protein hr^-1) a</th>
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<tr>
<td>9A</td>
<td>ME20</td>
<td>27 b</td>
</tr>
<tr>
<td>9B</td>
<td>ME20</td>
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<tr>
<td>8B</td>
<td>ME29</td>
<td>1,950</td>
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a FU, fluorescence unit.

b Corrected for background evolution of 4-methylumbelliferone (~8.3 FU hr^-1).

Figure 4. Fine-Scale Histochemical Localization of GUS Activity in Leaf Sections of ME29-Transformed F. bidentis.

(A) Bright-field view showing the concentration of the blue GUS product in bundle sheath cells. (B) to (D) Dark-field views. Under dark-field illumination, the crystalline GUS product is visible as bright pink spots (Peleman et al., 1989). Sections were taken from the leaf shown in Figure 3A. M, mesophyll; B, bundle sheath. Bars in (A) to (D) = 100 μm.
### Table 2. Measured Marker Enzyme and GUS Activities for Mesophyll and Bundle Sheath Cell Fractions

<table>
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<tr>
<th>Plant</th>
<th>Construct Used</th>
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<th>PEPC</th>
<th>NADP-ME</th>
<th>GUS (FU/hr⁻¹)</th>
<th>Protein (µg/L)</th>
<th>Fraction Purity (%)</th>
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<td>97</td>
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</table>

a MC1 and MC2 represent first and second mesophyll cell fractions, respectively. BSC represents the bundle sheath cell fraction.
b PEPC values represent $\Delta A_{340} \times 10^{-3}$ min⁻¹.
c NADP-ME values represent $\Delta A_{340}$ min⁻¹.
d FU, fluorescence unit.
e BSC purity represents the average of values obtained using MC1 or MC2 to calculate the proportion of protein deriving from MC versus BSC in each fraction.

¹ GUS activities in these mesophyll fractions were too low to permit accurate determination of fraction purity.

are under way to determine whether Me1 3' sequences have an enhancing effect on heterologous promoters.

### DISCUSSION

We have demonstrated that expression of the *F. bidentis* Me1 gene, which encodes the isoform of NADP-ME utilized in C₄ photosynthesis, is regulated by elements both upstream and downstream of the coding region of the gene. These elements were shown to have distinct functions. The Me1 downstream region was required for high-level expression in leaves but did not affect cell-type specificity. In contrast, the upstream region of the gene conferred bundle sheath cell preferential expression, even in the absence of the downstream element(s).

These findings indicate that regulation of Me1 expression differs from that seen for other C₄ genes studied to date. In studies similar to ours, 5' sequences from Flaveria genes encoding the C₄ isoforms of PEPC and pyruvate orthophosphate dikinase (PPDK) have been shown to be sufficient to direct high-level, mesophyll cell-specific expression of GUS in transgenic *F. bidentis* (Stockhaus et al., 1997; E. Rosche, J.A. Chitty, P. Westhoff, and W.C. Taylor, submitted manuscript). Similar conclusions have been reached for the maize genes encoding PPDK and the chlorophyll a/b binding protein of photosystem II by using transient expression assays (Matsuoka and Numazawa, 1991; Bansal and Bogorad, 1993). In none of these studies was the 3' region of the gene in question tested for activity. Therefore, the possibility remains that downstream elements could also be involved in regulating mesophyll cell-specific gene expression. However, it seems unlikely that any downstream regulation of
these genes would be as quantitatively significant as that observed for Mel. It seems more likely that downstream elements do not play a major role in regulating the expression of mesophyll cell–specific genes. It will be interesting to determine whether other bundle sheath cell–specific genes also require sequences at their 3' ends for expression.

Downstream elements have been implicated in the bundle sheath cell–specific expression of the small subunit of ribulose bisphosphate carboxylase (RbcS) in maize. Viret et al. (1994) have shown, using transient expression assays, that the 3' region of the RbcS-m3 gene is required to shift the balance of GUS expression from slightly mesophyll cell preferential to bundle sheath cell preferential. However, in contrast to the overall increase of leaf GUS activity conferred by the Mel 3' region, Viret et al. (1994) found that the absolute level of GUS expression in bundle sheath cells was only slightly affected by the inclusion of the 3' sequences. Their results indicate that the 3' region of the RbcS-m3 gene acts to suppress expression in mesophyll cells and therefore changes the proportion of expression in bundle sheath versus mesophyll cells.

Sequences at the 3' and have been shown to contribute to the regulation of a number of other plant genes, both qualitatively and quantitatively (Thornburg et al., 1987; Dean et al., 1989; Dietrich et al., 1992; Larkin et al., 1993; Fu et al., 1995a, 1995b). In several of these examples, the 3' sequences are required for correct spatial patterning of gene expression (Dietrich et al., 1992; Larkin et al., 1993; Fu et al., 1995a), and in one case, 3' sequences are required for wound-inducible expression (Thornburg et al., 1987). These results are in contrast with our finding that the Mel 3' fragment appears to act primarily by increasing the level of GUS expression and does not alter its cell specificity. Quantitative regulation of two petunia RbcS genes is accomplished by sequences at both the 5' and 3' ends (Dean et al., 1989).

The precise mode of action of the downstream region of Mel remains to be elucidated. The gusA-ocs 3' fragment combination results in high-level GUS activity in Flaveria leaves when driven by 5' sequences from the F. triervia Pdk gene, which encodes PPDK (E. Rosche, J.A. Chitty, P. Westhoff, and W.C. Taylor, submitted manuscript), as well as by the cauliflower mosaic virus 35S promoter (Chitty et al., 1994), indicating that the ocs 3' end functions as an efficient terminator and 3' untranslated region in Flaveria. Additional experiments are under way to determine whether the Mel 3' fragment affects transcription, mRNA turnover, or translation.

Another possible explanation for low GUS expression seen in leaves of ME20-transformed plants is that a negative regulatory element is present in the 5' region of Mel, and relief from the negative effects of this element requires downstream Mel sequences. However, analysis of the 5' deletion
Table 4. Transient GUS Expression Driven by a Heterologous Promoter with the Me7 3’ Fragment in N. plumbaginifolia Protoplasts

<table>
<thead>
<tr>
<th>Construct</th>
<th>GUS Activity (pmol mg⁻¹ protein min⁻¹)²</th>
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</thead>
<tbody>
<tr>
<td>pBS218 (ocs 3')</td>
<td>75</td>
</tr>
<tr>
<td>pBS237 (Me7 3')</td>
<td>117</td>
</tr>
</tbody>
</table>

² Each value represents the average of duplicate transient expression assays.

constructs ME25, ME26, and ME27 argue against the possibility of such a negative regulatory element >0.39 kb upstream of the Me7 start codon or within the first exon or first intron.

The 5’ region of Me7 was found to be sufficient to direct bundle sheath cell-preferential expression of GUS with either the Me7 3’ or ocs 3’ fragment. It is conceivable that this expression pattern could be a reflection of some nonspecific reduction of transgene expression in mesophyll cells relative to bundle sheath cells. However, F. bidentis plants transformed with the construct 35S-gusA-ocs 3’ show high levels of GUS activity in both cell types (cf. Figure 3A with Figure 2 in Chitty et al. [1994]), and F. bidentis plants transformed with PpcAl-gusA (which utilizes the promoter from the F. trinervia gene encoding the C4 isoform of PEPC) have been shown to drive detectable expression in leaves of transgenic tobacco (Matsuoka and Sanada, 1991). These three promoters are also active in transgenic rice, leading to high-level, mesophyll cell-specific expression (Matsuoka et al., 1993, 1994). Taken together, these results indicate that leaf-specific expression of Me7 may rely on C4-specific positive regulatory elements, whereas expression of the other genes mentioned above uses factors that are also present in C3 plants. One simple possibility is that the apparent downstream enhancer-like element present in Me7 interacts with C4-specific trans-acting factors, leading to high-level expression in leaves. The absence of such factors in tobacco might lead to the observed lack of GUS activity in this C3 plant.

GUS expression in stems and apices was controlled by the Me7 upstream region alone. High-level GUS activity in stems was found in the cambium and in parenchyma cells associated with phloem and xylem. It may be significant that these are the cells with the greatest number of chloroplasts. Although these cells are potentially active in photosynthesis, the absence of any C4 cellular differentiation suggests that the C3 pathway probably operates in stems. Therefore, it is curious that the promoter of a C4 gene is highly active in these cells. The F. trinervia Pdk promoter also directs a similar pattern of GUS expression in stems (E. Rosche, J. Chitty, P. Westhoff, and W.C. Taylor, submitted manuscript).

It is possible that GUS expression in stems and apices is not a true indication of the expression pattern of the Me7 and Pdk genes but is due to the absence in the constructs of cis-acting sequences responsible for suppressing nonleaf expression. Conversely, Berry and colleagues have demonstrated that several C4 genes are expressed in meristems and leaf primordia in amaranth (Ramsperger et al., 1996), indicating that at least part of the non-leaf-associated GUS activity observed in F. bidentis transformants may reflect actual expression of Me7.

The work presented here provides a range of interesting avenues for additional experiments. Of primary interest to us is finer scale mapping of both the 5’ and 3’ regions of Me7 to more precisely determine the location of the cis regulatory elements involved in high-level bundle sheath cell-specific expression. Experiments in which smaller fragments of both ends of Me7 are linked to GUS are in progress. In addition, we are interested in characterizing the nature of the downstream putative enhancer-like element. To this end, the Me7 3’ region is being analyzed in different positions relative to gusA and in conjunction with other promoters. Finally, we are interested in determining the mechanism by which cell-specific gene expression is determined. Some obvious possibilities include suppression of expression in mesophyll cells, the enhancement of expression in bundle sheath cells,
or a combination of these two. It seems likely, based on studies of 35S gene expression in other species (Sheen, 1990; Matsuoka and Numazawa, 1991; Bansal et al., 1992; Bansal and Bogorad, 1993; Wang et al., 1993b; Viret et al., 1994; Purcell et al., 1995; Ramsperger et al., 1996), that cell-specific expression involves a complex interplay between a number of different factors, including developmental stage, metabolic status, environmental signals, and cell type.

**METHODS**

**Construction of Chimeric Genes for Transformation**

For construction of ME20, a 3.5-kb BamHI-Xhol fragment of pKIWI101 (Janssen and Gardner, 1989) containing the β-glucuronidase gusA gene and octopine synthase (ocs) 3′ terminator was excised and ligated into BamHI-Sall-cut pUC18. This construct was then cut with BamHI plus Sall, and a 2.2-kb BamHI-Xhol fragment from the 5′ region of the Flaviera bidentis Mel gene (Marshall et al., 1996) was ligated in-frame to the 5′ end of the gusA gene. The resulting plasmid was cut with HindIII and ligated into the binary vector pGA470 (An et al., 1985), which was also cut with HindIII.

For construction of ME29, the 2.2-kb 5′ BamHI-Xhol fragment of Mel used in the construction of ME20 was ligated to the 5′ end of a 1.9-kb Sall-EcoRI fragment of pKIWI101 (Janssen and Gardner, 1989) containing the gusA gene. The chimeric Mel-gusA insert from this construct was excised with HindIII plus EcoRI and ligated to the 5′ end of a 5-kb EcoRI-SalI fragment from the 3′ end of Mel (beginning ~0.9 kb downstream of the Mel stop codon). The 0.9-kb Mel fragment from the stop codon to the 5′ end of the 5-kb EcoRI-SalI fragment was synthesized by polymerase chain reaction (PCR), using a 5′ primer containing a synthetic EcoRI linker, 5′-CCGAATTCTGTTTAGCCGGGAAAGGACAGC-3′. This primer was designed from the sequence immediately downstream of the F. bidentis Mel stop codon (Marshall et al., 1996). A 4.4-kb EcoRI subclone of ME7 (Marshall et al., 1996) containing the region of interest was used as a template, with the above-mentioned Mel-specific primer and the M13 – 21 primer. The resulting PCR product was cut with EcoRI and ligated into the EcoRI site separating the gusA gene and the 5-kb 3′ fragment from Mel. This ligation resulted in the reconstruction of the 3′ region of Mel from the stop codon extending 5.9 kb downstream. This construct was linearized with KpnI and ligated into KpnI-cut pGA482 (An, 1987).

The three 5′ deletion constructs were made by first generating exonuclease III deletions of the BamHI-XhoI Mel 5′ fragment. The deletion constructs were then amplified by PCR using a 5′ primer from pUC18 containing a synthetic HindIII site (CCAGTGTTAAAAACGACGGGCGAAG) and a 3′ primer that hybridizes immediately upstream of the start codon of Mel and containing an artificial PstI site (CCCTGCGGAGGGACAGAAGTGGAATGG). The PCR products were cut with HindIII plus PstI and cloned upstream of the gusA-ocs 3′ sequences from pKIWI101. The resulting plasmid was linearized with HindIII and cloned into pGA470.

The SC4 promoter of subterranean clover stunt virus was cloned by PCR amplification of a 539-bp region upstream of the component 4 coding region, using primers to introduce BamHI and Ncol restriction sites (Boevink, 1995). The BamHI-Ncol fragment was blunt ended with the Klenow fragment of DNA polymerase I and then ligated to the gusA-ocs 3′ sequences from pKIWI101 at the filled-in SalI site to make construct pBS218. In a second construct, pBS237, the ocs 3′ end was replaced by the 0.9-kb Mel 3′ EcoRI PCR fragment extending downstream from the translation stop codon.

**Plant Transformation**

Chimeric gene constructs in binary vectors were introduced into Agrobacterium tumefaciens and used to transform F. bidentis hypocotyls, as described by Chitty et al. (1994). All transgenic F. bidentis plants analyzed were progeny of self-pollinated primary transformants. Tobacco was transformed using standard procedures, and transformation was verified by assaying the selectable marker neomycin phosphotransferase.

**Histochemical Localization of GUS Activity**

Plant material was immersed in 50 mM sodium-phosphate buffer, pH 7.0, and cut into 1- to 2-mm sections. The sections were then incubated in 50 mM sodium-phosphate, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) at 37°C. For fine-scale analysis of GUS expression, histochemically stained material was fixed in 3% glutaraldehyde and embedded in LR White resin (London Resin Company, Basingstoke, UK), cut into 1- to 5-μm thick sections, and observed using dark-field illumination.

**Cell Separation Experiments and Enzyme Assays**

Preparations of mesophyll and bundle sheath cells were made using a modification of the method of Meister et al. (1996). Young leaves from greenhouse-grown progeny of selfed F. bidentis primary transformants were deribbed and chopped with a razor blade. Chopped leaves were suspended in 70 mL of buffer A (0.3 M sorbitol, 25 mM Pipes, pH 7.4, 10 mM DTT, and 1 mM MgCl2) and homogenized for 5 sec at low speed (setting 2) in a Sorvall Omnimixer (Du Pont, Newtown, CT). An aliquot representing unfraccionated leaves was removed and homogenized in a tissue homogenizer (Glasp-Col, Terre Haute, IN). A second aliquot, representing mesophyll cells, was removed and filtered through a 20-μm nylon mesh, and the filtrate was homogenized in a Dounce tissue homogenizer. The remaining unfiltred material was homogenized for an additional 5 sec at low speed, and a second mesophyll cell sample was removed, filtered, and homogenized as described above. Finally, the remainder was homogenized for 40 sec at full speed in the Omnimixer and filtered through a 20-μm nylon mesh. The material retained on the mesh (representing bundle sheath strands) was washed with 20 mL of buffer A, then suspended in 20 mL of buffer B (50 mM Pipes, pH 7.0, 10 mM MgCl2, 0.5 mM EDTA, 1% PVP, 5 mM DTT, 2 mM phenylmethylsulfonyl fluoride, and 2 mM d-amino-n-caproic acid), and homogenized in a Dounce tissue homogenizer. Aliquots of all fractions were removed for protein determination using the Bio-Rad protein assay reagent with BSA as standard, and the remainder of each fraction was adjusted to 0.1% (w/v) BSA and frozen in liquid N2. Samples were stored at ~80°C until assayed.
Calculation of Levels of GUS Expression in Mesophyll and Bundle Sheath Fractions

Net GUS activity present in mesophyll and bundle sheath cell fractions was calculated using an iterative process with the following assumptions:

(1) For any fraction, \( \text{GUST} = \text{GUS}_{\text{MC}} + \text{GUS}_{\text{BSC}} \).
(2) \( \text{GUS}_{\text{MC}}/\text{PEPC} \) is constant, and
(3) \( \text{GUS}_{\text{BSC}}/\text{ME} \) is constant.

where \( \text{GUST} \) is the measured GUS activity for the fraction, \( \text{GUS}_{\text{MC}} \) is the portion of \( \text{GUST} \) derived from mesophyll cells, \( \text{GUS}_{\text{BSC}} \) is the portion of \( \text{GUST} \) derived from bundle sheath cells, PEPC is the measured PEPC activity, and ME is the measured NADP-ME activity. Rates of activity were used for all enzymes. As an initial approximation, it was assumed that for mesophyll fractions, \( \text{GUST} = \text{GUS}_{\text{MC}} \).

Using this approximation and the measured PEPC activities for each fraction, a maximum value for \( \text{GUS}_{\text{MC}} \) present in the mesophyll fraction: \( \text{GUS}_{\text{MC}}/\text{Protein}_{\text{MC}} \) for mesophyll fractions and \( \text{GUS}_{\text{BSC}}/\text{Protein}_{\text{BSC}} \) for bundle sheath cell fractions. Purity of fractions was calculated as

\[
\text{Purity}_{\text{MC}} = \frac{\text{GUS}_{\text{MC}}/\text{Protein}_{\text{MC}}}{\text{GUS}_{\text{MC}}/\text{Protein}_{\text{MC}}} \quad \text{for mesophyll fractions}\]
\[
\text{Purity}_{\text{BSC}} = \frac{\text{GUS}_{\text{BSC}}/\text{Protein}_{\text{BSC}}}{\text{GUS}_{\text{BSC}}/\text{Protein}_{\text{BSC}}} \quad \text{for bundle sheath cell fractions.}
\]

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REFERENCES


Expression of the C4 Me1 Gene from Flaveria bidentis Requires an Interaction between 5[prime] and 3[prime] Sequences.
J. S. Marshall, J. D. Stubbs, J. A. Chitty, B. Surin and W. C. Taylor

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