The Gene for the P-Subunit of Glycine Decarboxylase from the C₄ Species Flaveria trinervia: Analysis of Transcriptional Control in Transgenic Flaveria bidentis (C₄) and Arabidopsis (C₃)

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Expression of a promoter-reporter gene fusion in transgenic plants of the transformable C₄ species Flaveria trinervia and vascular bundles. Interestingly, we found that the 1,571 bp of the GLDPA promoter in all photosynthetic cells, whereas in leaves of C₃-C₄ intermediate and C₄ species its occurrence is restricted to bundle-sheath cells. The specific expression of GLDP in bundle-sheath cells might have constituted a biochemical starting point for the evolution of C₄ photosynthesis. To understand the molecular mechanisms responsible for restricting GLDP expression to bundle-sheath cells, we performed a functional analysis of the GLDPA promoter from the C₄ species Flaveria trinervia. Expression of a promoter-reporter gene fusion in transgenic plants of the transformable C₄ species Flaveria bidentis (C₄) showed that 1,571 bp of the GLDPA promoter contain all the necessary information for the specific expression in bundle-sheath cells and vascular bundles. Interestingly, we found that the GLDPA promoter of F. trinervia exhibits a C₄-like spatial activity also in the C₃ plant Arabidopsis (Arabidopsis thaliana), indicating that a mechanism for bundle-sheath-specific expression is also present in this C₃ species. Using transgenic Arabidopsis, promoter deletion studies identified two regions in the GLDPA promoter, one conferring repression of gene expression in mesophyll cells and one functioning as a general transcriptional enhancer. Subsequent analyses in transgenic F. bidentis confirmed that these two segments fulfill the same function also in the C₄ context.

Net photosynthetic CO₂ assimilation rates in C₃ plants are reduced by photorespiration, a process that results from the oxygenase activity of Rubisco. C₄ plants usually show no apparent photorespiration, and this is achieved by splitting the photosynthetic reactions between two morphologically and biochemically distinct cell types, the mesophyll and the bundle-sheath cells. Initial CO₂ fixation in C₄ plants occurs exclusively in the mesophyll cells and is performed by the enzyme phosphoenolpyruvate carboxylase (PEPC) to form a C₄ acid, oxaloacetate. Depending on the C₄ subtype, oxaloacetate is converted to either malate or Asp, which subsequently move to the bundle-sheath and become decarboxylated, resulting in significant elevation of the CO₂ concentration in these cells. Final refixation of CO₂ is achieved by Rubisco, which in C₄ plants is only present in bundle-sheath cells. The enrichment of CO₂ in the vicinity of Rubisco effectively inhibits the enzyme’s oxygenase activity (Hatch, 1987).

In C₄ plants, the CO₂ assimilatory enzymes are compartmentalized into either mesophyll or bundle-sheath cells, and this is governed by differential gene expression. It has been shown that mesophyll-specific expression of C₄ cycle genes is mainly regulated at the transcriptional level (Schaffner and Sheen, 1992; Stockhaus et al., 1994; Rosche et al., 1998; Sheen, 1999), whereas bundle-sheath-specific expression is controlled at both transcriptional and posttranscriptional levels (Long and Berry, 1996; Marshall et al., 1997; Rosche et al., 1998; Stockhaus et al., 1994; Sheen, 1999; Patel et al., 2006).

There are indications that photorespiration also exists in C₄ plants, albeit at a much lower level than in C₃ plants (Osmond and Harris, 1971; Furbank and Badger, 1983; de Veau and Burriss, 1989). This is likely due to the fact that photorespiration in C₄ species is strictly confined to the bundle-sheath cells in the leaves, while in
C₄ plants photorespiration occurs in all photosynthetically active mesophyll cells (Ohnishi and Kanai, 1983). The mitochondrial multienzyme complex Gly dehydrogenase (GDC) plays a key role in the photosynthetic pathway. GDC is composed of four different subunits (P, H, T, and L) and catalyzes, in cooperation with Ser hydroxymethyltransferase, the oxidative decarboxylation of Gly that originates from the breakdown of photorespiratory phosphoglycolate. In the course of these reactions, two molecules of Gly are down-regulated in all photosynthetic cells of C₃ plant leaves, but strictly confined to the bundle-sheath cells in C₄ species (Ohnishi and Kanai, 1983). None of the GDC subunits has been detected in the mesophyll cells of C₄ plants (Morgan et al., 1993).

Plant species possessing a C₃-C₄ intermediate type of photosynthesis are of special interest for studying the evolution of C₄-characteristic traits. Some C₃-C₄ plants are to some extent able to fix CO₂ into malate and Asp (Monson et al., 1986), but most of these species do not have a C₄ metabolism. They can be characterized as "intermediate," for example, by their CO₂ compensation points, which are lower than those of C₃ plants but higher than those of C₄ plants (Edwards and Ku, 1987; Rawsthorne, 1992). As in C₃ plants, functional GDC occurs only in the bundle-sheath cells of the leaves of C₃-C₄ intermediate plants, as it was first demonstrated for Moricandia arvensis (Rawsthorne et al., 1988a, 1988b). Later on, it was discovered that the loss of GDC activity in the mesophyll is due to a lack of the P-subunit (GLDP). Because of the absence of GDC activity in mesophyll cells of M. arvensis leaves, photorespiratory Gly moves to the bundle-sheath cells to be processed by GDC. The bundle-sheath cells of M. arvensis contain a large number of mitochondria that are arranged at the centripetal cell wall adjacent to the vascular tissue, whereas the chloroplasts are located at the cell periphery. This special distribution of organelles and the restriction of Gly oxidation to the bundle-sheath compartment result in an efficient recapture of released photosynthetic CO₂, thereby lowering the CO₂ compensation point when compared to a typical C₃ plant (Rawsthorne et al., 1998). C₃-C₄ intermediate species are thought to represent a stage in the evolutionary transition from C₃ to C₄ photosynthesis (Edwards and Ku, 1987). It was therefore tempting to speculate that the confinement of GDC to the bundle-sheath cells has been one of the biochemical starting points for the evolution of C₄ plants (Morgan et al., 1993; Bauwe and Kolukisaoglu, 2003; Sage, 2004). However, the possible effects of this relocation for C₃ evolution are discussed controversially (Edwards et al., 2001). The loss of the P-subunit seems to be the initial step to inactivate GDC in the mesophyll, and the absence of all GDC subunits in the leaf mesophyll of other C₃-C₄ intermediate species suggests that they have developed further toward C₄ photosynthesis than M. arvensis (Morgan et al., 1993).

A well-established experimental system for investigating the evolution of C₄-characteristic traits is the genus Flaveria of the Asteraceae (Powell, 1978). This small genus comprising 23 known species includes both C₃ and C₄ species, but also a large number of C₃-C₄ intermediate species (Edwards and Ku, 1987). In this study we examined the promoter of the gene encoding GLDP from the C₄ species Flaveria trinervia to gain insight into the molecular basis of bundle-sheath-specific gene expression. Two genes encoding GLDP have been identified in F. trinervia, GLDPA and the pseudogene GLDPB (Cossu, 1997). The GLDPA promoter was fused to a GUS reporter gene and promoter activity was analyzed in transgenic Flaveria bidentis (C₄) and Arabidopsis (Arabidopsis thaliana; C₃). Similar expression patterns were observed in these two species, which allowed the use of Arabidopsis as a heterologous system for testing a series of promoter deletions to identify C₄-characteristic regulatory elements within the GLDPA promoter. These analyses resulted in the identification of regions within the GLDPA promoter that contribute mainly to the regulation of expression quantity or to the spatial expression pattern of the GLDPA gene, respectively.

RESULTS

In Situ RNA Hybridization

Immunolabeling studies have shown that, in C₄ plants, GLDP accumulates exclusively in bundle-sheath cells of leaves (Hylton et al., 1988; Morgan et al., 1993; Yoshimura et al., 2004). To examine whether this C₄-characteristic localization of the P-protein is due to specific accumulation of GLDPA mRNA in this compartment, we analyzed the expression pattern of the GLDPA gene in leaves of the C₄ species F. trinervia by in situ hybridization. As a probe we used a 2.4-kb fragment of the GLDPA cDNA from F. trinervia, and control hybridizations were performed with the corresponding sense probe.

In leaves of F. trinervia, transcripts of the GLDPA gene could only be detected in bundle-sheath and not in mesophyll cells (Fig. 1A). The GLDPA mRNA accumulated near the centripetal cell walls of the bundle-sheath cells due to the concentration of cytoplasm in this region. The confinement of the P-protein to the bundle-sheath cells therefore is controlled by the specific accumulation of GLDPA mRNA in this compartment. The same result was obtained by in situ hybridization of the GLDPA probe to leaf cross sections of the C₄ species F. bidentis (Fig. 1C).

Expression of a GUS Reporter Gene under the Control of the GLDPA Promoter from F. trinervia in Transgenic F. bidentis

The in situ RNA hybridization analysis showed that the occurrence of GLDPA transcripts is restricted to the
bundle-sheath cells in *F. trinervia* and *F. bidentis* (Fig. 1). To test whether the available 1,571 bp of the 5′ flanking region of the *GLDPA* gene (including the 5′ untranslated region upstream of the AUG start codon) harbor all the necessary information for this bundle-sheath-specific expression pattern, we fused this region to GUS reporter gene (construct GLDPA-Ft; Fig. 2A) and examined its expression behavior in transgenic *F. bidentis* plants. The C4 species *F. bidentis* is a close relative to *F. trinervia*, but unlike *F. trinervia* it is suitable for transformation by Agrobacterium tumefaciens-mediated gene transfer (Chitty et al., 1994).

Histochemical analysis of the expression of the *GLDPA*-Ft promoter-GUS construct revealed an intense blue staining in the bundle-sheath cells but not in the mesophyll cells (Fig. 2B). GUS activity could also be observed in most vascular bundles, with the degree of GUS expression varying with the size of the veins. The small minor veins usually exhibited a strong blue staining, while higher-order vascular bundles showed only moderate GUS activity. Additional weak *GLDPA* promoter activity was also detected in the guard cells of the stomatal complexes (Fig. 2C).

The Expression Pattern of the *GLDPA*-GUS Construct in Arabidopsis Is Similar to That in *F. bidentis*

Bundle-sheath cells are not a unique feature of C4 plants. They are also present in many C3 plants, but compared to the situation in C4 species, these cells exhibit fewer chloroplasts and mitochondria (Kinsman and Pyke, 1998; Leegood, 2002). To examine whether the *GLDPA* promoter of *F. trinervia* shows a cell-specific activity in a C3 background, we introduced the *GLDPA*-GUS construct into Arabidopsis.

The histochemical analysis revealed GUS expression in the vascular tissue and in the surrounding bundle-sheath cells (Fig. 3, C and D). Notably, very similar to the expression pattern in *F. bidentis*, no GUS activity could be detected in the mesophyll cells of transgenic Arabidopsis plants. The quantification of GUS levels showed that the median activity of the reporter protein was comparable in Arabidopsis and *F. bidentis* leaves (Figs. 2D and 3B).

To verify the results obtained from the histochemical GUS analysis, the *GLDPA* promoter was also fused to the GFP reporter gene *mgfp5-ER* (Siemering et al., 1996; Haseloff et al., 1997) and a histone 2B/yellow fluorescent protein (H2B:YFP) fusion gene (Boisnard-Lorig et al., 2001), which are targeted to the endoplasmic reticulum and the nucleus, respectively. These reporter proteins allow a nondestructive analysis by fluorescence or confocal laser microscopy, thereby avoiding any potential diffusion of the reporter protein that might occur during a histochemical staining procedure. In both cases, the reporter proteins could be detected in bundle-sheath cells and vascular bundles, but not in mesophyll cells (Fig. 3, E–L; Supplemental Videos S1 and S2).

Deletion Analysis of the *GLDPA* Promoter

The “C4-like” expression pattern of *GLDPA*-GUS in transgenic Arabidopsis provided the opportunity to functionally dissect the *GLDPA* promoter by using this C3 model organism as an experimental system. To identify cis-regulatory determinants that are responsible for the activity of the *GLDPA* promoter in bundle-sheath cells and the vascular bundle, we produced a set of 5′ deletions and analyzed their expression specificity and level in transgenic Arabidopsis. The *GLDPA* promoter was subdivided into seven fragments that are referred to as region 1 to region 7 in the following (Fig. 4A).

The removal of a 182-bp segment (region 1) from the 5′ end of the *GLDPA* promoter, resulting in construct *GLDPA*-Ft-Δ1 (Fig. 4A), did not alter the spatial expression pattern of the GUS reporter gene when com-
plants investigated (N) and the median values of GUS activity (M) are moles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

pared to the original full-length promoter, i.e. this promoter variant was still capable of directing GUS expression specifically in the vascular bundles and bundle-sheath cells of Arabidopsis (Fig. 4B). This indicates the absence of cis-regulatory elements conferring cell specificity in this most distal part of the GLDPA promoter. However, quantitative GUS assays revealed an approximately 20-fold lower GUS activity in leaves of transgenic plants expressing GLDPA-Ft-D1 relative to leaves of GLDPA-Ft plants (Fig. 4D). The transcriptional activity of the promoter further decreased when deleting another 251 bp (region 2) from the 5′ end of GLDPA-Ft-D1 (construct GLDPA-Ft-D2; Fig. 4A). GUS activity in leaves of Arabidopsis plants harboring the GLDPA-Ft-D2 transgene was about 60 times lower than that of GLDPA-Ft (Fig. 4D), but the spatial expression pattern in the Arabidopsis leaf was still identical to that of the full-length promoter construct (Fig. 4C). Region 3 of the GLDPA promoter included the sequences between −1,138 and −927. Deletion of this promoter fragment in construct GLDPA-Ft-D3 decreased the GUS activity below the sensitivity limit of the histochemical GUS assay. Hence, no GUS expression could be detected in leaf cross sections of plants carrying this promoter construct. Similar low GUS activities (using the quantitative GUS assay) were also observed for the constructs GLDPA-Ft-D4 and GLDPA-Ft-D5 (Fig. 4, A and D), while practically no GUS activity was observed for GLDPA-Ft-D6 (Fig. 4, A and D).

This deletion analysis clearly demonstrated the pronounced importance of regions 1, 2, and 3 for the transcriptional activity of the GLDPA promoter in the leaves of transgenic Arabidopsis plants. While truncation of regions 1 and 2 causes a dramatic decrease of transcriptional activity without affecting the spatial expression pattern, the additional deletion of region 3 results in a further reduction of promoter activity, which impeded further analysis of cell type-specific expression within the leaf.

Figure 2. Analysis of GLDPA-Ft promoter activity in transgenic F. bidentis. A, Structure of the chimeric GLDPA-Ft::GUS gene. B and C, Histochemical localization of GUS activity (blue staining) in leaf sections of transgenic F. bidentis transformed with GLDPA-Ft. The photograph (C) was taken from the leaf surface displaying GUS activity in the stomatal guard cells of F. bidentis leaves. Incubation times were 1 h. D, GUS activities in leaves of transgenic F. bidentis plants transformed with GLDPA-Ft. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The transformation of construct GLDPA-Ft-1-2-7 (Fig. 5A) into Arabidopsis caused a substantial level of GUS expression in mesophyll and bundle-sheath cells as well as in the vascular strands of the leaves (Fig. 5, B and C) and confirms that regions 1 and 2 contain transcriptional enhancers with no apparent cell type specificity within the leaf.

To test whether regions 1 and 2 of the GLDPA promoter function also in a heterologous promoter context, we fused this segment in front of the proximal promoter region of the ppcA gene of F. trinervia (Fig. 5A). The ppcA gene encodes the C₄ isozyme of PEPC (Hermans and Westhoff, 1992), and its complete 2,188-bp promoter directs high and mesophyll-specific GUS expression in transgenic F. bidentis (Stockhaus et al., 1997). In contrast, the activity of the 570-bp-long proximal ppcA promoter part (ppcA-PRᵥ) is extremely low and can hardly be visualized in histochemical GUS assays (Gowik et al., 2004). In plants in which the low activity permits a histological analysis, the ppcA-PRᵥ promoter fragment directs a uniform expression in all cells of the leaves of F. bidentis, including the vascular bundles (Akyildiz et al., 2007).

The fusion of regions 1 and 2 of the GLDPA promoter with the ppcA-PRᵥ promoter fragment resulted in strong GUS expression in leaves of Arabidopsis (Fig. 5B). The GUS reporter gene was active in both mesophyll and bundle-sheath cells as well as in the vascular bundles (Fig. 5D), and the expression profile of this chimeric promoter is thus indistinguishable

Regions 1 and 2 of the GLDPA Promoter Together Function as a General Enhancer of Transcription in the Arabidopsis Leaf

To investigate whether the GLDPA promoter fragment reaching from −1,571 to −1,139 (regions 1 and 2) was able to act as a transcriptional enhancer, we combined this segment of the promoter with region 7 of the GLDPA promoter (−298 to −1). Region 7 harbors a putative TATA box and the starting point of transcription, but as reported above this part of the promoter alone is not sufficient to drive GUS expression in the Arabidopsis leaf (Fig. 4D).

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from that of GLDPA-Ft-1-2-7. We conclude from these experiments that regions 1 and 2 of the GLDPA promoter constitute a general transcriptional enhancer module that, in combination with a basal promoter, stimulates the expression of a linked reporter gene in all types of interior leaf cells of Arabidopsis.

Region 3 of the GLDPA Promoter Acts as a Mesophyll-Specific Repressor of Gene Expression

The role of region 3 (−1,138 to −927) in regulating GLDPA promoter activity was investigated by introducing the relevant promoter fragment into construct GLDPA-Ft-1-2-7, resulting in the production of the chimeric promoter GLDPA-Ft-1-2-3-7 (Fig. 6A). The addition of region 3 to GLDPA-Ft-1-2-7 caused a significant change in the spatial expression pattern of the GUS reporter gene. While GLDPA-Ft-1-2-7 plants expressed the GUS reporter gene in mesophyll and bundle-sheath cells as well as in the vascular tissue (Fig. 5C), GUS activity of GLDPA-Ft-1-2-3-7 plants was strictly confined to the bundle-sheath cells and the vascular compartment (Fig. 6B). These observations indicate that region 3 of the GLDPA promoter from F. trinervia functions as a mesophyll-specific repressor of gene expression in the Arabidopsis leaf.

Analysis of GLDPA Promoter Regions 4, 5, and 6

We have shown that the GLDPA promoter fragment comprising base pairs −1,571 to −927 (regions 1–3) in combination with the most proximal promoter part (region 7) is sufficient to direct GUS expression in the bundle-sheath cells and vascular bundles of transgenic Arabidopsis plants. Nevertheless, additional cis-regulatory determinants that could be involved in the spatial regulation of transcriptional activity might also be present in promoter regions 4, 5, and 6. To investigate the occurrence of cis-regulatory elements within these promoter regions, it was necessary to raise the GUS expression levels of constructs GLDPA-Ft-Δ3 to GLDPA-Ft-Δ6 to a level that allowed a histochemical
analysis. Since regions 1 and 2 of the GLDPA promoter contain a general transcriptional enhancer with no apparent leaf cell specificity, we attached this GLDPA transcriptional enhancer module to the 5’ borders of the truncated promoters (Fig. 7A).

As expected, the transcriptional activity of these constructs was dramatically higher than that of their “enhancerless” counterparts and was therefore suitable for performing GUS stainings in situ (Figs. 4D and 7B). In construct GLDPA-Ft-1-2-4-5-6-7, only region 3 was removed from the original full-length GLDPA promoter. While about 50% of the transgenic lines displayed a uniform GUS expression in mesophyll and bundle-sheath cells and the vascular bundles (Fig. 7F), GUS expression in the other half of the plant lines was still restricted to the bundle-sheath cells and the vascular tissue (Fig. 7C). The same distribution of transgenic plants displaying either a uniform or restricted expression of the reporter gene was also observed for the promoter constructs GLDPA-Ft-1-2-5-6-7 and GLDPA-Ft-1-2-6-7 in which regions 4 and 5 were further deleted (Fig. 7, D, E, G, and H). In contrast, as already reported above, the additional deletion of region 6 in construct GLDPA-Ft-1-2-7 resulted in a uniform expression pattern in the leaf (Fig. 5C). These findings suggest that additional cis-regulatory elements conferring repression of gene expression in the mesophyll are located in region 6 of the GLDPA promoter. However, when compared to the highly effective repressor elements located in region 3, these additional elements in region 6 do not provide robust repression.

Analysis of Promoter Construct GLDPA-Ft-1-2-3-7 in Transgenic F. bidentis

A truncated promoter containing the transcription-enhancing regions 1 and 2, the mesophyll repressor region 3, and the basal expression segment 7 generated
were not essential for creating the C_4-characteristic i.e. the promoter regions 4, 5, and 6 (299 to 926) were not essential for creating the C_4-characteristic spatial expression pattern of a reporter gene. We now wished to examine whether this chimeric GLDPA-1-2-3-7 promoter is capable of providing this C_4 expression profile also in the C_3 background of F. bidentis. This chimeric promoter construct was therefore transformed into F. bidentis, and its expression was examined in the leaves of the transgenic plants (Fig. 8).

No differences between the spatial expression patterns of GLDPA-1-2-3-7 and the full-length promoter construct GLDPA-Ft were observed (compare Figs. 2 and 8). In both cases, GUS expression was found exclusively in the bundle-sheath cells and—with variable intensities—in the vascular strands. While GUS staining was strong in some minor veins, it was absent from other minor and all major vascular strands (Fig. 8, B and C). These results indicate that regions 1 to 3 in combination with the basal TATA box-containing segment 7 of the GLDPA promoter are sufficient to direct reporter gene expression in bundle-sheath cells and the vascular bundles of both the homologous C_4 species F. bidentis and the heterologous C_3 plant Arabidopsis.

**DISCUSSION**

The correct functioning of the C_4 photosynthetic cycle requires strict compartmentalization of C_4 enzymes in either mesophyll or bundle-sheath cells of the leaf. This cell type-specific accumulation of proteins is governed by differential gene expression (Sheen, 1999). To broaden our knowledge on the molecular basis of bundle-sheath-specific gene expression in C_4 plants, we have performed a functional analysis of the 5’ flanking sequences of the GLDPA gene from F. trinervia (C_4). The GLDPA gene encodes GLDP, which is specifically located in the bundle-sheath cells of C_4 species (Morgan et al., 1993). To determine whether the bundle-sheath-specific accumulation of the GLDP protein in the C_4 leaf is paralleled by the accumulation of the corresponding mRNA, we studied the occurrence of GLDPA transcripts within the leaves of F. trinervia and F. bidentis by in situ hybridizations. GLDPA RNA was exclusively found in the bundle-sheath cells of both C_4 species, indicating that the presence of this protein in bundle-sheath cells and its absence in mesophyll cells are caused by differential GLDPA mRNA accumulation.

We then investigated whether the available 1,571 bp of the 5’ flanking region of the GLDPA coding sequence harbor all the necessary information for this bundle-sheath-specific expression. Fusion of these sequences—including the 5’ untranslated segment of the GLDPA gene—to the GUS reporter gene resulted in reporter gene activity in the bundle-sheath but not in the mesophyll cells of transgenic F. bidentis plants. In addition, GUS activity could be detected in the vascular bundles. Here, GUS activity was clearly visible in minor veins but very low in major veins.

The expression of the reporter gene in the bundle-sheath cells and the absence of GUS activity in the mesophyll are consistent with the accumulation pattern of the GLDPA RNA. The additional activity of the GUS reporter gene in the vascular tissue, however, is in contrast to the lack of detectable GLDPA RNA in this tissue. There are two possible explanations that could account for this discrepancy in the patterns of RNA accumulation and reporter gene activity. First, there could be additional cis-regulatory sequences further upstream within the introns or even downstream of the GLDPA gene that might control GLDPA transcription in
Figure 6. Functional analysis of GLDPA promoter region 3 in transgenic Arabidopsis. A, Schematic structure of construct GLDPA-Ft-1-2-3-7. B, Histochemical localization of GUS activity in the leaf sections of a transgenic Arabidopsis plant transformed with GLDPA-Ft-1-2-3-7. Incubation time was 20 h. C, GUS activities in leaves of transgenic Arabidopsis plants transformed with promoter construct GLDPA-Ft-1-2-3-7. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferylone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

The exact physiological and biochemical functions of bundle-sheath cells in C3 species are poorly understood. They are involved in phloem loading and unloading (van Bel, 1993), and for tobacco (Nicotiana tabacum) it was shown that the bundle-sheath cells of stems and petioles exhibit high activities of enzymes characteristic of C4 photosynthesis, thus allowing the decarboxylation of four-carbon organic acids derived from the xylem and phloem (Hibberd and Quick, 2002). Additionally, a class of Arabidopsis mutants termed dov (differential development of vascular associated cells) demonstrates that differential chloroplast development occurs between bundle-sheath and mesophyll cells in the Arabidopsis leaf (Kinsman and Pyke, 1998).

These observations from tobacco and Arabidopsis provide some evidence that bundle-sheath cells in C4 plants are somehow predetermined to evolve C4-characteristic features. The special physiology of bundle-sheath cells in Arabidopsis and the fact that preexisting transcription factors in this C3 species are able to recognize heterologous C4-characteristic cis-regulatory elements in the correct fashion provide further evidence for the view that the evolution of C4 plants must have been relatively simple in genetic terms (Westhoff and Gowik, 2004). C4-like spatial activities of C4 promoters in transgenic C3 plants have also been reported for the C4 isoform of PEP carboxykinase of maize (Zea mays; Matsuoka et al., 1994; Nomura et al., 2000), the pyruvate orthophosphate dikinase gene of maize (Matsuoka et al., 1993), and the phosphoenolpyruvate carboxykinase of Zeysia japonica (Nomura et al., 2005). On the other hand, the C4-PEP promoter of F. trinervia loses mesophyll specificity when it is introduced in Arabidopsis (Akyildiz et al., 2007). Similarly, the NADP-dependent malic enzyme promoter from maize loses its bundle-sheath specificity in rice (Oryza sativa; Nomura et al., 2005). This shows that the functionality of C4-specific regulatory cis-elements in C3 plants cannot be generalized.

The C4-like expression pattern of the GLDPA-Ft promoter in Arabidopsis provided us with the possibility to dissect the functional organization of this promoter in Arabidopsis. A series of GLDPA promoter deletion and recombination constructs were analyzed, and two major functional modules were identified and localized, a non-cell-type-specific transcriptional enhancer and a segment that represses gene expression in mesophyll cells. The transcriptional enhancer is located within the outermost distal regions 1 and 2 of the GLDPA promoter comprising base pairs −1,571 to −1,139. The enhancer functioned in all interior leaf tissues of Arabidopsis, i.e. in mesophyll and bundle-sheath cells as well as in the vascular bundle. The transcription-enhancing activity of these regions was not restricted to the context of the GLDPA promoter but was also
functional when combined with the proximal part of the ppcA1 promoter of *F. trinervia*. The enhancer is thus not GLDPA gene specific but functions as a general enhancer module.

The quantitative analysis of promoter activities (Fig. 4D) indicates that region 1 has a higher potential for transcriptional enhancement than region 2. A search for known cis-regulatory elements (Prestridge, 1991; Higo et al., 1999) revealed the presence of a motif with similarity to the simian virus 40 enhancer core (GTGWWHG) at positions −1,455 to −1,448 in region 1. This motif is also present in a region of the GLDPA promoter of *Flaveria pringlei* that is associated with an increase in expression quantity (Bauwe et al., 1995).

Region 3 (−1,138 to −927) harbors cis-regulatory elements that confer cell specificity to the GLDPA promoter by repressing its activity in the mesophyll cells of the Arabidopsis leaf. A chimeric promoter consisting of the transcription-enhancing regions 1 and 2, region 3, and the proximal basal expression region 7 is also not active in the mesophyll cells of the *C₄* species *F. bidentis*. This indicates that region 3 can repress expression in mesophyll cells also in the *C₄* context, i.e. the mesophyll-repressing function of region 3 is conserved between the *C₃* and the *C₄* species. The lack of GLDPA expression in the mesophyll is thus caused by transcriptional regulation and not by posttranscriptional regulation as it was reported for the *FbRbcS1* gene of *F. bidentis*. *FbRbcS1* encodes the small subunit of Rubisco, and its bundle-sheath-specific expression is entirely established by selective *rbcS* transcript stabilization in the bundle-sheath cells (Patel et al., 2006).

Additional mesophyll-repressing cis-regulatory sequences are located in region 6 (−521 to −299). They can partially compensate for the lack of the mesophyll-repressing cis-regulatory sequences in region 3, when this segment is not present in the promoter construct. However, these cis-regulatory elements are not able to establish a robust repression of reporter gene activity in the mesophyll cells of Arabidopsis and appear to be of minor importance. This is documented by the cell type-specific expression of construct GLDPA-Ft-1-2-4-5-6-7 that consists of the transcription-enhancing regions 1 and 2, region 3, and the proximal basal promoter region 7. GLDPA-Ft-1-2-4-5-6-7 directs a *C₄*-characteristic expression profile in *F. bidentis*. This demonstrates that the cis-regulatory motives present in region 6 are not necessary for the repression of GLDPA expression in mesophyll cells of this *C₄* species. Moreover, we can infer from the expression profile of this truncated promoter that regions 4 and 5 are also not necessary to achieve a *C₄*-typical GUS expression pattern in both Arabidopsis and *F. bidentis*. Regarding the mechanism of mesophyll repression, no predictions can be made at this moment, since searching for known cis-regulatory elements in region 3 did not identify any robust candidate motifs.

The cis-regulatory determinants for the mesophyll-specific repression of GLDPA expression in the leaf have
D, GUS activities in leaves of transgenic *F. bidentis* each column. Values of GUS activity (M) are indicated at the top of transgenic plants investigated (N) and the median protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

Figure 8. Analysis of the promoter construct GLDPA-Ft-1-2-3-7 in transgenic *F. bidentis*. A, Schematic structure of construct GLDPA-Ft-1-2-3-7. B and C, Histochromial localization of GUS activity in leaf sections of transgenic *F. bidentis* plants transformed with GLDPA-Ft-1-2-3-7. Incubation times were 28 h. D, GUS activities in leaves of transgenic *F. bidentis* plants transformed with GLDPA-Ft-1-2-3-7. GUS activities are expressed in nanomoles of the reaction product 4-methylumbelliferone (MU) per milligram of protein per minute. The numbers of independent transgenic plants investigated (N) and the median values of GUS activity (M) are indicated at the top of each column.

not been determined yet, and no other gene system for bundle-sheath-specific expression has been investigated in such detail that its cis- and trans-regulatory elements are known. However, cis-regulatory elements for mesophyll-specific gene expression have recently been identified at the nucleotide level (Gowik et al., 2004; Akyildiz et al., 2007) for the C₄-PEPC gene and Russell (2001). Construct GLDPA is a gene of *Flaveria trinervia* from -1,571 to -1 (1 describes the first base of the translational start codon) was fused to the GUS cDNA in the binary plant transformation vector pBI121 (CLONTECH Laboratories). Different 5′-deleted fragments of the GLDPA promoter were generated by PCR amplification (Tables I and II). The primers added an XbaI restriction site at the 5′ border of the DNA fragments and an XmaI site at the 3′ end. Therefore, the deleted promoters could be inserted into XbaI/XmaI-cut pBI121, resulting in the formation of the constructs GLDPA-Ft-D1, GLDPA-Ft-D3, GLDPA-Ft-D4, GLDPA-Ft-D5, and GLDPA-Ft-D6. For the production of GLDPA-Ft,D2, plasmid GLDPA-Ft was digested with HindIII and Eco72I. The remaining plasmid was purified by gel electrophoresis and blunt ends were generated by treatment with the Klenow fragment of Escherichia coli DNA polymerase I. The vector was religated to form GLDPA-Ft-D2. The DNA fragment comprising regions 1 and 2 of the GLDPA-Ft promoter was generated by PCR amplification with primers GLDPA-5′-Xba and Eco72-R. XbaI sites were introduced at both ends of the PCR product, which allowed the insertion of the DNA fragment into XbaI-cut GLDPA-Ft-D3. GLDPA-Ft-D4, GLDPA-Ft-D5, GLDPA-Ft-D6, and ppcA-S-Ft (Stockhaus et al., 1994). The resulting plasmid constructs were named GLDPA-Ft-1-2-4-5-6-7, GLDPA-Ft-1-2-5-6-7, GLDPA-Ft-1-2-6-7, GLDPA-Ft-1-2-7, and GLDPA-Ft-1-2-ppcA-Pₜₜₜₜ. The cloning of construct GLDPA-Ft-1-2-3-7 involved PCR amplification of the promoter region between -1,571 and -927 using primers GLDPA-5′-Xba and gdc3-R. The PCR product was digested with XbaI and ligated with XbaI-cut GLDPA-Ft-D6. The correct orientation of the inserted DNA fragment in GLDPA-Ft-1-2-3-7 was shown by sequencing. To produce construct GLDPA-Ft::H2B-YFP, the H2B-YFP gene fusion was excised from plasmid pBI121-35S::H2B-YFP (Boisnard-Lorig et al., 2001) with BamHI and ScaI. The GLDPA promoter of *F. trinervia* was amplified from plasmid GLDPA-Ft (Cossu, 1997) with primers GLDPA5′-HindIII and GLDPA3′-BamHI. A pBI121 backbone was generated by removing the 35S::H2B-YFP insert from plasmid pBI121-35S::H2B-YFP via incubation with HindIII and SacI, and a triple ligation between pBI121 (HindIII/SacI), the GLDPA promoter (HindIII/BamHI), and the H2B-YFP fragment (BamHI/SacI) finally resulted in the formation of construct GLDPA-Ft::H2B-YFP.

The cloning of construct GLDPA-Ft::mGFP5-ER was achieved by PCR amplification of the mGFP5-ER gene (Haseloff et al., 1997) from genomic DNA of the Arabidopsis (*Arabidopsis thaliana*) enhancer trap line E2443 (generated by Scott Poethig, http://www.arabidopsis.org/abrc/poethig.jsp) with primes 5′-mGFP5ER-BamHI and 3′-mGFP5ER-SacI. The PCR product was then cloned into BamHI/SacI-cut GLDPA-Ft::H2B-YFP to yield GLDPA-Ft::mGFP5-ER.

MATERIALS AND METHODS

Construction of Chimeric Promoters

DNA manipulations and cloning were carried out according to Sambrook and Russell (2001). Construct GLDPA-Ft (Cossu, 1997) served as the basis for the series of GLDPA promoter deletions. In GLDPA-Ft, the 5′ upstream region of the GLDPA gene of *Flaveria trinervia* from -1,571 to -1 (1 describes the first base of the translational start codon) was fused to the GUS cDNA in the binary plant transformation vector pBI121 (CLONTECH Laboratories). Different 5′-deleted fragments of the GLDPA promoter were generated by PCR amplification (Tables I and II). The primers added an XbaI restriction site at the 5′ border of the DNA fragments and an XmaI site at the 3′ end. Therefore, the deleted promoters could be inserted into XbaI/XmaI-cut pBI121, resulting in the formation of the constructs GLDPA-Ft-D1, GLDPA-Ft-D3, GLDPA-Ft-D4, GLDPA-Ft-D5, and GLDPA-Ft-D6. For the production of GLDPA-Ft-D2, plasmid GLDPA-Ft was digested with HindIII and Eco72I. The remaining plasmid was purified by gel electrophoresis and blunt ends were generated by treatment with the Klenow fragment of Escherichia coli DNA polymerase I. The vector was religated to form GLDPA-Ft-D2. The DNA fragment comprising regions 1 and 2 of the GLDPA-Ft promoter was generated by PCR amplification with primers GLDPA-5′-Xba and Eco72-R. XbaI sites were introduced at both ends of the PCR product, which allowed the insertion of the DNA fragment into XbaI-cut GLDPA-Ft-D3. GLDPA-Ft-D4, GLDPA-Ft-D5, GLDPA-Ft-D6, and ppcA-S-Ft (Stockhaus et al., 1994). The resulting plasmid constructs were named GLDPA-Ft-1-2-4-5-6-7, GLDPA-Ft-1-2-5-6-7, GLDPA-Ft-1-2-6-7, GLDPA-Ft-1-2-7, and GLDPA-Ft-1-2-ppcA-Pₜₜₜₜ. The cloning of construct GLDPA-Ft-1-2-3-7 involved PCR amplification of the promoter region between -1,571 and -927 using primers GLDPA-5′-Xba and gdc3-R. The PCR product was digested with XbaI and ligated with XbaI-cut GLDPA-Ft-D6. The correct orientation of the inserted DNA fragment in GLDPA-Ft-1-2-3-7 was shown by sequencing.

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The cloning of construct GLDPA-Ft::mGFP5-ER was achieved by PCR amplification of the mGFP5-ER gene (Haseloff et al., 1997) from genomic DNA of the Arabidopsis (*Arabidopsis thaliana*) enhancer trap line E2443 (generated by Scott Poethig, http://www.arabidopsis.org/abrc/poethig.jsp) with primers 5′-mGFP5ER-BamHI and 3′-mGFP5ER-SacI. The PCR product was then cloned into BamHI/SacI-cut GLDPA-Ft::H2B-YFP to yield GLDPA-Ft::mGFP5-ER.
Plant Transformation

In all transformation experiments, the Agrobacterium tumefaciens strain AGL1 was used (Lazo et al., 1991). The promoter-GUS constructs were introduced into AGL1 by electroporation. Arabidopsis plants were transformed via the floral dip method according to Clough and Bent (1998). The transformation of Flaveria bidentis was performed as described by Chitty et al. (1994). The integration of the transgenes into the genome of regenerated F. bidentis or T1 Arabidopsis plants was proved by PCR analyses.

Measurement of GUS Activity and Histochemical Analysis of Reporter Gene Activity

F. bidentis T0 plants used for GUS analysis were 40 to 50 cm tall and before flower initiation; the Arabidopsis T0 plants were examined around 3 weeks after germination. Fluorometrical quantification of GUS activity was performed according to Jefferson et al. (1987) and Kosugi et al. (1990). For histochemical analysis of GUS activity, leaves were cut manually with a razorblade and the sections were transferred to incubation buffer (100 mM Na2HPO4, pH 7.5, 10 mM EDTA, 50 mM K4[Fe(CN)6], 50 mM K3[Fe(CN)6], 0.1% (v/v) Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl-D-glucuronide). After brief vacuum infiltration, the sections were incubated at 37°C for 1 to 2 h. After incubation, chlorophyll was removed from the tissue by treatment with 70% ethanol. Fluorescence microscopy was performed using a Zeiss Axioshot (Carl Zeiss AG) equipped with an Olympus DP50 camera (Olympus Optical) and a Zeiss GFP imaging filter system (BP 450–490, FT 510, BP 515–565). Bright-field and fluorescence images were overlaid with Adobe Photoshop 7.0 (Adobe Systems). For confocal laser microscopy, a Zeiss LSM 510 with a Plan-Neofluar 25× objective was used. Chlorophyll autofluorescence was visualized with a long pass 560-nm emission filter.

In Situ RNA Hybridization

Nonradioactive in situ hybridization experiments were performed according to the protocol described by Simon (2002). Embedded leaves of F. bidentis and F. trinervia were cut into cross sections of 20 μm thickness using a standard microtome. For the generation of the GLDPA probe, a 2.4-kb cDNA fragment of the GLDPA gene of F. trinervia was amplified by PCR (primers gdcS-F4 and gdcS-R4; see Table I). The PCR product was digested with BamHI and cloned into BamHI/XhoI cut pBluescript II KS+ (Stratagene). The use of two different RNA polymerases (T3 and T7) then allowed the production of both antisense and sense probes for in situ hybridization.

Supplemental Material

The following materials are available in the online version of this article.

Supplemental Video S1. Histochemical localization of H2B::YFP in Arabidopsis plants transformed with the construct GLDPA::H2B::YFP by confocal laser microscopy.

Supplemental Video S2. Histochemical localization of H2B::YFP in Arabidopsis plants transformed with the construct GLDPA::H2B::YFP by confocal laser microscopy.

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LITERATURE CITED


