Vascular-Specific Expression of the Bean GRP 1.8 Gene Is Negatively Regulated

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In French bean, the glycine-rich cell wall protein GRP 1.8 is specifically synthesized in the vascular tissue. To identify cis-acting sequences required for cell type-specific synthesis of GRP 1.8, expression patterns of fusion gene constructs were analyzed in transgenic tobacco. In these constructs, the uidA (β-glucuronidase) gene was placed under control of 5' upstream deletions as well as internal deletions of the GRP 1.8 promoter. Four different cis-acting regulatory regions, SE1 and SE2 (stem elements), a negative regulatory element, and a root-specific element, were found to control the tissue-specific expression. Deletion of the negative regulatory element resulted in expression of the uidA gene in cell types other than vascular cells. The SE1 region was essential for expression in several cell types in the absence of further upstream regulatory sequences. Full-length promoters having insertions between the negative regulatory element and SE1 strongly expressed the gene in nonvascular cell types in stems and leaves. Thus, vascular-specific expression of the GRP 1.8 promoter is controlled by a complex set of positive and negative interactions between cis-acting regulatory regions. The disturbance of these interactions results in expression in additional cell types.

INTRODUCTION

Glycine-rich proteins are a class of structural cell wall proteins that contain about 60% glycine on a molar basis. Glycine-rich proteins have been localized in cell walls of bean (Keller et al., 1988) and petunia (Condit et al., 1990). Proteins cross-reacting with antibodies against bean or petunia glycine-rich proteins were found in rape, soybean, maize, wheat, and barley (S.-H. Cheng, B. Keller, and C.M. Condit, unpublished data). In addition, genes encoding similar proteins have been described in Arabidopsis (de Oliveira et al., 1990) and barley (Rohde et al., 1990).

Glycine-rich protein 1.8 (GRP 1.8) was localized by antibody labeling in young tracheary elements (protoxylem) of developing tissue of seed coats and hypocotyls of French bean (Keller et al., 1989b). There, it is closely associated with the lignified secondary wall thickenings in xylem. Despite this close association with lignin, the bean GRP 1.8 is correctly deposited in cell walls of tracheary elements in the absence of any lignification (Keller et al., 1990). Recently, it was shown in bean and soybean that glycine-rich proteins are expressed in primary and secondary xylem as well as in primary phloem, i.e., in all tissues that are going to be lignified (Ye and Varner, 1991).

A 494-bp fragment of the GRP 1.8 promoter, including 427 bp of sequence upstream of the transcription start site, expressed the β-glucuronidase (GUS) reporter gene in transgenic tobacco plants (Keller et al., 1989b). There, vascular-specific expression was observed in all tissues examined. This cell type-specific activity of the GRP 1.8 promoter in vascular cells provides a defined system to study the molecular basis of gene expression in vascular tissue. Vascular cells that are determined to become tracheary elements undergo a very distinct cell differentiation. In contrast to many other plant cell types that can dedifferentiate and ultimately even form a new plant, cells determined to become tracheary elements are programmed to die. The analysis of the strong GRP 1.8 promoter should give us some understanding of the control of gene expression in those cells.

Whereas inducible promoters, particularly those that are light responsive and elicitor induced, have been studied in detail (reviewed by Benfey and Chua, 1989), cell type-specific gene expression in plants has been studied with only a few promoters. The best studied case is the CaMV 35S promoter of the cauliflower mosaic virus (CaMV). Benfey et al. (1989, 1990a, 1990b) and Benfey and Chua (1990) found a modular organization of the CaMV 35S enhancer, similar to the organization of other eukaryotic, e.g., mammalian, promoters (Dyan, 1989). Subdomains of this enhancer induced expression in only a subset of those cells that expressed the gene if a full-length promoter
was present. Combination and multiplication of these modules led to more complex expression patterns involving more cell types than one subdomain alone. In addition, synergistic effects were found between different elements as the expression pattern of two modules was more than additive. No negative interactions were found to be involved in the control of tissue-specific expression. These studies demonstrated that rather small elements can confer cell type-specific expression. However, the CaMV 35S promoter is a viral promoter. It remains to be seen whether endogenous plant promoters control tissue-specific expression in a similar way.

As a first step toward a molecular understanding of how cell type-specific expression of the GRP 1.8 gene is controlled, the function of cis regulatory elements was analyzed in transgenic tobacco. In this study, the regulatory sequences involved in the control of tissue-specific expression were found to be organized as distinct elements. A root-specific regulatory region is active in the absence of other cis elements. In addition, there are two partially redundant elements involved in vascular expression in stems. However, in contrast to the viral CaMV 35S promoter with its additive and synergistic interactions, tissue-specific expression in the GRP 1.8 promoter was found to be negatively controlled: removal of one cis-acting region both in internal and 5' upstream deletions of the promoter resulted in expression of the reporter gene in additional cell types. Topological constraints seem to be involved in inhibitory actions between cis elements, inasmuch as an insertion of 33 bp in the context of the full-length promoter also resulted in expression of the gene in additional cell types. The results obtained indicate a complex interplay of positive and negative regulatory elements for correct cell type-specific expression.

The different deletion end points in the 5' upstream region are shown in the sequence of the GRP 1.8 promoter in Figure 1. In this paper, the fragment starting at base pair -502 is referred to as the full-length promoter. Ten different 5' upstream deletions were analyzed in transgenic tobacco. Stems and roots of young plants (approximately 5 cm high, 3 weeks after cutting and rerooting) were used for analysis. For some constructs, plants from the F1 generation were analyzed. No difference was observed between them and their corresponding primary transformants. Fluorometric measurements of GUS activity in stems revealed strong activities in transformants with deletions up to base pair -121, as shown in Figure 2A. Only in the -94 deletion, expression was significantly lower (approximately 10-fold), indicating a positively acting regulatory element between -121 and -94 which is called stem regulatory element 1 (SE1). The values of GUS activities in individual transformants with a specific construct varied by a maximal factor of 18. In Table 1, GUS activities of stems from independent transformants are shown. This demonstrates that the 10-fold drop in activity upon removal of essential regulatory sequences was clearly detectable despite the variation among individual

RESULTS

Analysis of Progressive Deletions from the 5' End of the GRP 1.8 Promoter in Transgenic Plants

Previous studies (Keller et al., 1989b) have shown that an upstream deletion up to position -427 in the GRP 1.8 promoter can still confer vascular-specific expression to the uidA (GUS) reporter gene. To define in more detail the cis-acting regulatory elements involved in this tissue-specific expression, a set of deletions starting at position -502 was made. These constructs were translationally fused to the GUS encoding gene and transformed into tobacco using Agrobacterium-mediated gene transfer. In this study, between five and 19 independent transformants were analyzed for quantitative expression of each construct, whereas tissue specificity was determined in four to 12 independently transformed plants for each construct.

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Figure 1. Sequence of the GRP 1.8 Promoter from -620 bp to -40 bp. The 5' upstream deletion end points are indicated by arrowheads. The first four amino acids of the GRP 1.8 coding region are shown. The TATA box is underlined and the transcription start site is indicated by an arrow.
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Figure 2. GUS Activity of 5' Upstream Deletions of the GRP 1.8 Promoter in Transgenic Tobacco.

(A) Activity in stems.

(B) Activity in roots of plants grown under sterile conditions.

Five to 11 independent transformants with the deletions −502, −304, −205, −199, −186, −143, or −121 were analyzed. Only plants with GUS activities above background level had been selected for this analysis. For each of the deletions −94, −76, and −22, 10 to 15 independent transformants were analyzed. The values given represent the mean GUS activity of these transformants. The values in independent transformants with the same construct varied by a factor of maximally 18. The numbers in parentheses in (A) indicate the number of transformants that expressed GUS in nonvascular cells compared to the total number of histochemically analyzed plants. The expression of deletions −94, −76, and −22 was too weak to be analyzed for tissue specificity in stems.

Transformants. In roots, expression was still observed at a normal level in the −94 deletion (Figure 2B). A significant drop in the level of activity was only found if sequences up to −76 were deleted. Thus, there is an essential sequence element for root-specific expression between −94 and −76 that can confer enhanced root-specific expression in the absence of other upstream sequences.

Table 1. GUS Enzymatic Activities in Independent Transgenic Plants Containing Different 5' Upstream Deletion Constructs of the GRP 1.8 Promoter

<table>
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<th>Construct</th>
<th>0-400</th>
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<th>5,000-10,000</th>
<th>&gt;10,000</th>
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<td>1</td>
<td>1</td>
<td>3</td>
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<tr>
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<td>4</td>
<td>4</td>
<td>1</td>
<td>11</td>
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<td>8</td>
</tr>
<tr>
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<td>3</td>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
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<td>2</td>
<td>3</td>
<td>1</td>
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<td>15</td>
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</table>

*In the first column, the 5' upstream deletions are given (see Figure 1). In Columns 2 to 7, the number of independent transgenic plants for which the fluorometric assay of GUS activity was in the range indicated above the column is shown. GUS activity is for stems and is given in picomoles of 4-methylumbelliferone produced per milligram of protein per minute. In column 8 the total number of transgenic plants analyzed for each construct is given.

The same 5' upstream deletions were also analyzed for qualitative expression patterns in young primary transformants using histochemical staining to detect GUS activity. In most reactions, cycloheximide was added as a translational inhibitor to exclude a possible wound induction due to the staining conditions. This analysis demonstrated that, in contrast to quantitative expression levels, tissue specificity was altered in deletions starting at −199 or further downstream. In most plants of the −502, −304, and −205 deletions, the GRP 1.8 promoter activity was restricted to vascular tissue, as can be seen in Figure 3A. Only occasionally (e.g., in two of 11 plants in the −304 deletion), expression was also seen in cortical and epidermal cells (data not shown). However, the staining in those cells was very weak. In deletion −199, three of five plants showed relatively weak expression in cortical cells, whereas vascular expression remained strong (Figure 3B). The most dramatic change in tissue specificity of expression was found in plants containing constructs deleted up to −186. Eight of nine transgenic plants showed strong expression in nonvascular cells (Figures 3C and 3D), whereas expression in vascular tissue appeared to be weaker. The observed lower expression in vascular tissue is in agreement with quantitative measurements of GUS activity in these plants. The overall GUS activity was not higher despite the additional cell types that express the gene.

Two observations suggest that the expression in nonvascular cells is real and not an artifact of the GUS staining procedure. First, the cortical expression is not due to diffusion of the colored reaction product as only cortical
Figure 3. Histochemical Localization of GUS Expression in Stems and Roots from Representative Plants with 5' Upstream Deletions. Plants Were Analyzed 3 Weeks after Rerooting.
tissue was stained in short incubations with little infiltration (Figure 3E). Second, the observed expression is not due to generally higher expression levels that would result in detection of staining in cortical cells because expression is above the threshold of detectability. As an example, the stem of plant -186 (No. 6) is shown after histochemical staining in Figure 3D. GUS activity in this stem was 1600 pmol·mg protein⁻¹·min⁻¹, considerably less than the activity in stems of many other plants with longer promoter fragments that stain only in vascular tissue. Staining of pith parenchyma cells was often observed when cortical and epidermal cells showed GUS activity. However, the staining in pith parenchyma cells was more variable than in cortical cells and was sometimes difficult to distinguish from expression in inner phloem. Therefore, in this analysis, we focused on the pattern of expression in cortical and epidermal cells.

Thus, a loss of tissue specificity of expression was observed when a regulatory element between base pairs -199 and -186 was removed. We call this sequence the negative regulatory element. In the absence of the negative regulatory element, there was new expression in nonvascular cells, whereas expression in vascular tissue appeared to be weaker. Deletions up to -143 bp and -121 bp (Figure 3F) showed a similar expression pattern, whereas deletions at -94 bp and -76 bp only occasionally showed any detectable staining (Figure 3G).

Overviews are given in Figures 3A to 3G to show the distribution of GUS activity across a whole stem section. More details on the cell types expressing the gene are shown in Figures 3J to 3L. In thin sections of stems stained for GUS activity (deletions at -143 bp and -121 bp), strong expression is generally seen in cortical cells (Figures 3A, 3J, 3K, and 3L) and sometimes also in epidermal cells (Figure 3J). In thin sections of stems, the staining of cortical cells occurred in patches. This is probably because in sections only a part of the larger cortical cells remain intact after sectioning.

In confirmation of our GUS activity results, which showed a root-specific element located between -94 bp and -76 bp, histochemical staining of roots showed no change in the expression pattern of plants with deletions to -121 bp or -94 bp. Only when the promoter was deleted to -76 bp no root activity was observed. In fact, both in the -121 bp and -94 bp deletions, strong expression was observed in root tips (Figures 3H and 3I). A detailed analysis of expression of these deletions in roots will be described elsewhere (D. Heierli and B. Keller, unpublished data). Thus, the analysis of 5′ upstream deletions revealed three regulatory regions involved in gene regulation: SE1, between -121 bp and -94 bp, is required for strong expression in stems; the root-specific element, between -94 bp and -76 bp, is essential, at least in the absence of further upstream sequences, for expression in root tips. A third region, the negative regulatory element, plays an important role in restricting expression to vascular tissue, as well as to enhance expression in that tissue.

Analysis of Internal Deletions

To study the role of the regulatory elements defined by the 5′ upstream deletions in the context of the full-length promoter, a series of internal deletions was made. Between 25 bp and 48 bp were internally deleted and partially substituted by linker sequences used for cloning. The structure of the six internal deletions analyzed is shown in Figure 4A. They span the region from -229 bp to -76 bp. The level of expression was studied quantitatively in stems of young plantlets (Figure 4B). Internal removal of both the SE1 and the root-specific element in construct G resulted in a dramatically lower expression level. Deletion of SE1 alone in deletion F, however, gave an expression lower than in deletions A, B, C, and D, but still more than sixfold higher than deletion G. This indicates at least a partial redundancy of SE1. We named this second element SE2. That interaction between SE2 and the root-specific element is necessary for strong expression in stems in the absence of SE1 is demonstrated by deletion G, which is lacking the root-specific element.

Tissue-specific expression of the internal deletions was analyzed by histochemical staining. Results from the 5′ deletion analysis indicated a negative regulatory element for tissue specificity between -199 bp and -186 bp. In

Figure 3. (continued).

(A) Expression in the stem of a plant with deletion -502. Bar = 1 mm.
(B) Stem of a plant with deletion -199.
(C) Stem of a plant with deletion -186.
(D) Stem of plant -186 (No. 6).
(E) Stem of a plant with deletion -186, brief incubation in substrate solution without prior infiltration.
(F) Stem of a plant with deletion -121.
(G) Stem of a plant with deletion -94.
(H) Expression in root tips in a plant with deletion -121. Bar = 100 μm.
(I) Expression in root tips in a plant with deletion -94. Bar = 100 μm.
(J) Cross-section 20 μm thick through the stem of a plant with deletion -143. Bar = 100 μm.
(K, L) Cross-sections 20 μm thick through the stems of plants with deletion -121. Bars = 100 μm.
Magnifications vary slightly between micrographs (A) to (G). Abbreviations: Epi, epidermis; V, vascular tissue; Co, Cortex; Ph, Phloem; X, xylem.
A series of deletions starting from construct F (Figure 4A) was made to localize the redundant element SE2. The structure of these constructs is shown in Figure 6A. GUS activity in stems of transgenic plants containing these constructs was analyzed (Figure 6B). No significant change in activity when compared to construct F (=F1) was seen if sequences up to −192 bp were deleted. Further removal of sequences up to −293 bp reduced expression to the level of the −94 deletion. Thus, SE2 is localized between −192 and −293. This was further confirmed by the construct F6 (Figure 6A), in which SE1 is deleted as well as all the sequences upstream of −205 bp. This deletion also has a low activity, confirming the localization of SE2 upstream of −205. Histochemical staining confirmed the vascular-specific expression in plants with constructs F3 or F4 (Figures 6D and 6E).

Insertions into the Full-Length Promoter Change Tissue-Specific Expression

5′ upstream and internal deletions revealed the presence of the cis-acting negative regulatory element important for tissue-specific expression. However, even a construct leaving this element intact (internal deletion D) strongly expressed the gene in cortical cells. This suggested that the distance between different regulatory elements may be critical for correct cell type specificity. To investigate this possibility, two constructs, I1 and I2, were made. Their structure is given in Figure 7A. In I1, 33 bp are inserted at position −121, whereas 89 bp are inserted in construct I2. In fact, I2 has a duplication of promoter sequences from base pairs −121 to −63 in addition to 32 bp of linker DNA. Both these mutated promoters gave strong expression in stems and leaves of transgenic plants. Histochemical localization of this expression in stems showed that both constructs expressed the reporter gene in vascular and cortical cells and also often in epidermal and pith parenchymal cells (Figures 5G, 5H, 5J, 5K, and 5L). In leaves (Figures 5I and 5M), strong expression was also observed in nonvascular cells.

These data show that both an insertion and a duplication (in which all promoter sequences are present in an intact form at least once) resulted in the loss of vascular-specific expression. In Figure 5K, stems from transgenic plants with a promoter with an insertion of 33 bp (left) and with the normal full-length promoter (right) are shown to allow a direct comparison of tissue-specific GUS expression.
DISCUSSION

Control of Tissue-Specific Expression of the GRP 1.8 Promoter

The quantitative expression of the GRP 1.8 promoter is controlled by at least four regulatory regions that act differentially in different organs. Although we have not yet studied each element in the absence of the other sequences, several conclusions can already be drawn. The root-specific element was localized within 18 bp between -94 bp and -76 bp. In the absence of other upstream sequences, it still enhanced expression in roots. SE1 is essential for strong expression in stems in the absence of SE2. SE2 itself can partially substitute for SE1. Thus, these two sequences are at least partially redundant. Whereas SE2 is dependent on the root-specific element for promoter activation, it remains to be seen whether this is true also for SE1. A schematic representation of the different regulatory elements and their interactions is given in Figure 7B. It is interesting to note that the cis element that is essential for expression in roots in the absence of further upstream elements is the last distinguishable element in front of the TATA box. This is similar to the situation in the CaMV 35S promoter (Benfey et al., 1989).

In addition, in both the GRP 1.8 and the CaMV 35S promoters, this root element is synergistically interacting with other elements that are located further upstream.

Removal of the negative regulatory element region both in 5’ deletions as well as in an internal deletion resulted in activity of the promoter in the cortical and sometimes the epidermal and pith parenchymal cells. The expression in cortical cells was very strong. This is indicated by the fact that color development was easily detectable. It also has to be considered that these cells are large compared to other, e.g., vascular, cells and thus need high GUS activities to be stained. The nonvascular expression was not due to the diffusion of the colored product out of the vascular tissue, inasmuch as short incubations with no infiltration only stained cortical, but not vascular, cells. Expression in cortical cells was also detected in transformants having a relatively weak expression of the reporter gene. This shows that the observed nonvascular expression was not due to a generally strong expression in these plants. Nevertheless, it is possible that the full-length promoter expresses the gene normally in cortical cells, although at low, nonhistochemically detectable levels. In fact, in some plants containing the negative regulatory element and all sequences further downstream in an intact form, very weak expression in the cortex was observed.

However, this weak expression was not correlated with the absolute level of expression and it is likely that these variations are due to positional effects. Positional effects have also been shown to result in variation of the expression pattern in transformants with individual cis-regulatory elements of the CaMV 35S promoter (Benfey et al., 1989, 1990a, 1990b). Chromatin structure at the integration site could influence the tissue-specific control mechanism of the GRP 1.8 promoter, considering the importance of correct arrangement of the promoter elements for the inhibitory action of the negative regulatory element.

Interestingly, the removal of the negative regulatory element region and the subsequent expression in additional cell types does not result in an decrease of quantitative expression in 5’ upstream deletions. Histochemical staining of these plants revealed that their vascular tissue was stained less than that in plants with the full-length promoter. Thus, it appears that the negative regulatory element is not only repressing expression in nonvascular cells but also activating expression in vascular cells. There are other examples where the same regulatory element can either activate or repress a gene depending on the cell context, e.g., it has been shown that a 25-bp sequence of the prolifiner promoter confers hormone-dependent positive or negative regulation depending on the cell type (Miesfeld et al., 1987; Sakai et al., 1988). Our observations best fit with the hypothesis that the negative regulatory element binds a repressing/activating protein, depending on the cell type. Some transcriptional regulators, among them the rat glucocorticoid receptor (Diamond et al., 1990), stimulate gene expression in some genetic and cellular contexts and repress expression in others. Alternatively, the negative regulatory element might contain two different binding sites that were not separated in our constructs. We have preliminary data that at least one protein is specifically binding to or near the negative regulatory element (D. Heierli and B. Keller, unpublished data).

Interaction between Regulatory Elements Confers Tissue-Specific Expression

Internal and 5’ deletions of the GRP 1.8 promoter demonstrated that SE1 is involved in the expression of the GUS reporter gene in several nonvascular cell types in the absence of the negative regulatory element. These experiments indicated that inhibition of SE1 by the negative regulatory element was found to be necessary for specific vascular expression. However, construct D, which has both SE1 and the negative regulatory element intact, still shows a strong expression in cortical cells. This could be due to either the destruction of an additional sequence required for repression in the cortex or, as we have shown, reflects a requirement for a certain distance or structural arrangement of SE1 (and possibly elements further downstream, e.g., the TATA box) and the negative regulatory element to give a functional negative interaction. The analysis of the two constructs 11 (33 additional bp) and 12 (duplication of 57 bp of promoter sequence and insertion of an additional 32 bp, all promoter elements are present in an intact form at least once in this construct).
Figure 5. Histochemical Localization of GUS Expression in Representative Plants with Internal Deletions of the GRP 1.8 Promoter. Plants Were Analyzed 3 Weeks after Rerooting.
Figure 6. Localization of the SE2 Element in the GRP 1.8 Promoter.

(A) Fragments from the GRP 1.8 5' upstream region that were used in expression constructs F1 to F6.
(B) Analysis of GUS activity in stems of sterilely grown transformants with construct F1 to F6. Four to nine independent transformants were analyzed for each construct.
(C) to (E): Histochemical analysis of GUS expression in stems of representative plants with construct F5 (C), F4 (D), and F3 (E).

demonstrated that the arrangement of the different control elements is critical. This indicates that cooperative interactions of protein factors binding to the negative regulatory element and SE1 are involved in the control of activity. Whenever this interaction is disturbed, SE1 activates the promoter in nonvascular cells. Such a control mechanism represents a system for tissue-specific expression by negative control. In addition, it provides flexibility for relieving tissue specificity under certain conditions. It is possible that there is a natural situation where the restriction of expression of the GRP 1.8 promoter to vascular cells is derepressed. Induction by pathogens, as well as other environmental stimuli, could trigger such a relaxed specificity. Signal transduction pathways originating from such stimuli could act on the negative regulatory element binding factor, interfere with SE1-negative regulatory element interactions, and thus activate the gene in other cell types.

This type of control mechanism for tissue specificity is different from the one in the CaMV 35S promoter, the best studied promoter active in plants (Benfey et al., 1990a, 1990b; Benfey and Chua, 1990). There, combinations of subdomains of the promoter resulted in additive expression patterns as well as in synergistic interactions between modules. Such an exclusively additive or synergistic interaction contrasts to the negative control between regulatory elements described here. Negative interactions, although not defined at the level of cell type-specific expression, have also been found in a combination of two plant promoters, rbcS3A and CaMV 35S (Poulsen and Chua, 1988). Recently, negative regulation of tissue specificity was found in Arabidopsis. There, the apetala2 gene product was shown to be a negative spatial regulator of agamous RNA accumulation. Mutations in apetala2 resulted in expression of the agamous gene in cell types that normally do not express it (Drews et al., 1991). Altered tissue specificity was also observed in mutated forms of the developmentally regulated bean phenylalanine ammonia-lyase 2 promoter. There, the deletion of an internal element...
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Figure 7. Interactions between Regulatory Elements Are Required for Vascular-Specific Expression of the GRP 1.8 Promoter.

(A) Mutations of the full-length GRP 1.8 promoter. Construct I1 has an insertion of 33 bp between −120 bp and −121 bp (hatched area). Construct I2 has the same insertion in addition to a duplication of the promoter sequence between −121 bp and −63 bp (crosshatched area).

(B) Regulatory elements involved in tissue-specific expression of the GRP 1.8 promoter. The four elements characterized in this study are indicated with their positions as defined by the deletion analysis. SE2 and the root-specific element positively interact to give expression in vascular tissue, whereas the negative regulatory element represses gene expression induced by SE1 in nonvascular cell types.

Abbreviations: v+, stimulation of expression in vascular cells; c+, in cortical cells; r+, in root cells; c−, repression of expression in cortical cells; NRE, negative regulatory element; RSE, root-specific element.

activates the gene in cell types that normally do not express the gene (C.J. Lamb, personal communication).

METHODS

Plasmids and Gene Constructs

A PstI fragment containing the GRP 1.8 promoter and most of its coding region (Keller et al., 1988) was progressively deleted from the 5′ end using Bal31 nuclease (Maniatis et al., 1982). A BamHI site containing linker was ligated to the fragments, and the BamHI-ScaI promoter fragment was cloned into pBl101.1 cut with BamHI and ScaI (Jefferson et al., 1987, for the location of the Scal site see Keller et al., 1988). The translational fusion with the −502 deletion was sequenced with a GUS sequence-specific primer (Clontech, Palo Alto, CA) using the dideoxy sequencing method (Sanger et al., 1977). The BamHI-EcoRI fragment of this plasmid (GRP 1.8 promoter and GUS coding region) was isolated and cloned in the BamHI-EcoRI plasmid pB125 to give pBK550. This BamHI-EcoRI cassette was used for all further experiments. For 5′ deletions, pBK550 was opened at the BamHI site and deletions were made with Bal31 nuclease. Deletions were cloned into pBl25 (XhoI cut and filled with the Klenow fragment of DNA polymerase I, cut with EcoRI) and sequenced. They were then transferred into the plasmid pBl101.1 (Jefferson et al., 1987) by replacing the plasmid BamHI-EcoRI fragment by the new constructs. For 3′ deletions, pBK550 was opened at the MluI sites of the GUS gene and deletions were made with Bal31 nuclease. After nuclease digestion, octamer linkers (Boehringer Mannheim) with an XhoI site were ligated to the fragments which were then sequenced. To create appropriate internal deletions, 5′ and 3′ deletions were ligated in a triple ligation: A BamHI-XhoI fragment from a 3′ deletion and an XhoI-EcoRI fragment from a 5′ deletion of pBK550 were ligated into pBl101.1 cut with BamHI and EcoRI. Plasmids were analyzed by restriction digestion and partially by sequencing. In cases where the XhoI site was lost in the 5′ deletion, the HindIII-EcoRI plasmid fragment containing these deletions as well as the GUS coding region were inserted into pSP70 (Promega) cut with HindIII and EcoRI. Then again, an XhoI-EcoRI fragment was cut out and used for triple ligations. In addition, the Scal site at position −204 was used to make some constructs (F6 and 5′ deletion −205).

Plant Transformation

The 5′ and internal deletions were mobilized from Escherichia coli HB101 into Agrobacterium tumefaciens LBA 4404 as described by Bevan (1984). Leaf discs of Nicotiana tabacum cv Xanthi were transformed as described by Horsch et al. (1984). Transformed plants were selected on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 150 µg/mL kanamycin and 500 µg/mL carbenicillin or cefotaxime. After rooting, plantlets were both propagated as cuttings under sterile conditions and transferred to soil and grown in a greenhouse. Most of the analysis was done with sterilely grown primary transformants when they were approximately 5 cm high. Greenhouse plants were allowed to self-fertilize and seeds were collected. Seeds from several primary transformants were sterilized and seedlings were grown and analyzed after 6 weeks. No differences to the expression pattern of primary transformants were observed in this F1 generation.

GUS Assay and Histochemical Localization

Tissue extracts were prepared and analyzed for the fluorescent reaction product 4-methylumbelliferylglucuronide as described by Jefferson et al. (1987), using 4-methylumbelliferylglucuronide (Sigma) as a substrate. The reaction was performed in 200 µL volume at 37°C for 60 min. It was stopped with 800 µL of ice-cold 0.2 M Na2CO3. Histochemical assays were also performed as described by Jefferson et al. (1987) and Jefferson (1987). After fixation with formaldehyde (0.3%, 45 min at room temperature, infiltrated twice), the tissue was incubated with X-gluc (United States Biochemicals). In most reactions, a possible induction of GUS expression due to the staining conditions was inhibited by 1.8 mM...
cycloheximide as a translational inhibitor (Siebertz et al., 1989). Staining was allowed to proceed until a similar strength of blue stain had developed in the different samples (1 hr to overnight). Weak staining was always allowed to proceed overnight. Stem sections were cleared of chlorophyll by incubation in 70 to 100% ethanol and were observed in a stereo microscope (Wild-Leitz, Rockleigh, NJ). Cell type specificity was analyzed in a Zeiss microscope either after sectioning the stained tissue with a hand microtome or by directly staining hand microtome-made sections (thickness of about 20 μm). Incubation of thin sections was performed at 37°C in a humidified chamber. Micrographs were taken on Kodak Tungsten 50 film.

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