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Distinct *cis*-Acting Elements Direct Pistil-Specific and Pollen-Specific Activity of the Brassica *S* Locus Glycoprotein Gene Promoter

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The promoter of the *S* Locus Glycoprotein (*SLG*) gene of Brassica is a tightly regulated promoter that is active specifically in reproductive organs. In transgenic tobacco, this promoter is active exclusively in cells of the pistil and in pollen. We transformed tobacco with truncated versions of the SLG_{13} promoter fused to the β-glucuronidase reporter gene. We show that the promoter has a modular organization and consists of separable DNA elements that independently specify pistil- and pollen-specific expression. A 196-bp region (−339 to −143) is sufficient to confer stigma and style specificity to the marker gene. Two distinct, but functionally redundant, domains (−415 to −291 and −117 to −8) allow specific expression of the gene in pollen. The functional domains identified within the SLG_{13} promoter contain sequence elements that are highly conserved in different alleles of the *SLG* gene and in the *S* Locus Related *SLR1* gene.

INTRODUCTION

The *S* Locus Glycoprotein (*SLG*) gene, which is derived from the self-incompatibility locus of Brassica, is a tightly regulated gene that is expressed specifically in two organ systems of the flower: the pistil and the anther. Evidence for the specificity of *SLG* promoter activity derives from genetic ablation studies in which a chimeric gene construct consisting of the *SLG* promoter fused to the diphtheria toxin subunit A (*DTA*) gene was introduced into tobacco (Thorsness et al., 1991), Brassica (Kandasamy et al., 1993), and Arabidopsis (Thorsness et al., 1993). Transformation of these plants with the *SLG*::*DTA* gene fusion resulted in the high-frequency production of transgenic plants that underwent normal differentiation and produced flowers in which only specific cells of the pistil and anther were ablated.

The analyses of the *SLG*::*DTA* fusion and of a reporter gene fusion consisting of the *SLG* promoter fused to the *Escherichia coli uidA* reporter gene that encodes β-glucuronidase (GUS) have identified the cell types of the pistil and anther in which the *SLG* promoter is active. In the pistils of transgenic Brassica and Nicotiana, the promoter is active in cells of the stigma and in the transmitting tissue of the style and ovary (Sato et al., 1991; Thorsness et al., 1991). In the anthers of transgenic Brassica, promoter activity is evident in the tapetum, a sporophytic tissue of the anther, and in microspores (Sato et al., 1991). On the other hand, in transgenic tobacco anthers, the *SLG*::*uidA* fusion exhibits strict gametophytic expression; GUS activity is detected in pollen grains and not in the sporophytic tissues of the anther (Thorsness et al., 1991).

Because of its dual activity in two organ systems of the flower, the *SLG* promoter presents an interesting problem in plant gene regulation. We have demonstrated that the DNA sequences required in *cis* for the expression of the *SLG* gene in pistils and anthers lie within 3.65 kb upstream of the gene's coding region (Sato et al., 1991; Thorsness et al., 1991). In this report, we examine the minimal promoter elements required for the expression of the *SLG* gene in the pistil and anther of transgenic tobacco. Although there are species-dependent differences in the activity of the *SLG* promoter, tobacco is well suited for the functional dissection of this promoter because the dual activity of the promoter in pistil and pollen is maintained in this easily transformable plant.

To separate pistil- and pollen-specifying domains of the *SLG* promoter, several truncated versions of the *SLG* promoter were constructed. Expression of the *SLG*::*uidA* reporter gene in transgenic tobacco was directed either by sequential 5′ deletions of the *SLG* promoter or by short *SLG* promoter modules inserted upstream of a minimal cauliflower mosaic virus (CaMV) 35S promoter (Benfey et al., 1990a). We show that distinct promoter elements direct GUS activity in pistil and pollen. A 196-bp region (−339 to −143) is sufficient to direct expression of the marker gene in the stigma and style. Two distinct,

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but functionally redundant, domains (−415 to −291 and −117 to −8) allow expression of the gene in pollen.

RESULTS

SLG Promoter-Reporter Gene Fusions

To analyze the *cis*-acting elements of the *SLG* promoter, we used the *Brassica oleracea* SLG_{13} allele to generate two sets of *SLG* promoter::*uidA* fusions. The first set consisted of sequential deletions of the 5′ upstream region of the SLG_{13} allele. The end points of these derivatives (numbered from the translation initiation codon) and their respective orientations are given in Figure 1A. Each of these promoter constructs retained the putative TATA binding site found in the *SLG* promoter and was joined to the coding region of the GUS indicator gene, as described in Methods.

In the second set of promoter fusions, individual promoter modules were synthesized from the 5′ upstream region of the SLG_{13} allele by the polymerase chain reaction (PCR) and joined to the −46 to +8 region of the CaMV 35S promoter. This 35S promoter fragment provides the TFIID binding (TATA box) and transcription initiation sites and has been used as a minimal promoter in studies of plant gene activity (Benfey et al., 1990a). The primers used for PCR amplification were selected based on an assessment of regions lacking repeated motifs. In addition, a subset of primers was selected to include or exclude sequence motifs found to be highly conserved in several alleles of the *SLG* and *S Locus Related SLR1* genes (see below). The SLG_{13} promoter modules were linked to the *uidA* coding region and introduced into a binary vector for plant transformation, as described in Methods. The end points of this second set of constructs are diagrammed in Figure 1B. In both sets of plasmids, a polyadenylation site is included at the terminus of the *uidA* coding region. The constructions were stably introduced into tobacco by Agrobacterium-mediated gene transfer.

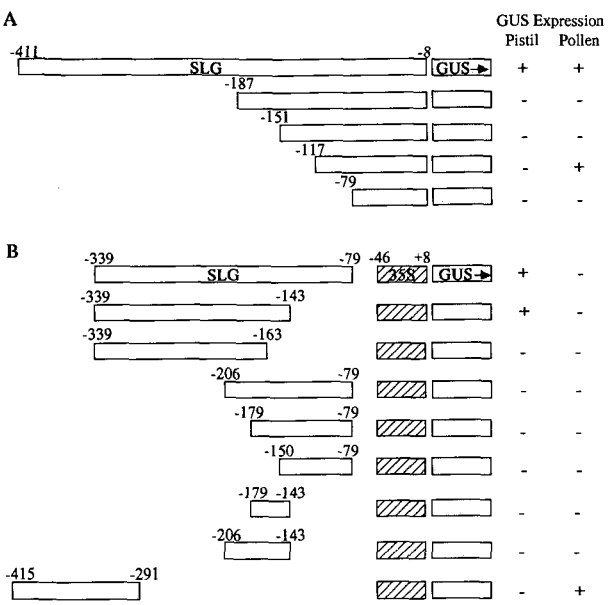

Figure 1. Summary of the SLG_{13} Promoter::*uidA* Constructs and Their Expression in Transgenic Tobacco Pistils and Pollen.

(A) Constructs containing sequential 5′ deletions of the SLG_{13} promoter (open boxes) fused to the GUS indicator gene (stippled boxes). The deletion end points are numbered from the translation initiation codon of the SLG_{13} promoter.

(B) Constructs containing PCR-generated promoter modules derived from the SLG_{13} gene. The promoter modules were joined to the "TATA box" region of the CaMV 35S (−46 to +8, striped boxes) promoter and to GUS. The 5′ and 3′ end points of the promoter modules are numbered from the translation initiation codon of the SLG_{13} promoter. (+), GUS activity; (−), no GUS activity.

In the resulting transgenic plants, GUS activity was monitored histochemically by applying the chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-gluc) to freshly excised tissues of the transgenic plants, and the levels of GUS activity were quantitated in fluorometric GUS assays using 4-methylumbelliferyl β-D-glucuronide as substrate. The results of this analysis are summarized in Figure 2 and Tables 1 and 2 and are described in detail in the following sections. The number of integrations in independent transformants varied from one to five, as demonstrated by DNA gel blot analysis (data not shown). The variable number of transgene integrations in different transformants had no effect on the qualitative aspects of GUS activity specified by each of the constructs. In addition, there was no strict correlation between insert number and the level of GUS activity directed by any one construct (see Table 2), presumably due to position effects that resulted in unequal activity of transgenes and to the silencing of transgenes in some cases.

Pistil-Specific Expression

A photograph of a longitudinal section through the upper region of a tobacco pistil is shown in Figure 2A. At the apex of the pistil is a bilobed stigma consisting of a papillate epidermis, a subepidermal zone, and parenchymatous ground tissue. The secretory zone of the stigma converges into the style as a central region of transmitting tissue surrounded by the cortex and the epidermis. During pollination, pollen germinates at the stigma surface, and the pollen tubes extend into the transmitting tissue of the style as they make their way toward the ovary. Untransformed tobacco pistils do not contain detectable endogenous GUS activity and do not stain with X-gluc (Figure 2B; Table 1).

Tobacco plants transformed with three of the SLG_{13} promoter constructs exhibited GUS activity in the stigma and style (Table 1). From the first set of sequential 5′ promoter deletions, only one of the fragments, that having an end point at −411, directed GUS expression in the pistil (Figure 2C). Activity was

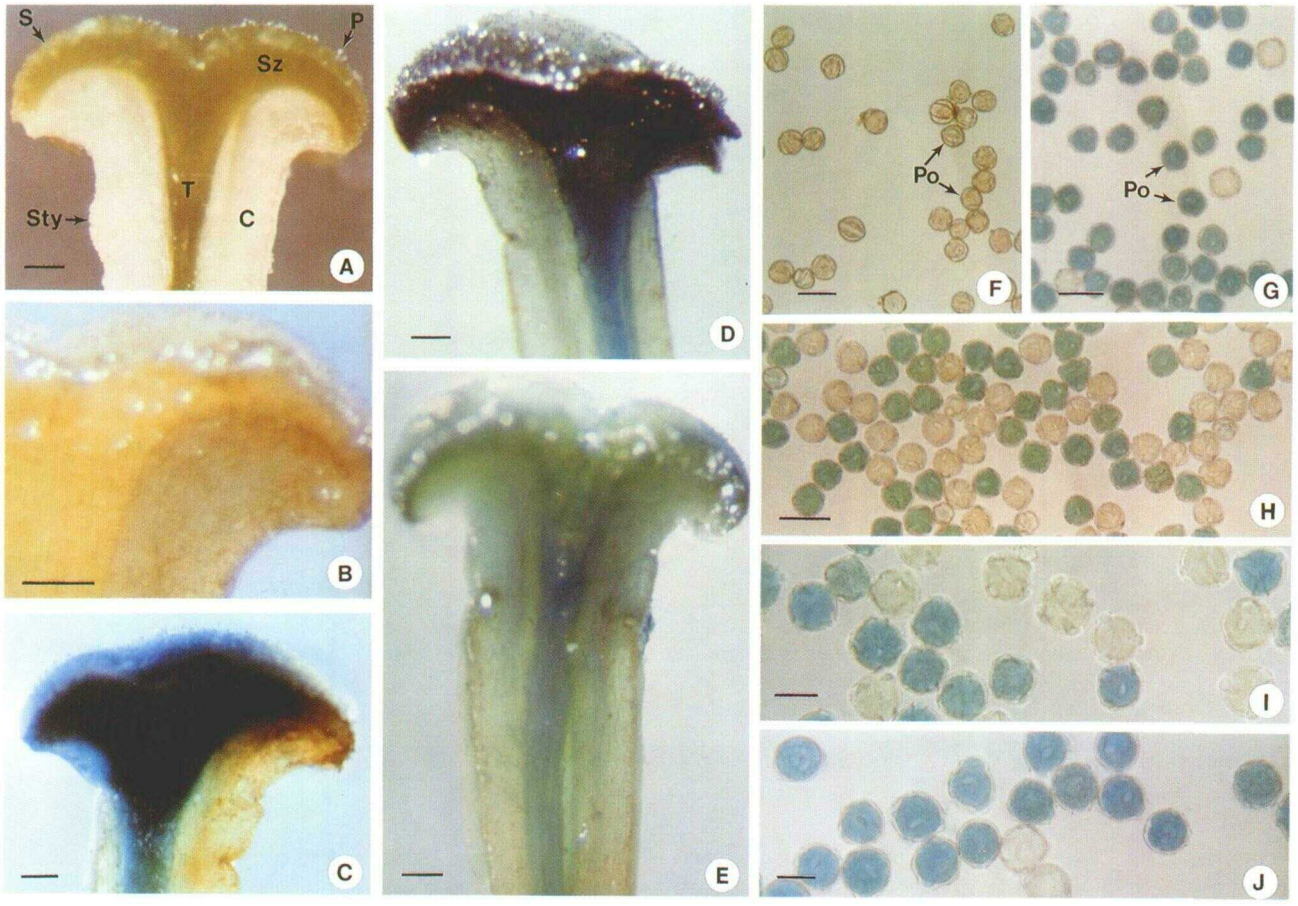

Figure 2. Histochemical Analysis of GUS Activity Conferred by *SLG* Promoter Fragments in Pistils and Pollen of Transgenic Tobacco.

(A) to **(E)** GUS activity in pistils. Bars = 600 μm.

(A) Longitudinal section through a tobacco pistil showing the stigma and upper portion of the style. C, cortex; P, papillar cell; S, stigma; Sty, style; Sz, secretory zone; T, transmitting tissue.

(B) to **(H)** *Longitudinal sections of tobacco pistils following histochemical staining for GUS activity.*

(B) Pistils from an untransformed control plant exhibiting no blue staining.

(C) Pistils from plants transformed with the −411 sequential deletion.

(D) Pistils from plants transformed with the (−339 to −79)::35S::*uidA* construct.

(E) Pistils from a plant transformed with the (−339 to −143)::35S::*uidA* construct exhibiting blue staining of the secretory zone of the stigma and the transmitting tissue of the style.

(F) to **(J)** GUS activity in pollen. Bars = 50 μm in **(F)**, **(G)**, and **(H)**; 25 μm in **(I)** and **(J)**.

(F) Pollen from an untransformed plant in which no blue staining is detected. Po, pollen grains.

(G) Pollen from a plant transformed with the −411 5′ deletion construct in which ~90% of the pollen grains exhibit GUS activity.

(H) Pollen from a plant transformed with the −117 5′ deletion construct in which ~40% of the pollen grains exhibit GUS activity.

(I) and **(J)** Pollen from two plants transformed with the (−415 to −291)::35S::*uidA* promoter construct.

(I) In this plant, ~45% of the pollen grains are stained.

(J) In this plant, ~90% of the pollen grains are stained.

apparent along the length of the transmitting tissue of the style, in the stigma, and in papillar cells. GUS activity was detected in these tissues in each of the flower bud stages examined, ranging from buds 1.2 cm in length to open flowers. No activity was detected in leaf, root, anther, petal, sepal, or stem (data not shown). The −411 construct also directed GUS expression in mature pollen grains (Figure 2G). The pattern of expression observed with the −411 construct was thus qualitatively similar to that seen when a 3.65-kb *SLG* promoter::*uidA* fusion was introduced into tobacco (Thorsness et al., 1991). In addition, no significant quantitative differences beyond the plant-to-plant variability expected in transgenic plant experiments were observed between plants transformed with the −411 construct and those transformed with the 3.65-kb

Table 1. Summary of Histochemical Localization of GUS Activity Conferred by *SLG* 5′ Promoter Deletions and *SLG* Promoter Modules

Construct End Points[a]	TATA Element[b]	Pistil Expression[c]	Pollen Expression[c]
−411	*SLG*	5/7	5/7
−187	*SLG*	0/18	0/18
−151	*SLG*	0/20	0/20
−117	*SLG*	0/18	13/18
−79	*SLG*	0/5	0/5
−339 to −79	35S	20/24	0/24
−339 to −143	35S	10/21	0/21
−339 to −163	35S	0/17	0/17
−206 to −79	35S	0/17	0/17
−179 to −79	35S	0/7	0/7
−150 to −79	35S	0/20	0/20
−179 to −143	35S	0/22	0/22
−206 to −143	35S	0/16	0/16
−415 to −291	35S	0/10	9/10
Controls			
CaMV 35S minimal promoter[d]		0/6	0/6
Untransformed tobacco		0/4	0/4

[a] The end points of promoter fragments are numbered from the translation initiation codon of the SLG_{13} promoter.
[b] The TATA element used in a particular construction is indicated. *SLG*, native *SLG* TATA element; 35S, −46 to +8 CaMV 35S minimal promoter.
[c] The number of plants exhibiting GUS activity following staining with X-gluc is given over the number of independently isolated transgenic plants analyzed.
[d] Plants transformed with the −46 to +8 CaMV 35S minimal promoter fused to GUS.

promoter fusion. Both promoter fragments directed equally strong GUS activities in pistils and relatively weaker GUS activities in pollen, which ranged from 10 to 30% of pistil levels (Table 2).

These data suggested that the DNA elements required for stigma- and style-specific expression were located downstream of the −411 end point. The ability of fragments with end points within this region to direct pistil-specific expression was assayed in the second set of constructions. Of the fragments joined to the minimal promoter and GUS, two expressed GUS activity. Constructs that contained fragments spanning a 260-bp sequence from −339 to −79 (Figure 2D) or a 196-bp sequence from −339 to −143 (Figure 2E) exhibited GUS activity patterns that were qualitatively similar to the −411 construct described above. Expression was detected in the stigma secretory zone, stylar transmitting tissue, and in stigma papillar cells. However, GUS activity in plants transformed with the −339 to −143 construct was appreciably lower than in plants transformed with either the −411 or the −339 to −79 constructs (Table 2). The reduced activity of the 196-bp promoter fragment was clearly evident in the lower intensity of staining in histochemical GUS assays (Figure 2E). In addition, a smaller proportion of the plants transformed with the −339 to −143 construct exhibited GUS activity: blue staining was noted in five of seven plants transformed with the −411 fragment and in 20 of 24 plants transformed with the −339 to −79 fragment but in only 10 of 21 plants transformed with the shorter 196-bp fragment (Table 1). With both the 196-bp and 260-bp promoter fragments, GUS activity was detected in pistils of open flowers and in buds as small as 1.6 cm in length and was not detected in other floral or vegetative tissues. Deletions of the 3′ end of the 196-bp fragment by only 20 bp to −163 or of the 5′ end to −206 led to the loss of GUS expression (Tables 1 and 2). Plants transformed with a construct containing the −46 to +8 CaMV 35S minimal promoter region linked alone to GUS did not show GUS activity in pistils (Tables 1 and 2).

Pollen-Specific Expression

Three of the constructs directed consistent GUS expression in pollen (Tables 1 and 2). Figure 2 shows the results obtained with representative plants. GUS activity was not observed in pollen of untransformed plants (Figure 2F; Table 1) or in pollen of plants transformed with the 35S minimal promoter fused alone to GUS (Tables 1 and 2). Of the sequential promoter deletions, both the −411 and −117 constructs showed activity in pollen (Figures 2G and 2H), while constructs with end points in between were not active (Table 1). Of the 35S minimal promoter fusions, one construct, with end points of −415 and −291, exhibited GUS activity in pollen (Figures 2I and 2J). Other than the −411 deletion, none of these constructs conferred expression in the pistil. Conversely, the pistil-specific −339 to −143 and −339 to −79 gene fusions did not exhibit

pollen expression (Tables 1 and 2). On occasion, a small number of plants containing the −150 to −79 construct exhibited weak light blue staining in pollen. This erratic staining was noted for only five of 20 plants containing this construct and only under greenhouse conditions. No blue staining of pollen was evident after the same plants were moved to a growth chamber and grown under controlled conditions (20°C, 16-hr/8-hr light–dark cycle, 50% humidity).

Fluorometric GUS assays indicated that the −117 and −415 to −291 constructs directed higher levels of GUS activity in pollen than the −411 deletion (Table 2). Qualitatively, however, the patterns of expression directed by the three constructs in pollen were identical. GUS activity was noted only in pollen of open flowers and was not seen in pollen from flowers at earlier stages of development. Staining was limited to pollen and was not detected in the sporophytic tissue of the anther. In some of the plants, approximately one-half of the pollen grains showed GUS activity (Figures 2H and 2I). This observation is consistent with the introduction of a single copy of the gene into the genome of these plants, as was verified by DNA gel blot analysis (data not shown).

Promoter Sequences of *SLG* and *SLR1* Alleles

The nucleotide sequences of the promoter regions of three alleles of the Brassica *SLG* gene—SLG_{13} and SLG_2 from *B. oleracea* and SLG_8 from *B. campestris*—and one allele of the *SLR1* gene were determined. The *S Locus Related* gene *SLR1* is a member of the *S* gene family that is unlinked to the *S* locus and is expressed in a manner similar to *SLG* (Lalonde et al., 1989; Trick and Flavell, 1989; Hackett et al., 1992). The four promoter regions share a high degree of sequence similarity with five elements being particularly well conserved. These five boxes are shown aligned in Figure 3 and have the following consensus sequences: box I, GACNAATGATA; box II, GTTTGT; box III, TGANTTAATCG; box IV, TGAAAAAGTCATNGA; and box V, ATTTTNCTTGTCTGCT. In addition, several stretches of sequence similarity are found between individual genes. The SLG_{13} and SLG_8 alleles are the most similar and are 85% identical over 202 bp, whereas the SLG_2 allele is only 66% identical to SLG_{13} over 289 bp. The *SLR1* sequence is the least conserved and shares ~40% sequence identity with the SLG_2 and SLG_{13} alleles.

DISCUSSION

Previous studies have indicated that sequences upstream of the *SLG* coding region are capable of directing pistil- and pollen-specific gene expression in transformed tobacco plants (Thorsness et al., 1991). In this report, we have established the minimal sequence elements of the *SLG* promoter necessary for this expression pattern and have provided evidence that distinct *SLG* promoter modules account for pistil- and pollen-specific gene expression. Figure 4 summarizes, in diagrammatic form, the arrangment of these functional modules in the SLG_{13} promoter.

We found that a 411-bp fragment of DNA adjoining the translation initiation codon of *SLG* is sufficient for expression in stigma and style and at a lower level in pollen. The pattern

Table 2. Fluorometric Analysis of GUS Activity in Transgenic Plants Containing Active *SLG* Promoter Constructs

Construct End Points[a]	Pistil Expression[b]	Pollen Expression[b]	Number of Integrations[c]
−3650	5279 ± 1117	1448 ± 150	5
	3278 ± 1834	480 ± 40	1
−411	2056 ± 923	352 ± 96	1
	3429 ± 873	374 ± 73	2
−117	36 ± 13	736 ± 90	2
	27 ± 9	664 ± 83	1
−339 to −79	2300 ± 884	5 ± 3	2
	1597 ± 400	20 ± 7	3
−339 to −143	830 ± 62	36 ± 10	1
	616 ± 156	49 ± 5	3
−415 to −291	12 ± 3	848 ± 120	2
	30 ± 10	1186 ± 315	2
Control			
CaMV 35S minimal promoter	47 ± 27	38 ± 25	3

[a] The end points of the promoter fragments are numbered from the translation initiation codon of the SLG_{13} promoter.
[b] The levels of GUS activity were determined on samples from flowers by the fluorometric GUS assay using 4-methylumbelliferyl β-D-glucuronide as substrate and are expressed in picomoles of 4-methylumbelliferone per milligram protein per minute. For each construct, values represent the averages of determinations for six flowers from two of the most highly expressing plants.
[c] The number of integrations was determined by DNA gel blot analysis.

Figure 3. *Nucleotide Sequence and Alignment of the Promoter Regions of the SLG_{13}, SLG_2, SLG_8, and SLR1 Genes.*

The boxes outline five regions that show particular conservation among the four sequences. The sequences are numbered from the translation initiation codon of each gene.

of GUS activity directed by this fragment was qualitatively and quantitatively similar to that directed by a 3.65-kb *SLG* promoter::*uidA* construct in transgenic tobacco (Thorsness et al., 1991), indicating that the major control elements of the *SLG* promoter are contained within 411 bp immediately upstream of the coding region of the gene. The activity of the −411 *SLG* promoter fragment in pistils and pollen is also consistent with the detection of endogenous *SLG* transcripts in Brassica pistils and developing pollen grains (Sato et al., 1991). It should be noted, however, that the steady state levels of endogenous Brassica *SLG* transcripts in developing pollen are three to four orders of magnitude lower than in pistils (Sato et al., 1991). By contrast, the analysis of the transgenic tobacco plants described here indicates that the levels of GUS activity from the 411-bp or 3.65-kb promoter fragments in pollen were 10 to 30% of those in pistils. A similar discrepancy was noted between the levels of transcripts encoded by the 3.65-kb *SLG* promoter::*uidA* construct and endogenous *SLG* transcripts in transgenic Brassica (Sato et al., 1991) and was ascribed to the possible existence of quantitative *cis*-acting elements not included in the construct or to differential stabilities of reporter and *SLG* transcripts in pollen and pistils.

In addition to the −411 construct, expression in the pistil was also noted for two of the SLG_{13} promoter modules that were joined to the 35S minimal promoter and GUS: the −339 to −79 and the −339 to −143 modules. The −339 to −143 construct defined a 196-bp region that, when coupled to the minimal promoter, was sufficient to confer stigma and style specificity to the marker gene. Because further deletion at either the 5′ or 3′ end of this fragment abolished expression in the pistil, it is suggested that one or more *cis*-acting elements within this fragment is required for the proper expression of *SLG* in the stigma and style. This 196-bp region conferred an expression pattern similar to that specified by the longer SLG_{13} promoter fragments tested. Activity in expressing plants was apparent in the transmitting tissue along the length of the style, in the stigma secretory zone, and in papillar cells. In developing floral organs, expression was detected in the pistil of flowers and in the stigmas of flower buds as small as 1.2 cm in length, but was not seen in other floral or vegetative tissues, including leaf, petal, anther, and root. The 196-bp pistil-specific fragment, however, was appreciably less active than the −339 to −79 pistil-specific fragment in directing pistil expression, as evidenced by lower levels of histochemically and fluorometrically detectable GUS activity and by the relatively high frequency (11 of 21) of transgenic plants that did not exhibit GUS expression. In promoter dissection studies, diminished activity of promoter fragments is often associated with a reduced proportion of transformants that express the reporter gene as well as with a reduced level of reporter expression (for example, see Benfey et al., 1990b). In any event, the diminished activity of the −339 to −143 fragment relative to the −339 to −79 fragment suggests that the region between −143 and −79 contains sequences that are required for high-level pistil activity of the *SLG* promoter.

The 196-bp stigma/style domain did not direct expression in pollen; only the pistils of these plants acquired GUS activity. Two distinct, but functionally redundant, domains allowed expression of the gene in pollen: −415 to −291 and −117 to +8. Two of the sequential promoter deletions (5′ end points at −411 and −117) showed activity in pollen, whereas constructs with end points in between did not. Of the 35S minimal promoter fusions, one construct, with promoter end points of −415 and −219, exhibited GUS activity in pollen. The absence of pollen expression in the sequential deletion construct with 5′ end points downstream of the −415 to −219 domain, but upstream of the pollen-expressing −117 end point, suggests that a silencer *cis*-acting element that negatively modulates expression of the −117 pollen-specifying domain might be located upstream of the −117 end point. The presence of such a silencer element is also suggested by the higher levels of

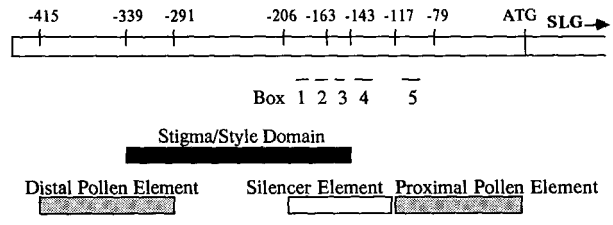

Figure 4. Diagrammatic Representation of the Arrangement of Functional Elements within the SLG_{13} Promoter.

The deletion end points of the constructs used in this study are shown at the top of the figure. The positions of the stigma/style domain (black box), the two pollen elements (shaded boxes), and the pollen silencer element (white box) are shown relative to the positions of the sequence motifs found to be highly conserved in *SLG* and *SLR* genes (boxes I to V).

GUS activity directed by the −415 to −219 and −117 constructs relative to the −411 construct. Further, the low-level and erratic GUS activity that we detected under the relatively uncontrolled greenhouse plant growth conditions in pollen from a small proportion of plants containing the −150 to −79 construct may reflect marginal activity of this promoter fragment resulting from the incomplete inactivation of the silencer element.

For each of the pollen-expressing constructs, GUS activity was noted only in pollen of flowers and was not detected in pollen from flower buds at earlier stages of development. Staining was limited to pollen and was not detected in the sporophytic tissue of the anther. The gametophytic nature of pollen expression was clearly evident in the transformants that contained a single integration of the transgene and in which approximately one-half of the pollen grains showed GUS activity.

The high degree of sequence conservation that exists among the promoters of the *SLG* and *SLR1* genes supports the notion that promoter elements important to the expression of these genes are located within these regions. Among *SLG* alleles, SLG_{13} was reported to be 92% identical to SLG_8 over its protein coding region (Dwyer et al., 1991) but only 70% identical to SLG_2 (Chen and Nasrallah, 1990). Similarly, the promoter region of the SLG_{13} allele is 85% identical to that of the SLG_8 allele and 66% identical to that of the SLG_2 allele. The *SLR1* gene, which shares ~68% sequence identity to *SLG* genes over its protein coding region (Lalonde et al., 1989; Trick and Flavell, 1989), is only ~40% identical to the SLG_2 and SLG_{13} alleles within the 5' regulatory region. Nevertheless, significant stretches of sequence similarity were observed among the promoters of the four genes analyzed. Five short elements are particularly well conserved among the *SLG* and *SLR1* genes. Three of these motifs, boxes I through III, are found within the 196-bp stigma/style element (−339 to −143 of the SLG_{13} allele) identified in this study. When the 3' end point of this element is reduced by 20 bp, box III is deleted and pistil expression is lost. Boxes IV and V, on the other hand, are not essential for pistil expression but may modulate the level of *SLG* expression or may specify *cis*-acting elements whose functions are furnished by the −46 to +8 element of the CaMV 35S promoter. In addition, an upstream promoter element, located between −339 and −206, is required for stigma/style expression. However, there are no conserved sequence elements between the *SLG* and *SLR1* promoters in this region. Box V is also found within the −117 promoter fragment that shows pollen-specific activity and may play a role in directing pollen-specific expression. Despite the high degree of sequence conservation in the similarly expressed *SLG* and *SLR1* promoters, no significant regions of sequence similarity exist between these promoters and the reported promoters of other genes that are expressed specifically in the pistil or in pollen.

The gametophytic activity of the *SLG* pollen-specific promoter elements together with the activity of the stigma/style–specific promoter element in the transmitting tissue of the pistil are consistent with the results obtained previously with a 3.65-kb SLG_{13} promoter fragment. We had previously noted that the different behavior of the *SLG* promoter in transgenic tobacco and Brassica suggests that the transcription factors that recognize *SLG* promoter elements have different distributions in the floral tissues of the two species (Sato et al. 1991; Thorsness et al., 1991). In particular, the tapetal cells of Brassica, but not those of tobacco, are predicted to contain protein factors that interact specifically with appropriate elements in the *SLG* promoter. Whereas the two pollen-specific elements identified in this study behave as functionally redundant elements in transgenic tobacco, it is possible that in Brassica one of the two pollen elements is interpreted as a tapetal-specific element. Alternatively, each of the pollen elements may specify both tapetal (sporophytic) and gametophytic expression.

METHODS

Construction of 5' Promoter Deletions

Our starting point for the construction of sequential 5' deletions of the *S* Locus Glycoprotein SLG_{13} promoter (Figure 1A) was an SLG_{13} promoter::*uidA*::nopaline synthase (*nos*) gene fusion consisting of a 3.65-kb promoter fragment fused to the *uidA* reporter gene and to the polyadenylation signal and transcription termination region of the *nos* gene of *Agrobacterium tumefaciens* (Thorsness et al., 1991). The promoter fragment in this gene fusion extends from a natural BamHI site 3.65 kb 5' of the SLG_{13} coding region to a BamHI site that was introduced by site-directed mutagenesis at nucleotide −8 (8 bp upstream of the ATG initiating codon) in the SLG_{13} gene (Thorsness et al., 1991). This SLG_{13}::*uidA*::*nos* fusion was cloned into the pBluescript SK+ (Stratagene) plasmid to generate plasmid pJSS1. pJSS1 was used to generate a deletion derivative to a natural HpaI site found in the SLG_{13} promoter at nucleotide −411. The resulting plasmid, pMKT29, was in turn used to generate two additional deletion derivatives: one in which sequences were deleted to the ClaI site at −151 and another in which sequences were deleted to the DraI site at −79.

Additional 5' promoter deletions were generated by the polymerase chain reaction (PCR) with pMKT29 as template. Deletions were directed to positions −187 and to −117 in the SLG_{13} promoter by using, as 5' amplimers, synthetic oligonucleotides complementary to sequences at these positions and, as 3' amplimer, an oligonucleotide complementary to a sequence located within the *uidA* coding region. The BamHI site (at position −8 from the ATG) in the PCR-amplified fragments was used to insert the promoter fragments upstream of *uidA*::*nos*. The correct orientation and sequence of the fusions were verified by DNA sequence analysis, and the gene fusions were cloned into the binary vector pBIN19 (Bevan, 1984).

Construction of Promoter Fragment/Minimal Promoter/Reporter Gene Fusions

Promoter fragments were generated by PCR utilizing the SLG_{13} promoter as a template. The end points of these fragments relative to the translation initiation codon are shown in Figure 1B. The PCR-amplified products were cloned into the linearized plasmid vector pCR1000 (Invitrogen, San Diego, CA) and sequenced. Based on the orientation of the promoter fragments relative to the restriction endonuclease sites

of the vector, one of two strategies was taken to couple the *SLG* promoter domain to the minimal promoter and *uidA*.

Plasmids that contained inserts facing "left" (i.e., toward the SpeI site of pCR1000) were opened at the SpeI site and joined to the intermediate vector pEMBL-(GUS-46)-pBluescript at a corresponding SpeI site. In the resulting plasmid, the *SLG* promoter fragment is linked to the cauliflower mosaic virus (CaMV) 35S minimal promoter::*uidA* fusion. The region of interest was excised from the plasmids as an EcoRI fragment and cloned into the corresponding site of pBIN19. (The intermediate vector pEMBL-[GUS-46]-pBluescript contains the −46 to +8 region of the CaMV 35S promoter joined to the coding region of the *uidA* gene followed by the polyadenylation and transcription termination region of the ribulose bisphosphate carboxylase small subunit [*rbcS*] gene. This plasmid was generated by cloning a BglII-EcoRI fragment of pEMBL-[GUS-46], containing the CaMV 35S minimal promoter::*uidA*::*rbcS* terminator fusion, into the BamHI-EcoRI sites of the pBluescript SK+ vector).

Plasmids that contained PCR-amplified products facing "right" were opened at a SacI site and fused at this site with pEMBL-(GUS-46)-pBluescript. The resulting plasmids were digested with NotI and religated to place the promoter fragments adjacent to the CaMV 35S::*uidA*::*rbcS* fusion. These constructs were opened at an EcoRI site and fused at the corresponding site to pBIN19.

Plant Transformation

The aforementioned SLG_{13} promoter::*uidA* fusions were introduced into the Agrobacterium strain pCIB542/A136, which is derived from pEHA101 (Hood et al., 1986), and the resulting bacterial strains were used to transform axenic excised leaf tissue of *Nicotiana tabacum* cv Petit Havana, as described by Horsch et al. (1988). Kanamycin-resistant plantlets resulting from independent transformation events were propagated and analyzed. The presence of inserted DNA sequences in the putative transgenic plants was verified in representative numbers of plants for each of the constructs by DNA gel blot analysis.

Histochemical and Fluorometric Assays of GUS Activity

GUS activity was visualized histochemically using the chromogenic substrate 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-gluc), as described by Jefferson et al. (1987). Pistil sections that included the stigma and style were dissected longitudinally and incubated at 37°C in 2 mM X-gluc, 0.1 M $NaPO_4$, pH 7.0, 0.5% (v/v) Triton X-100 for 16 to 18 hr. The tissue was destained in increasing concentrations of ethanol to a final concentration of 95% (v/v) ethanol and observed under a dissecting microscope. Due to oxidative discoloration of the tissue surface, some of the samples were further dissected. Pollen grains were taken from several anthers of an open flower by tapping the grains into the well of a microtiter plate. Pollen was incubated in the above assay buffer at 37°C for 4 to 8 hr, rinsed in 50% (v/v) ethanol, and viewed under a dissecting microscope. Sections of leaf, stem, root, petal, sepal, anther, and filament tissue were analyzed as was done with pistil sections. Representative pollen and pistil sections were photographed under a phase-contrast and dissecting microscope, respectively.

For fluorometric analysis, the extracts of freshly harvested tissue were incubated with the 4-methylumbelliferyl β-D-glucuronide substrate, as described by Jefferson et al. (1987).

Determination of the Nucleotide Sequence of *SLG* and *SLR1* Promoter Regions

The isolation of *SLG* alleles has been previously described. SLG_{13} was isolated from a *B. oleracea* var *acephala* (kale) S_{13} homozygote (Nasrallah et al., 1988), SLG_2 from a *B. oleracea* var *alboglabra* S_2 homozygote (Chen and Nasrallah, 1990), and SLG_8 from the *B. campestris* S_8 homozygote (Dwyer et al., 1991; Toriyama et al., 1991). The *SLR1* gene was isolated from a *B. oleracea* var *acephala* S_{22} homozygote as follows: Genomic DNA was prepared from leaf tissue, purified on CsCl gradients, digested with EcoRI, and fractionated on agarose gels. The region of the gel that contained the *SLR1* gene, previously identified by DNA gel blot analysis, was excised from the gel. The DNA was eluted and used to construct a subgenomic library in the bacteriophage λGEM11 (Stratagene). *SLR1*-containing clones were identified by hybridization to an *SLR1* cDNA probe (Lalonde et al., 1989).

For nucleotide sequence analysis, restriction fragments containing sequences 5′ of the initiating ATG codon were subcloned into appropriate plasmid vectors. For the SLG_{13} and SLG_2 genes, restriction fragments were subcloned into the M13 vectors mp18 and mp19 (Yanisch-Perron et al., 1985), and single-stranded DNA templates were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977). For the SLG_8 and *SLR1* genes, restriction fragments were subcloned into the pUC118 and pUC119 vectors (Vieira and Messing, 1987). A series of nested deletions was generated by ExoIII digestion using the Erase-A-Base kit (Promega), and the DNA sequence was determined on double-stranded plasmid templates (Chen and Seeburg, 1985).

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