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RESEARCH ARTICLE

Activity of an *S* Locus Gene Promoter in Pistils and Anthers of Transgenic Brassica

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The pollen-stigma interaction of self-incompatibility in crucifers is correlated with glycoproteins localized in the cell wall of the stigmatic papillae that are encoded by the *S* locus glycoprotein (*SLG*) gene. When fused to the β-glucuronidase (*GUS*) reporter gene, the 5′ upstream regulatory region of *SLG* directed high level expression in the papillae of transgenic Brassica plants. Histochemical and fluorometric assays revealed that, in addition to its primary site of expression in the stigmatic papillae, the *SLG-GUS* fusion was also expressed in the transmitting tissue of stigma, style, and ovary, and in anthers. This conclusion was verified by the detection of transgene-encoded *GUS* transcripts and endogenous *SLG*-homologous transcripts by RNA gel blot analysis. Significantly, in anthers, the *SLG* promoter was active not only sporophytically in the nurse cells of the tapetum, but also in the haploid microspores. Because self-incompatibility systems exhibiting sporophytic control of pollen phenotype are thought to have evolved from systems with gametophytic control, we suggest that sporophytic control was acquired without loss of gametophytic function.

INTRODUCTION

Self-incompatibility is a genetic block to self-fertilization that is controlled in several plant species by a single polymorphic Mendelian *S* locus (Bateman, 1955). The genetic model for self-incompatibility predicts the arrest of pollen germination and/or subsequent pollen-tube development when pollen and pistil carry identical alleles at the *S* locus. Based on this single-locus control, it is assumed that the products of the *S* locus would be present in cells of the pistil at the site of the self-incompatible reaction and in pollen. The timing and site of *S* locus gene expression during pollen development has been the subject of much speculation because of the occurrence of distinct self-incompatibility systems in which the self-incompatibility phenotype of pollen is determined either gametophytically (as in the Solanaceae) or sporophytically (as in the Brassicaceae). Whereas gametophytic control is thought to result from action of the *S* locus within the haploid microspores or mature pollen after meiosis (for a review, see Heslop-Harrison, 1975), two hypotheses have been presented to explain sporophytic control. The first is based on activity of the *S* locus premeiotically in the pollen mother cells (Pandey, 1960). The second proposes *S* gene action in the tapetum, a sporophytically derived cell layer that surrounds the developing microspores, with subsequent transfer of the *S* gene products onto the surface of microspores after the degeneration of the tapetum (Heslop-Harrison, 1967).

In the crucifer Brassica, the self-incompatibility response has been correlated with the production by the stigma of glycoproteins (Nasrallah and Wallace, 1967a, 1967b; Hinata and Nishio, 1978; Nasrallah and Nasrallah, 1984) encoded by the *S* locus-derived *SLG* genes (*S* locus glycoprotein genes). In the stigma, maximal expression of *SLG* genes coincides with the acquisition of self-incompatibility competence 1 day before anthesis, and the site of expression is consistent with the site of inhibition of pollen germination and pollen tube growth at the stigmatic surface in incompatible pollinations. In situ hybridization experiments have shown that the *SLG* genes are expressed in the papillar cells of the stigma (Nasrallah et al., 1988), and immunogold labeling of thin sections has further demonstrated that *S* locus-specific glycoproteins are secreted into the papillar cell walls where they accumulate (Kandasamy et al., 1989). These localization methods have not

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however allowed the detection of *SLG* gene expression in anthers. To further investigate the question of anther expression, we used a chimeric gene consisting of the 5′ upstream regulatory region of an *SLG* gene fused to the reporter gene β-glucuronidase (*GUS*). The analysis of transgenic plants of the heterologous hosts tobacco, a self-fertile member of the Solanaceae (Thorsness et al., 1991), and Arabidopsis, a self-fertile member of the Brassicaceae (Toriyama et al., 1991), demonstrated that the *SLG* promoter was active during pollen development as well as in pistil tissue. However, two drastically different patterns of promoter activity were observed in the genera *Nicotiana* and *Arabidopsis* (Thorsness et al., 1991; Toriyama et al., 1991), and as a result, the pattern of *SLG* promoter activity in the homologous host Brassica could not be directly inferred.

In this paper, we demonstrate that in Brassica, the *SLG* promoter exhibits yet a third pattern of activity that differs qualitatively and quantitatively from, and is a composite of, the activity patterns previously described in tobacco and Arabidopsis. By a combination of *SLG-GUS* reporter gene analysis in transgenic plants and RNA gel blot analysis, we show that in addition to its activity in the papillar cells of the stigma, the *SLG* promoter is also active in the tapetal cells of the anther, in the transmitting tissue of the style and ovary, and in microspores. These observations have important implications that relate not only to the evolution of self-incompatibility systems, but also, in a more practical context, to the use of heterologous plant systems for inferring promoter activity patterns.

RESULTS

Production of Transgenic Brassica Plants

A chimeric gene consisting of an *SLG* promoter fused to the *Escherichia coli GUS* gene and carried on vector pMKT8 (Thorsness et al., 1991) was used to describe the activity of the *SLG* promoter in various Brassica tissues. A partial restriction map of the T-DNA in pMKT8 is shown in Figure 1. The *SLG* promoter-*GUS* gene fusion was introduced by Agrobacterium-mediated transformation into a self-incompatible kale strain and into a self-compatible oilseed rape strain. The stable integration of the *SLG-GUS* fusion into the genome of transgenic plants was demonstrated by DNA gel blot hybridization and analysis of transgenic progeny. As illustrated in Figure 2, a *GUS* probe did not hybridize to DNA from untransformed controls (lanes 1 in Figures 2A, 2B, and 2C), but identified a restriction fragment of the expected (2 kb) length in genomic DNA isolated from primary kale (Figure 2A, lane 2) and oilseed rape (Figure 2B, lanes 2 to 5) transformants, and from their antibiotic-resistant selfed progenies that inherited the transgene (Figure 2A, lanes 3 to 12, and Figure 2C, lanes 3 to 12).

An initial survey for GUS activity identified the transgenic plants that expressed the *SLG-GUS* gene fusion and indicated that transgene expression was limited to specific tissues of the flower. The fluorometric data in Table 1 show that five independent transgenic plants exhibited high levels of GUS activity in the stigma. With the exception of plant 84-2, the transgenic plants also exhibited GUS

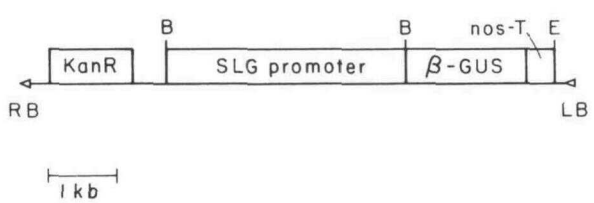

Figure 1. The T Region of the pMKT8 Plant Transformation Vector.

Abbreviations used are as follows: KanR, kanamycin resistance cassette consisting of the neomycin phosphotransferase II gene fused to promoter and terminator sequences from the nopaline synthase gene; nos-T, 3′-terminal region of the nopaline synthase gene; RB, right border; LB, left border; B, BamHI; E, EcoRI.

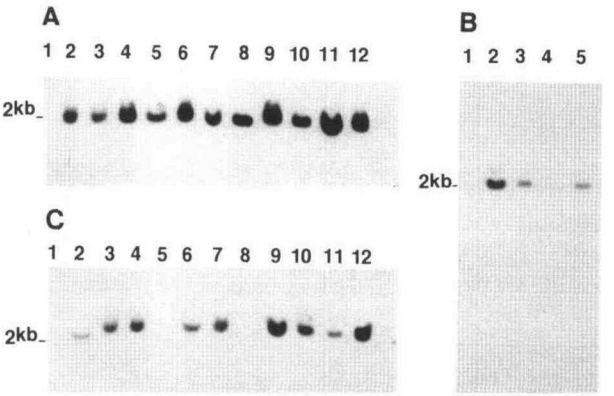

Figure 2. DNA Gel Blot Analysis of Brassica Plants Transformed with the *SLG* Promoter-*GUS* Fusion.

(A) Kale untransformed control (lane 1), primary transformant E29 (lane 2), and progeny plants (lanes 3 to 12). All of the progeny plants shown have inherited the transgene.
(B) Oilseed rape untransformed control (lane 1) and primary transformants (lanes 2 to 5).
(C) Oilseed rape untransformed control (lane 1), primary transformant 84-4 (lane 2), and its progeny plants (lanes 3 to 12). Plants 5 and 8 did not inherit the transgene.
DNA was digested with a combination of BamHI and EcoRI. The blots were hybridized to a ^{32}P-labeled *GUS* probe. The expected 2.0-kb fragment containing the *GUS* gene is detected in transformed plants and not in untransformed controls.

Table 1. Tissue Distribution of *SLG-GUS* Expression in the Transgenic Brassica Flower[a]

Plants	Stigma	Style/Ovary	Anther	Filament	Petal/Sepal	Pedicel
Kale						
Control	0.1	1	3	4	5	2
Transgenic						
E29	4309	42	195	7	8	1
Oilseed rape						
Control	6	2	11	4	11	3
Transgenic						
84-1	1312	48	19	11	10	5
84-2	1737	7	25	19	15	10
84-3	3991	30	15	9	16	6
84-4	1072	34	40	16	19	6
35S-GUS	3638	3409	1908	2668	5168	2452

[a] All values were determined on samples from open flowers by the fluorometric GUS assay using 4-methylumbelliferyl β-D-glucuronide as substrate and are expressed in picomoles of 4-methylumbelliferone per minute per milligram protein.

activity in the style and ovary. Of the five plants, transgenic plant E29, and to a lesser extent plant 84-4, exhibited GUS activity in the anther. The observation that some transgenic plants did not exhibit GUS activity in style/ovary and in anthers is attributed to the relatively low level of *SLG* promoter activity in these structures (see below), combined with differences in site of integration and copy number of the transgene. For example, based on the number of border fragments that hybridized to the *GUS* probe on DNA blots (data not shown) and on the segregation of progeny plants for kanamycin resistance and for inheritance of the *SLG-GUS* transgene (see Figures 2A and 2C), plant E29 carried three T-DNA integrations and plant 84-4 carried one integration. The anther filaments, petals, sepals, and pedicels of transgenic plants and the tissues of control untransformed kale and oilseed rape plants produced only background levels of GUS activity. In Table 1, and for reference, we also show the cauliflower mosaic virus 35S promoter-driven GUS activity values obtained for the tissues of an oilseed rape plant transformed with vector pBI121 (Jefferson et al., 1987).

In the following, we will describe in detail the expression pattern of the *SLG-GUS* fusion in primary transformant E29 and its progeny plants, a self-incompatible transgenic line that exhibited GUS activity in stigma, style/ovary, and anther tissues.

Histochemical and Fluorometric Analysis of *SLG-GUS* Expression in Pistil and Anther Tissue

Transgenic progeny plants were generated by self-pollination of primary transformant E29 at the immature bud stage before the onset of the self-incompatibility response. Because plant E29 carried three unlinked T-DNA integrations, all of the progeny plants analyzed were kanamycin resistant and were shown by DNA gel blot analysis to have inherited the *SLG* promoter-*GUS* fusion (Figure 2A). The pattern of GUS activity described below was derived from the analysis of plant E29 and 13 progeny plants.

The histochemical distribution of *SLG* promoter-driven GUS activity is illustrated in Figure 3. Stigma sections showed intense staining in the papillar cells, and lower levels of activity in the subepidermal cells (Figure 3A). Below the stigma, GUS staining was evident in the central cells of the solid style (Figures 3A and 3B) within a zone defined cytologically as the transmitting tissue (Hill and Lord, 1987) and in the septum that runs through the center of the ovary (Figure 3C). In anthers (Figures 3D to 3F), staining was detected simultaneously in the cells of the tapetum and in the microspores, starting approximately at the late uninucleate microspore stage (Figure 3D). The staining of both cell types intensified progressively during development and was clearly visible in anthers at the early binucleate and mid binucleate microspore stages (Figures 3E and 3F). After the degeneration of the tapetum, GUS staining of the microspores persisted into the mature trinucleate pollen stage. GUS staining was not visible in other cells of the anther or in other tissues of the plant.

The fluorometric quantitation of GUS activity in primary transformant E29 (Table 1) indicated that GUS activity levels were approximately 40,000-fold higher than control levels in the papillar/subepidermal cells of transgenic stigmas, 100-fold higher than control levels in the tapetum/microspores of transgenic anthers, and 40 times higher than control levels in the transmitting tissue of the styles and ovaries. A similar pattern of *SLG-GUS* gene expression was also evident in the progeny of plant E29. Table 2 shows that all progeny plants exhibited above-background GUS activity levels in the stigmas, styles/ovaries, and pollen of open flowers. It can be seen that considerable

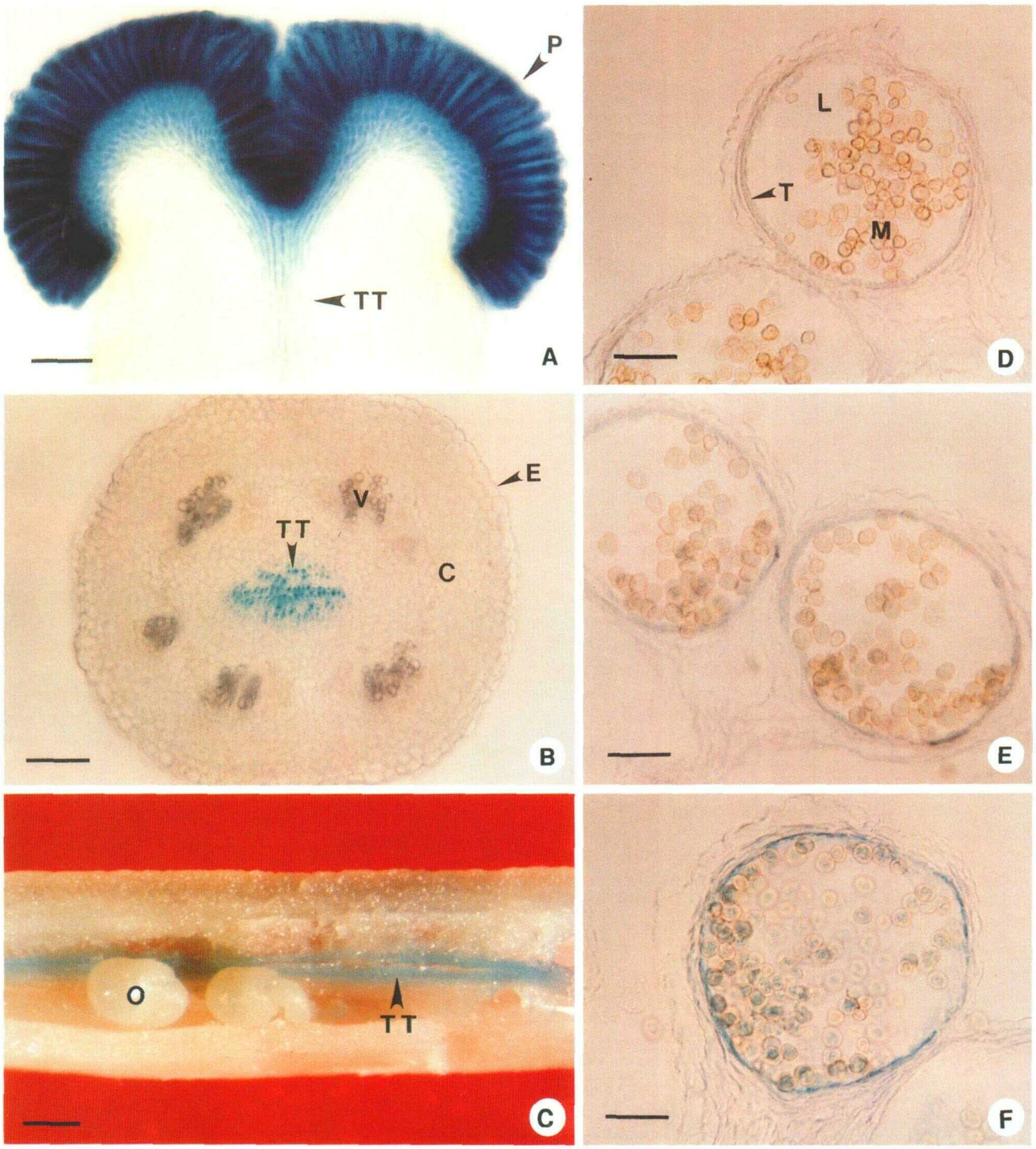

Figure 3. Histochemical Localization of GUS Activity in the Pistil and Anther of Transgenic Kale.

(A) Longitudinal section of stigma and upper portion of style. The single-layered crown of papillar cells (P) at the stigma surface shows intense blue staining. The subepidermal cells of the stigma and the transmitting tissue (TT) of the style show light blue staining. Bar = 0.1 mm.

(B) Cross-section of the style. The central transmitting tissue (TT) of the style is stained blue. E, epidermis; C, cortex; V, vascular bundle. Bar = 0.1 mm.

(C) Longitudinal section through the ovary. The transmitting tissue (TT) in the middle of the septum is stained blue. O, ovule. Bar = 0.3 mm.

Table 2. GUS Activity in Stigma, Style/Ovary, and Pollen of the Progeny Plants of Transgenic Kale[a]

Organ/ Cell Type	Plant Number												
	1	2	3	4	5	13	19	25	42	52	53	58	70
Stigma	3108	942	2591	204	1269	2474	2633	1521	4842	793	1391	835	1185
Style/Ovary	57	125	51	52	101	156	66	146	291	65	155	105	134
Pollen	227	227	463	194	417	329	252	241	375	606	295	180	280

[a] All values were determined on samples from open flowers by the fluorometric GUS assay using 4-methylumbellifery β-D-glucuronide as substrate and are expressed in picomoles of 4-methylumbelliferone per minute per milligram protein. The values for samples from untransformed control plants were equivalent to those in Table I.

plant-to-plant variation was observed in the absolute fluorometric values and in the relative levels of GUS activity between organs. This quantitative variability is ascribed to the differences in the number and nature of T-DNA integrations inherited and to differences in homozygosity for one or more integration. Because all of the progeny plants exhibited GUS activity in stigma, style/ovary, and pollen, we infer the presence of at least one active *SLG-GUS* transgene at each site of T-DNA integration. However, the site of integration apparently had a significant influence on the relative activities of the transgene in cells of the pistil and in pollen. In different transgenic progenies, GUS activity levels ranged between 2 to 25% of stigma values in style/ovary and between 7 to 95% of stigma values in pollen, and the highest activity in stigma was not always correlated with the highest activity in style/ovary and in pollen.

We quantitated the developmentally regulated changes in *SLG-GUS* expression in primary transformant E29 by performing fluorometric GUS assays on stigmas and anthers from buds at different stages of development. Figure 4 shows that GUS activity was first detected in stigmas and anthers from buds approximately 5 mm in length that contained microspores at the late uninucleate/early binucleate stage. Thereafter, the level of GUS activity in stigmas increased several thousandfold and was highest at maturity in the open flower. The induction of GUS activity was more modest in anthers than in stigmas, but this activity persisted throughout subsequent development even after the degeneration of the tapetum and into the mature pollen stage. The activity of the *SLG* promoter in styles and ovaries was also developmentally regulated and was first detected at later stages of development in 9- to 10-mm buds (data not shown). No GUS activity was detected in pistils and anthers of untransformed control plants at any stage of development.

RNA Gel Blot Analysis of *SLG* Promoter Activity

The tissue distribution of GUS activity in pistils and anthers prompted us to characterize *SLG* promoter activity by gel blot analysis of poly(A)$^+$ RNA from hand-dissected styles and ovaries and from isolated microspores. Our aim was to ascertain that GUS activity in cells of the transmitting tissue of the style and ovary and in microspores was due to transcription of the chimeric *SLG-GUS* gene in these cells, and not to artifactual translocation of GUS enzyme, substrates, and/or reaction products from the primary sites of *SLG* promoter activity in the papillar cells of the stigma and the tapetal cells of the anther.

Previous RNA gel blot analysis of Brassica had shown that *SLG* transcripts were easily detected in stigmas even when small quantities of poly(A)$^+$ RNA (<0.1 μg) were analyzed. On the other hand, the GUS activity levels observed in transgenic styles, ovaries, and microspores were in general indicative of a much lower level of *SLG* promoter activity in these structures. We, therefore, performed our gel blot analysis with approximately 100 and 50 times

Figure 3. (continued).

(D) to (F) Cross-sections through anthers demonstrating the developmental increase of GUS activity in the tapetum and microspores. Bars = 50 μm.

(D) Uninucleate microspore stage (bud size, 5.3 mm); the blue staining of the tapetum and microspores begins at this stage. T, tapetum; L, locule; M, microspores.

(E) Early binucleate microspore stage (bud size, 6.1 mm). Note the increase in staining intensity of the tapetum. The microspores also show light blue staining.

(F) Mid binucleate microspore stage (bud size, 7.0 mm). Blue staining is clearly evident in the tapetum and the microspores.

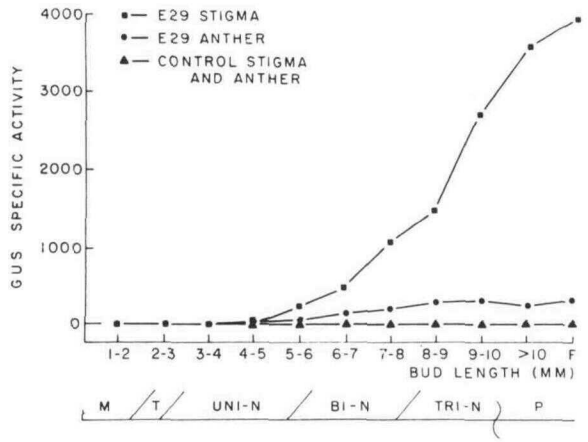

Figure 4. Expression of the *SLG-GUS* Fusion in Developing Stigmas and Anthers of Transgenic Kale.

GUS activity was determined fluorometrically on extracts of stigmas and anthers dissected from the buds of transgenic and untransformed control plants. The numbers on the x axis indicate bud length in millimeters, and F designates the open flower stage. **Bottom**, the developmental stage of microspores within these buds is indicated. M, meiosis; T, tetrad stage; Uni-N, uninucleate microspore stage; Bi-N, binucleate microspore stage; Tri-N, trinucleate microspore stage; P, mature pollen. The increase in GUS activity occurred simultaneously in stigmas and anthers from 5-mm buds at the late uninucleate microspore stage.

more style/ovary and microspore poly(A)$^+$ RNA, respectively, than stigma poly(A)$^+$ RNA (0.02, 2, and 1 μg of stigma, style/ovary, and microspore poly(A)$^+$ RNA, respectively). The "stigma" tissues included the stigma proper and the underlying 1-mm segment of stylar tissue, and the "style/ovary" tissues consisted of 2-mm segments of the pistil immediately below the "stigma" tissues. The microspores were isolated as described in Methods from anthers at the binucleate microspore stage, a stage at which GUS staining of microspores was clearly visible.

To detect *SLG-GUS* transcripts in transgenic tissues, we used a probe derived from the protein-encoding region of the *GUS* gene, and to detect endogenous *SLG* transcripts in kale plants homozygous for the S_6 allele, we used BOS6, a stigma *SLG*-cDNA probe derived from the kale S_6 homozygote (Nasrallah et al., 1987). The results of this analysis are shown in Figure 5. Three different transgenic progeny plants (plants 1, 25, and 52) were analyzed with the results illustrated in Figure 5A. *GUS* transcripts were detected in dissected styles/ovaries (lane 1) and in microspores (lanes 3 to 5) from transgenic progeny plants, but were absent in tissues of untransformed control plants (lane 2). Figure 5B shows that in kale S_6S_6 plants, endogenous *SLG*-homologous transcripts were detected not only in stigmas (lane 1), but also in styles/ovaries (lane 2) and microspores (lane 3). Significant differences in the steady-state levels of endogenous *SLG*-homologous transcripts were observed between the three samples. Stigma RNA produced an intense hybridization signal with the *SLG* probe even after short (2 to 3 hr) exposure to x-ray film. Style/ovary RNA produced a lower but detectable signal with similar exposure times, but the visualization of hybridizing transcripts in microspore RNA required much longer (2 to 3 days) exposure times. These observations were all the more striking because much less stigma RNA was analyzed relative to style/ovary and microspore RNA. Moreover, the differences in hybridization signal were specific to the *SLG*-homologous transcripts because subsequent hybridization of the same blot to an actin probe resulted in hybridization signals consistent with the quantity of RNA analyzed (Figure 5B). After normalizing for the amount of poly(A)$^+$ RNA contained in each sample, we estimate that the steady-state level of endogenous *SLG* mRNA in stigmas is three orders of magnitude higher than in styles/ovaries and four orders of magnitude higher than in microspores.

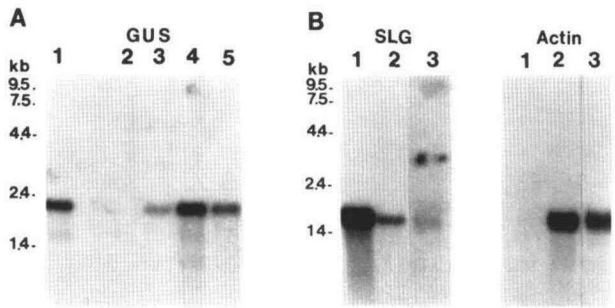

Figure 5. RNA Blot Analysis of *SLG* Promoter Activity.

(A) Transcripts in transgenic kale encoded by *SLG-GUS*. Poly(A)$^+$ RNA was isolated from styles/ovaries of transgenic progeny plant 52 (lane 1), from microspores of an untransformed control plant (lane 2), and from microspores of transgenic progeny plants 1 (lane 3), 52 (lane 4), and 25 (lane 5). A 2.0-kb transcript was detected by the *GUS* probe in transgenic tissues.

(B) Endogenous *SLG* transcripts in the kale S_6S_6 homozygote. *SLG* transcripts were detected in poly(A)$^+$ RNA from stigmas (lane 1), styles/ovaries (lane 2), and microspores (lane 3). Approximately 0.02, 2, and 1 μg of poly(A)$^+$ RNA were loaded, respectively, in the three lanes. The hybridization signals obtained with the actin probe are correlated with the relative amounts of RNA loaded.

The times of exposure to x-ray film at −70°C were 24 hr for the *GUS* probe in **(A)**; 3 hr for the stigma and style/ovary (lanes 1 and 2) and 4 days for the microspore (lane 3) with the *SLG* probe in **(B)**; 3 hr for the actin probe in **(B)**.

DISCUSSION

We have described the pattern of *SLG* promoter activity in two *Brassica* species, the self-incompatible *B. oleracea* and the self-compatible *B. napus*. Our analysis of reporter gene expression and endogenous *SLG*-homologous transcripts have shown that, in addition to the major site of promoter activity in the papillar cells of the stigma, expression was also evident at lower levels in the transmitting tissue of the style, in the septum of the ovary, and in anthers, specifically in cells of the tapetum and in the haploid microspores.

In the pistil, *SLG* promoter activity was observed along the path of pollen tube growth as described by the study of gynoecial morphology in a crucifer (Hill and Lord, 1987). Promoter activity in the stigmatic papillar cells was consistent with the results of transcript localization (Nasrallah et al., 1988) and immunolocalization (Kandasamy et al., 1989), which demonstrated that these cells were the primary site of *SLG* gene expression. However, the activity of the *SLG* promoter in the stylar transmitting tissue and in the ovarian septum of Brassica was only revealed in this study. The reporter enzyme-based assays and the analysis of poly(A)$^+$ RNA reported here were clearly more sensitive than the earlier RNA and protein localization studies and allowed the detection of the relatively low level of *SLG* promoter activity in styles and ovaries. We have shown not only that transgene-encoded *GUS* transcripts were present in isolated styles and ovaries of transgenic plants, but also that endogenous *SLG*-homologous transcripts were detected in these structures, provided sufficient quantities of poly(A)$^+$ RNA were analyzed.

The observation that the *SLG* gene was expressed during pollen maturation in the cells of the tapetum and in haploid microspores shed new light on the molecular basis of sporophytic control of self-incompatibility phenotype. Tapetal cell expression was consistent with the view that sporophytic control of pollen self-incompatibility phenotype is based on *S* gene action in the tapetum followed by transfer of *S* gene products onto the surface of microspores after tapetal cell degeneration (Heslop-Harrison, 1967). However, the observation of GUS staining in the haploid microspores after the completion of meiosis was indicative of gametophytic promoter activity. In addition, the possibility of earlier but lower level activity in the premeiotic pollen mother cells was not excluded by our data. GUS staining represented bona fide *SLG* promoter activity in microspores as indicated by the detection of *GUS* transcripts in these cells. Furthermore, isolated microspores contained transcripts that hybridized to an *SLG* probe. Although we cannot rule out the possibility that the activity of related members of the self-incompatibility gene family (Dwyer et al., 1989; Stein et al., 1991) contributes to the *SLG* hybridization signal on RNA blots, at least a fraction of these transcripts is encoded by the *SLG* gene. Independent confirmation of the expression of the *SLG* gene in anthers was obtained through the isolation of *SLG*-encoded cDNAs from a λgt10 cDNA library constructed with RNA isolated from kale S_6S_6 anthers at the binucleate microspore stage (S.-M. Yu and J. B. Nasrallah, unpublished observations). Consistent with the low level of *SLG* promoter activity in cells of the anther, only six cDNA clones were recovered in a screen of more than 10^6 recombinant phage in this anther library with the BOS6 *SLG*-cDNA probe. The nucleotide sequence of the cloned inserts, which ranged in length from approximately 500 to 1600 bp, was identical to the corresponding sequence of the *SLG* gene from the S_6 homozygous strain (Nasrallah et al., 1987).

It should be noted that the results of the analysis of the *SLG-GUS* gene fusion were qualitatively similar to, but differed quantitatively from, those obtained by endogenous *SLG* transcript analysis. In particular, when the relative levels of promoter activity in styles and ovaries on the one hand and microspores on the other hand were compared, reporter gene analysis suggested that promoter activity was higher in microspores than in styles and ovaries, whereas the reverse was indicated by endogenous transcript analysis. One explanation for this discrepancy is that *SLG* promoter activity is subject to quantitative regulation by *cis*-acting elements that lie outside the *SLG* 5′ fragment used in our *SLG-GUS* construct. Alternatively, it is possible that differences exist in the relative stabilities of *GUS* and *SLG* transcripts in microspores and styles/ovaries. Thus, our simultaneous analysis of the expression of the *SLG-GUS* fusion and of the endogenous *SLG* gene, although underscoring the usefulness of reporter-gene analysis as a method for describing cell type-specific patterns of gene expression in a qualitatively faithful manner, may point to some limitations of this method for deriving quantitative information on relative levels of gene expression.

Another and perhaps more significant limitation of reporter gene analysis, at least when performed in heterologous hosts, as is often the practice, is emphasized by our analysis of the same *SLG-GUS* construct in three different transformation hosts, Arabidopsis (Toriyama et al., 1991), tobacco (Thorsness et al., 1991), and Brassica (this paper). The overall conclusion from the analysis in the three genera, *Arabidopsis*, *Nicotiana*, and *Brassica*, is that the *SLG* promoter is active only in pistil and anther tissue. However, the patterns of *SLG* promoter activity in these tissues differed qualitatively and quantitatively between species, with the Brassica pattern being a composite of the patterns described in the heterologous hosts Arabidopsis and tobacco. In transgenic Arabidopsis, *SLG* promoter activity was detected in only two cell types, the papillar cells of the stigma and the tapetal cells of the anther (Toriyama et al., 1991). In transgenic tobacco on the other hand, the *SLG* promoter was maximally active throughout the transmitting tissue of the stigma and style, with lower activity observed in the placental epidermis of the ovary, and was active gametophytically in mature

pollen grains (Thorsness et al., 1991). Significantly, however, no *SLG* promoter activity was detected in the tapetum of transgenic tobacco anthers (Thorsness et al., 1991). As has been already noted (Thorsness et al., 1991), the pattern of expression of the *SLG* promoter in transgenic tobacco is consistent with the known features of self-incompatibility in the self-incompatible solanaceous relatives of tobacco, which exhibit stylar inhibition of self-pollen and gametophytic control of pollen incompatibility phenotype. The observed species-specific expression patterns thus point to the existence in each species of endogenous genes that are similarly regulated in pistil and anther, and would reflect the distribution of endogenous *trans*-acting factors available for interaction with the *SLG* promoter elements. Such differences in the interpretation of *cis*-regulatory elements in different species are not restricted to the system reported here. A recent study of the cauliflower mosaic virus 35S promoter has shown differences in the activity of this viral promoter in transgenic plants of the two closely related genera *Petunia* and *Nicotiana* (Benfey and Chua, 1990).

The evolutionary relationships suggested by the analysis of the *SLG-GUS* fusion in transgenic tobacco and Brassica have been previously considered. In particular, it has been proposed that systems with sporophytic control of pollen self-incompatibility phenotype evolved from systems with gametophytic control by a shift in the timing of *S* gene action from haploid microspores to the premeiotic pollen mother cells (Pandey, 1960). The possibility of dual sporophytic and gametophytic activity of an *S* locus gene had not been considered by theoretical models of sporophytic control, however, perhaps because the gametophytic activity would be redundant, in effect being masked by the associated sporophytic activity. Nevertheless, genetic evidence has been recently presented in the genus *Cerastium* (Caryophyllaceae) for a one-locus sporophytic *S* gene system reinforced with back-up "traces of gametophytic pollen control" (Lundqvist, 1990). It can thus be argued that the expression of the *SLG* gene in microspores and in the transmitting tissue of the style and ovary of Brassica may constitute remnants of a primitive pollen recognition system with gametophytic control and associated stylar inhibition, and suggests that sporophytic control may have been acquired without loss of elements of gametophytic function.

METHODS

Plant Transformation Vectors

The construction of vector pMKT8 (see Figure 1) that carries *SLG-GUS*, a chimeric gene consisting of the promoter region of the *SLG*-13 gene fused to the *GUS* reporter gene (Jefferson et al., 1987), and a chimeric nopaline synthase-neomycin phosphotransferase II gene for selection of transformed shoots was described in Thorsness et al. (1991). The vector was mobilized into *Agrobacterium tumefaciens* pCIB542/A136 (derived from pEHA101; Hood et al., 1986) by direct transformation (An et al., 1988).

Plant Material and Transformation

Two strains of *Brassica* were used in plant transformation experiments: the self-compatible *B. napus* spp *oleifera* cv Westar (oilseed rape) and the self-incompatible *B. oleracea* var *acephala* (marrow stem kale; designated "kale" for brevity) S_6S_{14} heterozygote.

Flowering stem discs were used for *A. tumefaciens*-mediated transformation essentially as described by Fry et al. (1987). Kale plants required vernalization, and were, therefore, subjected to a 2-month treatment at 4°C to induce flowering. Flowering stems were precultured on medium containing 1/10 strength MS salts (Murashige and Skoog, 1962), 1/1 B5 vitamins, 1% (w/v) sucrose, 1 mg/L 6-benzylamino purine, 0.8% (w/v) Phytoagar (GIBCO), and adjusted to pH 5.8. Preculture was for a period of 2 days in the case of oilseed rape and overnight for kale. Cocultivation with *A. tumefaciens*, selection, and shoot induction were on media consisting of standard MS salts and vitamin mixtures (Murashige and Skoog, 1962), 0.5 mM arginine, 3% (w/v) sucrose, 1 mg/L 6-benzylamino purine, 0.8% Phytoagar, and adjusted to pH 5.8, with the appropriate antibiotics added for selection. Transgenic shoots were selected on medium containing 100 mg/L kanamycin for kale and 10 mg/L geneticin (G418, Sigma) for oilseed rape. Antibiotic resistance was confirmed by culturing leaf segments on medium containing antibiotic (Fry et al., 1987). In this leaf test, untransformed tissue was bleached or turned brown within 10 to 14 days, whereas transformed tissue remained green and produced callus. The transgenic shoots thus identified were transferred to the soil after rooting and maintained in the greenhouse.

GUS Assays

Fluorometric and histochemical GUS assays were as described by Jefferson et al. (1987). For fluorometric analysis, the extracts of freshly harvested tissue were incubated with the 4-methylumbelliferyl β-D-glucuronide substrate. For histochemical analysis, the substrate was 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc; Research Organics, Inc., Cleveland, OH). Pistils were dissected from buds and flowers at different stages of development, and hand sections from different regions of the pistils were obtained and immediately processed for histochemical staining. Tissue sections were incubated at 37°C in 2 mM X-gluc in 0.1 M $NaPO_4$ (pH 7.0) and 0.5% Triton X-100 for 24 hr. The sections were dehydrated in ethanol, treated with xylene, and mounted in Permount (Fisher Scientific). Portions of anthers at different stages of development were stained with X-gluc as above, rinsed in 100 mM $NaPO_4$ (pH 7.0), and processed for cryosectioning. The samples were embedded and frozen in Tissue-Tek OCT compound (Miles Inc., Elkhart, IN) and sectioned at −20°C using a SLEE Cryostat Microtome. Ten- to 12-μm sections were placed on polylysine coated slides. After drying at room temperature for a few hours, the slides were washed in distilled water to remove the embedding medium, dehydrated, treated with xylene, and

mounted in Permount. The sections were observed with a Zeiss optical microscope.

DNA Gel Blot Analysis

DNA was extracted from leaves of transgenic plants by a mini-preparation procedure (Mettler, 1987). The DNA was digested with restriction enzymes, fractionated on 0.7% (w/v) agarose gels, and transferred to GeneScreen-*Plus* membranes (Du Pont-New England Nuclear). Prehybridization and hybridization were conducted at 65°C in a solution containing 10% (w/v) dextran sulfate, 330 mM sodium phosphate (pH 7.0), 10 mM EDTA, 5% (w/v) SDS, and 150 μg/mL calf thymus DNA. DNA probes were labeled with ^{32}P by the random-primed labeling procedure (Feinberg and Vogelstein, 1983) using a commercial kit (Boehringer Mannheim). The presence of the *SLG-GUS* transgene was detected with a probe consisting of the *GUS* coding sequence (Jefferson et al., 1987). Filters were washed at 65°C in 0.3 M NaCl, 40 mM Tris (pH 7.8), 2 mM EDTA, and 0.5% (w/v) SDS. The blots were exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

RNA Gel Blot Analysis

Pistils were hand dissected to obtain a "stigma" fraction that consisted of the stigma proper and 1 mm of underlying stylar tissue, and a "style/ovary" fraction which consisted of segments that included portions of the style and ovary. To isolate microspores, anthers were cut transversely into 1-mm segments and placed in a spun column. Microspores were pelleted in a buffer consisting of 10 mM Tris-HCl, pH 7.2, and 1 mM EDTA by centrifugation at 1,000*g*. Poly(A)$^+$ RNA was extracted from Brassica tissues with the FastTrack kit (Invitrogen, San Diego, CA) and fractionated on 1% (w/v) agarose in the presence of formaldehyde. The RNA was transferred to GeneScreen membranes. The membranes were prehybridized and hybridized as described above for DNA gel blots. *GUS* transcripts were detected with a probe consisting of the *GUS* coding sequence, and *SLG* transcripts were detected with BOS6, a stigma cDNA clone derived from the *SLG* gene and isolated from the kale S_6S_6 homozygote (Nasrallah et al., 1987).

Segregation Analysis of Transgenic Progeny

Seeds obtained from the selfing of antibiotic-resistant transgenic plants were surface-sterilized and sown in Petri plates containing MS salts and vitamins, 3% (w/v) sucrose, 0.8% (w/v) Phytoagar, and 100 mg/L kanamycin. The numbers of green kanamycin-resistant seedlings and bleached antibiotic-sensitive plants were scored 1 week after sowing.

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