Plant Physiol. (1996) 112: 1601–1607

Functional Analysis of a *Brassica oleracea SLR1* Gene Promoter[1]

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The *Brassica oleracea* S-locus-related gene 1 (*SLR1*) is expressed in the papillar cells of *Brassica* stigmas from a few days before anthesis. We have previously shown that a 1500-bp fragment of the *SLR1* gene promoter is sufficient to direct high-level, temporally regulated expression of the β-glucuronidase reporter gene in the pistils of transgenic tobacco. We have carried out a deletion analysis of the *SLR1* promoter and found that elements required for pistil expression are located between −258 and −327 bp (relative to the translation start site). Furthermore, specific binding of pistil nuclear factors to sequences within this region was demonstrated by gel retardation analysis. Sequences between −1350 and −1500 were found to be required for high-level expression.

SLR1 of *Brassica oleracea* is a member of the *Brassica* self-incompatibility (*S*) multigene family. It is coordinately expressed with the *SLG* gene, exhibiting a tightly regulated pattern of expression, with *SLR1* transcripts being detectable in the papillar cells of *Brassica* stigmas from 3 d before anthesis (Lalonde et al., 1989). Since genetic studies indicate that *SLR1* is not linked to the *S* locus, it is unlikely to have a direct role in controlling the specificity of the self-incompatibility reaction. Nevertheless, it is thought to contribute some other key function in the pollen-pistil interaction, because it is highly conserved among the Brassicaceae and a homolog has been detected in Arabidopsis (Dwyer et al., 1992).

We have previously carried out an analysis of the *SLR1* promoter-directed gene expression in transgenic tobacco (Hackett et al., 1992). The promoter that was used was obtained from *B. oleracea* plants that were homozygous for the S_{63} allele (Trick, 1990). A chimeric gene construct that contained 1.5 kb of the *SLR1* promoter fused to the *GUS* reporter gene (Jefferson et al., 1987) was introduced into tobacco plants, and the resulting transformants expressed the *GUS* gene in stigmas, styles, and pollen. Furthermore, expression was temporally regulated, showing a pattern of regulation similar to that of the *SLR1* gene in *Brassica*. GUS activity in pistils was first detectable at 5 d prior to anthesis, before rising sharply at 3 d before flowering and then remaining high as the buds opened.

We report here the results of a deletion analysis of the 1.5-kb *SLR1* promoter region aimed at delineating sequences within the promoter that control aspects of the expression pattern of this *S* gene family member.

MATERIALS AND METHODS

SLR1 Promoter Constructs

A nested set of deletions in the *SLR1* promoter was generated by synthesizing a set of oligonucleotide primers and using PCR to amplify the corresponding fragments. The template for PCR reactions was the plasmid pBluescript (Stratagene) containing 2.3 kb of S63 SLR1 promoter, extending up to the SLR1 translation initiation codon. This plasmid was kindly provided by Dr. M. Trick (John Innes Centre, Norwich, UK). *Hin*dIII and *Bam*HI restriction sites were incorporated into the 5′ and 3′ primers, respectively, allowing the fragments to be cloned into these sites of the plasmid pUC18. Following verification of the nucleotide sequence of the cloned fragments, they were subcloned into the *Hin*dIII and *Bam*HI sites of pBI101. The 5′ end points of PCR fragments are shown in Figure 1. The 3′ PCR primer corresponded to the region of the SLR1 promoter shown in Figure 1 and an additional 11 bp of the pBluescript multiple cloning site.

A further set of promoter deletions was obtained by Bal 31 exonuclease digestion of the 687-bp *SLR1* promoter described in Figure 4a, following cleavage at the single *Xho*I site. Digested ends were made blunt by the addition of nucleotide triphosphates using the Klenow fragment of DNA polymerase, and then religated. The nucleotide sequence of the deleted constructs was analyzed and end points of the internal deletions were determined.

Transformation of Tobacco

Constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Wen-jun and Forde, 1989). Bacteria containing the required construct

[1] This work was supported by the European Community Bridge Program and a European Molecular Biology Organization short-term fellowship to R.M.H.

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Abbreviations: *SLG*, *S*-linked glycoprotein gene; *SLR1*, *S*-locus-related gene 1.

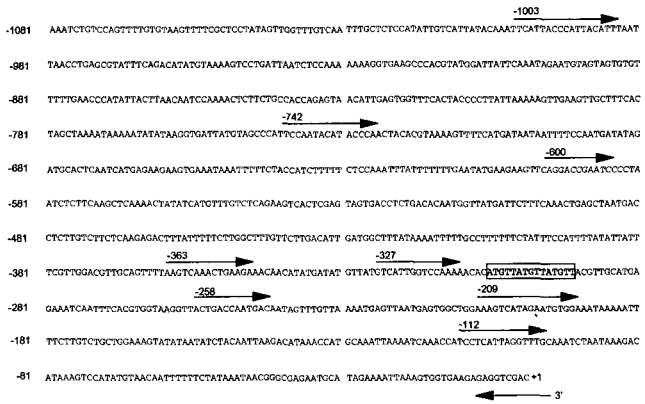

Figure 1. Nucleotide sequence of the S_{63} *SL21* 5′ region. All positions are relative to the translation start site. The positions of oligonucleotide primers used for PCR amplification are indicated by arrows. The position of the translation start site is indicated by +1. The position of the ATGTT tandem repeat is indicated by a box around the sequence.

were then used to infect leaf explants of *Nicotiana tabacum* cv Xanthi. Plantlets were regenerated on 100 mg/L kanamycin and grown to maturity. Primary transformants were used in the analyses; each plant contained a variable number of inserts of the transgene as determined by Southern analysis using the GUS coding sequence as a probe (data not shown).

Fluorometric GUS Assays

Extracts were prepared by homogenizing single stigmas or styles in 100 μL of extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 1 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100). The stigma was dissected from the pistil tissue immediately below the stigmatic lobes. The style included tissue from below this point down to, but not including, the ovary.

For the assays, 1 to 10 μL of extract was added to assay buffer (1 mM 4-methylumbelliferyl β-D-glucuronide [Sigma] in extraction buffer) and incubated at 37°C. One-hundred-microliter aliquots were removed at set time points and the reaction was stopped by addition of stop buffer (0.2 M Na_2CO_3) to 900 μL. Relative fluorescence was read on a TKO fluorometer (Hoefer, Newcastle under Lyme, UK) and standardized with 50 nM methylumbelliferone (Sigma) in stop buffer. Protein content of the extracts was determined using the protein assay from Bio-Rad. Results obtained for each set of transformants (3–15 plants per construct; replicate assays were made on stigmas and styles from each individual transformant) were analyzed by two-way analysis of variance and a variation of the Student's *t* test, which allows comparison of the means of two small samples (Bailey, 1959).

Histochemical GUS Assays

Tissue was incubated in a 1 mM solution of 5-bromo-4-chloro-indolyl β-D-glucuronide (Clontech, Palo Alto, CA) in 50 mM phosphate buffer, pH 7.0, at 37°C overnight. Following incubation in 5-bromo-4-chloro-indolyl β-D-glucuronide solution, tissue was washed in ethanol to clear off chlorophyll. Where histochemical analysis showed an absence of GUS activity in transgenic pistil tissue, one to four fluorometric assays were carried out to confirm the absence of GUS activity by a further test.

Preparation of Pistil Nuclear Extracts

Tobacco pistils were ground in liquid nitrogen and transferred to a beaker containing ice-cold extraction buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Hepes, pH 7.9, 0.1% Nonidet P-40, 10% glycerol, 0.5 mM DTT, and 1 mM PMSF). The suspension was briefly homogenized with a Polytron before being filtered through nylon mesh. Nuclei were pelleted by centrifugation at 500*g*, washed in extraction buffer, and repelleted. Nuclei were then resuspended in dialysis buffer (20% glycerol, 40 mM KCl, 25 mM Hepes, pH 7.9, 5 mM $MgCl_2$, and 5 mM DTT). Ammonium sulfate was added to a final concentration of 0.4 M. The solution was incubated on ice for 30 min before centrifugation at 4°C in a microfuge. The supernatant was dialyzed against dialysis buffer overnight. Insoluble matter was pelleted in a microfuge at 4°C. Protein concentration was determined using the Bio-Rad protein assay (as above). Preliminary purification of the extract was carried out by gel filration using a Pharmacia LKB fast protein liquid chromatography system with a Superose 12 HR 10–30 column. Five hundred microliters of crude extract was injected into the column and eluted in dialysis buffer at a flow rate of 0.25 mL min^{-1}. The fractions were monitored by measuring A_{280} and 1-mL fractions were collected using a fraction collector (Frac-100, Pharmacia) every 4 min. Protein concentrations of the resulting fractions were measured using the Bio-Rad protein assay.

Gel Retardation Assays

Promoter fragments that were amplified by PCR were digested with *Hin*dIII to give overhanging ends, which were then filled in using DNA polymerase I (Klenow fragment), [^{32}P]dCTP, and nucleotide triphosphates, to a specific activity of approximately 1.8×10^8 cpm/μg.

In a typical reaction 0.1 to 0.2 ng of probe was added to 1 to 3 μg of protein of the appropriate fast protein liquid chromatography fraction in a 25-μL reaction volume containing 10% glycerol, 40 mM KCl, 25 mM Hepes, pH 7.9, and 5 mM $MgCl_2$. One to 2 mg of the synthetic copolymers p(dA-dT)·p(dA-dT) or p(dI-dC)·p(dI-dC) was added to the reaction, which was incubated at 4°C for 15 min before being loaded onto a 7% polyacrylamide gel containing 6.75 mM Tris-HCl, pH 8.0, 3.3 mM sodium acetate, pH 7.9, and 1 mM EDTA. Electrophoresis was carried out in buffer containing 13.5 mM Tris, pH 8.0, 6.6 mM sodium acetate, pH 7.9, and 2 mM EDTA, pH 8.0, at 4°C for 2 h. Gels were then dried onto filter paper and exposed to autoradiography film or PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) overnight.

RESULTS

SLR1:GUS Constructs

A 1.5-kb fragment of the *SLR1* 5′ flanking region, hereafter referred to as the full-length promoter, was previously shown to direct *GUS* gene expression in pistils of transgenic tobacco (Hackett et al., 1992). To determine the position of the elements that are responsible for the tissue-specific activity of the *SLR1* promoter in transgenic tobacco, a nested set of deletions was constructed. PCR primers were synthesized so that fragments of the promoter could be amplified with 5′ end points at positions −1003, −742, −600, −363, −327, −258, −209, and −112 bp relative to the translation start site (+1) (Fig. 1). These promoter fragments were then ligated upstream of the *GUS* reporter gene in the binary vector pBI101 and introduced into tobacco. The resulting transformants were grown to flowering and analyzed for expression of the reporter gene.

Expression Pattern of SLR1 Promoter Constructs

Transformants were initially tested for GUS activity using the histochemical assay. Reporter gene expression was detected in stigmas and styles of plants transformed with all of the constructs with promoter deletions between −1003 and −327. GUS activity was not, however, detected in pistil tissue of plants transformed with the −258, −209, and −112 *SLR1:GUS* fusions following analysis of buds from 33, 8, and 19 transformants, respectively. Neither was it detected in vegetative tissues of any transformants. GUS activity was measured in −1003 to −327 deleted constructs using the fluorometric assay for GUS. Mean levels of GUS activity in stigmas and styles of transformants at flowering are shown in Table I.

The analysis indicated that a key promoter element (or elements) responsible for expression of the *GUS* gene in tobacco pistils is localized between −258 and −327 nucleotides upstream of the start of translation. To examine the temporal pattern of promoter activity, stigmas and styles from transformants that expressed the reporter gene were collected at the following stages of pistil maturation: flowering, −3, and −5 d relative to flowering, corresponding to buds of length 44, 31, and 16 mm, respectively. The stigmas and styles were then analyzed fluorometrically. Analysis of the stigma tissue revealed that there were no significant differences (P > 5%) in the maximal level of GUS activity between any of the truncated promoter constructs (Fig. 2a). However, it was particularly noticeable that, whereas the maximum GUS activity for the −327 to −1003 constructs fell within the range of 300 to 750 pmol min^{-1} mg^{-1} protein, this was at least 1 order of magnitude lower than that directed by the full-length *SLR1* promoter (Fig. 3, a and b), which gave mean GUS activities of 20,883 and 2,006 pmol 4-methylumbelliferone min^{-1} mg^{-1} protein in stigmas and styles, respectively. The overall level of reporter gene activity was lower in styles than in the corresponding stigma tissue (Fig. 2b), although the −1,003 transformants showed significantly greater GUS activity in this tissue than the other sets of transformants (P < 2.5%).

Regarding temporal regulation, expression of the *SLR1* gene is first detected in stigma cells shortly before anthesis and then rapidly accumulates until flowering. Our earlier work has shown that this expression pattern is reproduced in the pistils of transgenic tobacco carrying the full-length *SLR1:GUS* promoter fusion (Hackett et al., 1992). Stigmas and styles from plants transformed with the deleted *SLR1* promoter series were analyzed for evidence of similar temporal regulation of GUS activity. Between the −5 stage and flowering at most a 1.5- to 3-fold increase in GUS activity was detected. This marginal increase in GUS activity is significantly lower in magnitude than the 12-fold increase detected over the same period in stigmas of plants transformed with the full-length promoter construct.

Primary transformants were used to analyze *SLR1*:GUS constructs, and the number of copies of the the transgene per

Table I. *GUS activity in SLR1:GUS transformants at flowering*

Mean GUS activity (pmol 4-methylumbelliferone min^{-1} mg^{-1} protein) was determined for each set of transformants using the fluorometric assay.

Construct	Stigma	Style	No. of Transformants
−1003	774 ± 223	273 ± 40	8
−742	550 ± 185	98 ± 11	5
−600	347 ± 106	58.6 ± 4	5
−363	337 ± 289	62 ± 4	5
−327	662 ± 68	68 ± 5	3
BalΔ1	7842 ± 1877	1507 ± 279	9
BalΔ2	3750 ± 997	1130 ± 224	10
−537	407 ± 120	93 ± 39	5

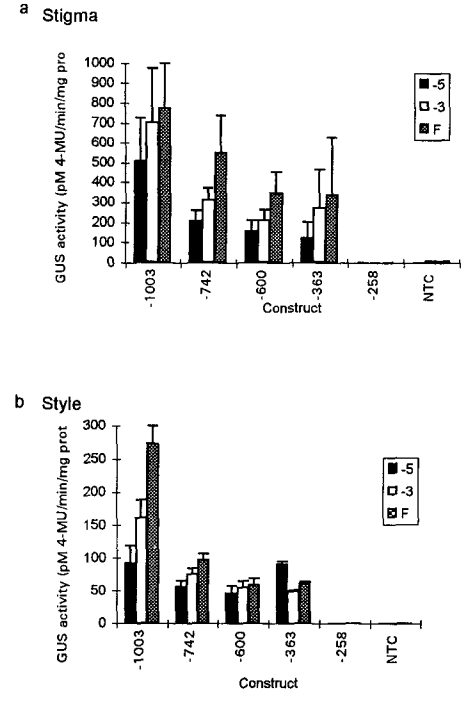

Figure 2. Fluorometric determination of GUS activities in stigmas (a) and styles (b) of plants transformed with *SLR1:GUS* constructs at flowering (F), 3 d before anthesis (−3), and 5 d before anthesis (−5). NTC, Nontransformed control. GUS activity is in pmol 4-methylumbelliferone min^{-1} mg^{-1} protein.

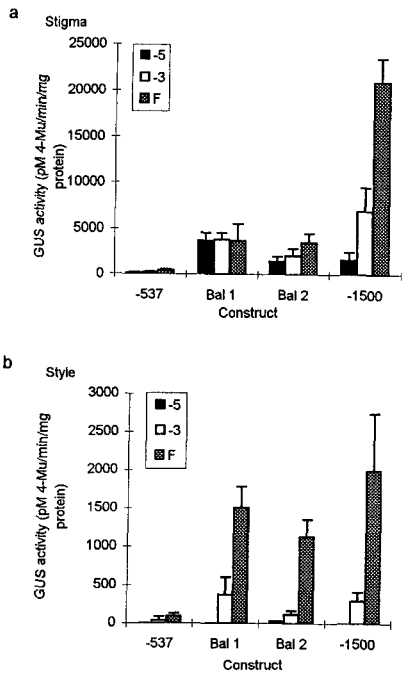

Figure 3. Fluorometric determination of GUS activities in stigmas (a) and styles (b) of plants transformed with *SLR1:GUS* constructs at flowering (F), 3 d (−3), and 5 d (−5) before anthesis. GUS activity is in pmol 4-methylumbelliferone min^{-1} mg^{-1} protein.

transformant varied. The transgene copy number and GUS activities measured in stigmas and styles of all −1003:GUS transformants are shown in Table II. GUS activity varied independently of the transgene copy number for this and all other constructs tested (Hackett, 1992; Cadwallader, 1994).

Sequences Located between −1350 and −1500 Modulate Promoter Activity

Although promoter constructs with end points from −327 to −1003 exhibited pistil-specific expression, in all cases the maximal level of expression was significantly lower than that obtained from the full-length *SLR1* promoter. This suggested that sequences located between −1003 and −1500 may enhance promoter activity. To address this possibility a construct was produced in which a section of DNA covering the region +1 to −537 was fused to a 150-bp fragment from between −1350 and −1500 to form a composite 687-bp promoter (Fig. 4a). This promoter was then linked to the *GUS* gene and expression was determined in transgenic tobacco plants. None of the 15 transformed plants generated expressed the reporter gene in any tissue. However, expression was restored if the region corresponding to −1350 to −1500 was deleted from the composite promoter. At 407 pmol min^{-1} mg^{-1} protein (mean value) in stigmas at flowering, the level of GUS activity was of the same order as that obtained for the −327 to −1003 promoter constructs (Fig. 3a). The composite 687-bp promoter was then used to produce other derivatives using Bal 31 exonuclease (Fig. 4b). Two constructs from this deletion set were analyzed in transgenic plants: BalΔ1 contained *SLR1* DNA from the region −1416 to −1500 ligated to the section from +1 to −474, and BalΔ2 comprised −1408 to −1500 ligated to the +1 to −432 region. Both deletion derivatives were found to direct expression of the reporter gene in pistils of transgenic tobacco at flowering. Furthermore, fluorometric analysis (Table I) revealed the mean level of GUS expression directed by the BalΔ1 and BalΔ2 promoter constructs in stigmas to be greater than that obtained for the −537 construct by a factor of 19 and 9, respectively (Fig. 3, a and b), and particularly in the case of the style, approached the same order of magnitude as that obtained with the full-length promoter. Although mean GUS activity was higher in stigmas of plants transformed with the BalΔ1 construct compared with the BalΔ2 transformants, this difference was not statistically significant at the 5% level. Analysis of reporter gene expression in the BalΔ1 and BalΔ2 transformants at the various developmental stages revealed that, in the case of stylar tissue, it clearly peaked at flowering. This pattern was less pronounced in stigma tissue, although in the case of the BalΔ2 transformants expression was maximal at flowering. Although this is reminiscent of the expression

Table II. *GUS activity in stigmas and styles of −1003* SLR1:GUS *transformants at flowering*

Values for two replicates (pmol 4-methylumbelliferone min^{-1} mg^{-1} protein) are given per transformant.

Transformant	Transgene Copy No.	Stigma	Style
A2	2	304	184
		200	100
A4	3	1800	548
		1037	402
A5	1	1820	1000
		2128	537
A6	1	300	165
		308	124
A9	1	507	146
		350	234
A10	2	410	196
		383	95
A12	2	373	159
		458	136
A15	1	1563	91
		443	256

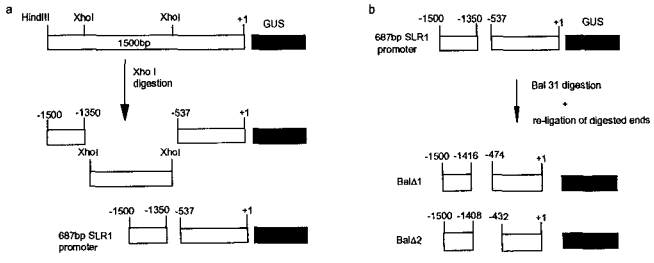

Figure 4. SLR1 promoter constructs containing −1350 to −1500 sequences. a, The composite 687-bp promoter was produced by removal of an internal *Xho*I fragment and religation of the free ends, resulting in a plasmid containing the −1350 to −1500 section fused to the −537 to +1 region. b, The 687-bp promoter was linearized by digestion at its single *Xho*I site and digested with Bal 31 exonuclease. Deleted ends were then religated, and two of the resulting constructs, BalΔ1 and BalΔ2, were selected for analysis in transgenic plants.

pattern seen with the full-length promoter, the overall magnitude of increase is not as great (Fig. 3, a and b).

GUS activity in styles was again lower than that in stigma tissue for the −537, BalΔ1, and BalΔ2 transformants. There was a 16- and 12-fold increase in GUS activity in styles of BalΔ1 and BalΔ2 transformants, respectively, compared with −537 transformants, a similar magnitude of increase as that measured in the stigmas of these plants. Again, there was no significant difference ($P > 5\%$) in GUS activity in BalΔ1 compared with BalΔ2 styles.

These results indicate that there are sequences located between −1416 and −1500 that significantly enhance activity of the *SLR1* promoter in pistil tissue. Moreover, they provide an initial suggestion that a silencer element may be located around −1350 to −1408.

Detection of a Binding Activity That Interacts with the Pistil-Specific Region of the *SLR1* Promoter

The deletion analysis of the *SLR1* promoter clearly suggested that one or more *cis*-acting elements located between −258 and −327 nucleotides upstream of the translation start site contribute to pistil-specific expression of the *GUS* reporter gene in transgenic tobacco. The implication of this result was that these sequences would be specifically recognized by DNA-binding proteins present in nuclear extracts prepared from pistil cells. Nuclear extracts were prepared from tobacco pistil cells and used in gel retardation analyses, with a 48-bp fragment constituting the region −279 to −327 to test for binding of putative *trans*-acting factors to the short promoter fragment.

When added to the pistil nuclear extract, the labeled 48-bp fragment produced a single retarded band, and binding was not competed by addition of p(dI-dC)·p(dI-dC) or p(dA-dT)·p(dA-dT). The specificity of the binding activity for the 48-bp probe was tested by addition of increasing molar excess of an unlabeled, 51-bp *SLR1* fragment from −207 to −258, which had no homology to the 48-bp retarded fragment. Addition of this nonspecific competitor had no effect on the retarded fragment (Fig. 5a). In contrast, and most significantly, addition of the unlabeled 48-bp fragment did effectively complete the binding of the labeled probe (Fig. 5b), so that when the competitor was added to 150-fold molar excess, the retarded complex was virtually undetectable.

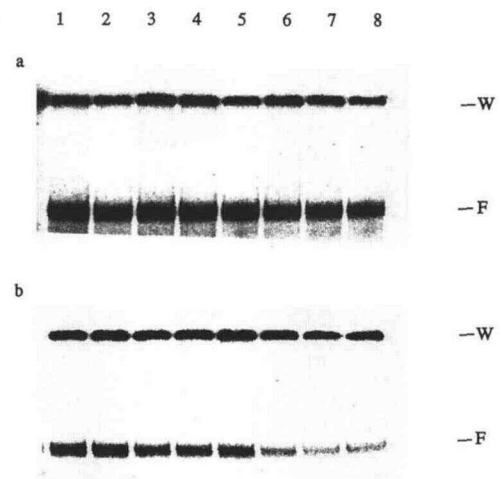

Figure 5. Gel retardation assays using the 48-bp *SLR1* fragment as a probe. a and b show phosphor images of the gel retardation assay. W indicates the position of the loading well, and F indicates the position of the retarded fragment. a, Addition of the 51-bp unlabeled competitor fragment. Lane 1, 48-bp probe + p(dA-dT)·p(dA-dT) only; lanes 2 to 8, same as lane 1 with the addition of 4 fmol (lane 2); 20 fmol (lane 3); 40 fmol (lane 4); 80 fmol (lane 5); 160 fmol (lane 6); 320 fmol (lane 7); and 600 fmol (lane 8) unlabeled 51-bp competitor. b, Addition of unlabeled 48-bp fragment. Lane 1, 48-bp probe + p(dA-dT)·p(dA-dT) only; lanes 2 to 8, same as lane 1 with addition of 4 fmol (lane 2); 20 fmol (lane 3); 40 fmol (lane 4); 80 fmol (lane 5); 160 fmol (lane 6); 320 fmol (lane 7); and 600 fmol (lane 8) unlabeled 48-bp fragment.

DISCUSSION

We have carried out deletion analysis of a *Brassica oleracea SLR1* gene promoter in transgenic tobacco, which has delineated *cis*-acting elements that are needed to direct gene expression in stigmas and styles of the heterologous host species.

Analysis of plants transformed with a set of sequential 5′ deleted constructs showed that sequences located between positions −258 and −327 relative to the translation start site are required to direct reporter gene expression that is specific to the pistil cells. Stylar expression was lower than that detected in stigmas, which is consistent with results obtained with the full-length promoter.

Gel retardation analysis provided further confirmation that sequences around −258 to −327 play an important role in the regulation of *SLR1* promoter activity. A 48-bp PCR fragment incorporating the region from −279 to −327 was shown to specifically interact with pistil extracts to produce a single retarded complex. These preliminary results lend support to those from the deletion analysis, and provide a basis for further work involving isolation and characterization of the binding factor. Inspection of the nucleotide sequence of the 48-bp fragment reveals three copies of the motif ATGTT, arranged in tandem (Fig. 1). Although the significance of this sequence requires clarification, it is interesting to note that a single copy of the motif is present within the phytochrome *A3* gene promoter, where it interacts with the oat DNA-binding protein PF1 (Nieto-Sotelo et al., 1994). Moreover, another motif, TGTCATT, which appears 13 nucleotides upstream of the ATGTT sequence in the *SLR1* gene promoter, is similarly positioned and also bound by PF1 in the oat phytochrome *A3* gene. These sequences have been shown to be involved in directing high-level expression of the phytochrome *A3* gene (Bruce and Quail, 1990), thus implicating PF1 in transcriptional activation.

Our localization of sequences involved in directing pistil-specific expression is in broad agreement with studies of another member of the *Brassica S* gene family, the *SLG13* (S-locus glycoprotein 13) promoter, which has been shown to direct a similar pattern of expression in transgenic tobacco (Thorsness et al., 1991). A 3.65-kb fragment of

this promoter directed GUS gene expression in the stigmas, styles, and pollen of transgenic tobacco plants, with low levels of the reporter gene product also detected in ovary epidermal tissue. Dissection of this promoter revealed sequences between −79 and −339 able to confer pistil-specific activity on a minimal cauliflower mosaic virus 35S promoter (Dzelzkalns et al., 1993). Further studies are required to determine whether the −258 to −327 section of the S63 SLR1 promoter could similarly activate transcription; therefore, the existence of additional regulatory elements downstream of the −258 position cannot be ruled out.

Direct quantitative comparisons of the GUS activities for the two promoters are not possible because only data for the two highest-expressing plants were presented for *SLG13* (Dzelzkalns et al., 1993), but it does appear likely that some differences in functional organization exist. In the case of *SLG13* no significant increase in promoter activity was found when the full-length promoter was compared with a −411 promoter construct (Dzelzkalns et al., 1993). Similarly, in the case of *SLR1*, addition of sequences up to the −1003 position did not have a great impact on the level of reporter gene activity detected in pistil tissues. However, the GUS activity measured in plants transformed with all of the sequentially deleted promoters ranged from 1.7 to 3.7% of that directed by the −1500 *SLR1* promoter, suggesting the existence of sequence elements upstream of −1003 with a role in high-level expression of the *SLR1* gene. Consistent with this hypothesis, analysis of plants expressing the BalΔ1 and BalΔ2 constructs (Fig. 4, a and b) revealed a substantial increase in expression levels, approaching those obtained for the full-length promoter.

In addition to the identification of nucleotide sequences within the −1350 to −1500 region of the *SLR1* promoter that activate expression, analysis of this region also suggested the presence of an element that appeared to silence pistil-specific transcription when fused to an otherwise functional −537 promoter. Further studies will be required to confirm this observation, since the possibility that silencing occurred as a consequence of fusing the two promoter fragments was not excluded. Silencing of a GUS transgene has been shown to occur when very high levels of the transcript were produced by a cauliflower mosaic virus 35S promoter (Elmayan and Vaucheret, 1996). This was observed when a single copy of the transgene was present. An alternative explanation for the lack of GUS activity in plants transformed with the composite 687-bp *SLR1* promoter may be silencing due to exceptionally high levels of GUS gene transcript. However, it is difficult to explain why this type of gene silencing should occur so effectively in plants transformed with this construct, since it was not observed in the −1500 *SLR1*:GUS transformants, where levels of GUS activity reached 75 nmol methylumbelliferone min^{-1} mg^{-1} protein in some plants (Hackett et al., 1992).

Thus, this study has shown that the −1350 to −1500 region of the *SLR1* promoter may play an important role in regulation. Furthermore, preliminary studies have revealed that this 150-bp segment does specifically interact to form several complexes with nuclear extracts from pistil cells (F.C.H. Franklin and G. Cadwallader, unpublished observations).

In all of the constructs tested GUS activity was higher in stigmas than in styles. It is evident that transcription factors that are capable of activating the *SLR1* promoter are present in both stigmas and styles, but the GUS activities recorded may reflect a difference in abundance of these factors in the two tissues. However, none of these *SLR1*:GUS constructs has been tested in transgenic *Brassica*; therefore, it is unclear whether a promoter element that would normally completely suppress *SLR1* expression in styles has been removed, thus allowing a degree of transcriptional activity.

Temporal regulation of expression by the *SLR1* promoter was tested by measurement of GUS activity in both stigmas and styles of developing pistils. The analysis showed that all of the deleted promoters that directed GUS activity in pistils did so from at least 5 d before flowering. However, although the full-length *SLR1* promoter produced a large increase in GUS activity between −5 d and flowering, this was not the case for deleted promoters, in which developmental differences were much less marked. Similarly, in the case of the BalΔ1 and BalΔ2 derivatives it did appear that expression was maximal at flowering, but, again, this was not as dramatic as that observed for the full-length promoter; hence, it is feasible that further sequences with a role in developmental control of expression remain to be identified.

ACKNOWLEDGMENTS

We are indebted to Dr. Richard Thompson (Max Planck Institut für Züchtungsforschung, Köln, Germany) for his help and advice relating to the gel-shift experiments. We wish to thank John Martin and colleagues for horticultural support and Antony Jones for technical assistance.

Received May 28, 1996; accepted August 28, 1996.

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