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A *Brassica* Self-Incompatibility Gene Is Expressed in the Stylar Transmitting Tissue of Transgenic Tobacco

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Tobacco was transformed with a gene coding for an S-locus-specific glycoprotein of *Brassica oleracea*. The resulting transgenic plants showed tissue-specific and developmentally regulated expression of the introduced gene. Immunolocalization experiments showed that the *Brassica* gene was expressed in the stylar transmitting tissue of the transgenic plants. The pattern of expression of the introduced gene was more similar to that of the S-associated genes of *Nicotiana alata* than to expression in *Brassica*. Self-incompatibility was not conferred by the introduced gene.

INTRODUCTION

Many plants possess self-incompatibility mechanisms that act to prevent fertilization of a fertile hermaphrodite plant by pollen of the same plant (de Nettancourt, 1977; Richards, 1986). The specificity of the self-incompatibility interaction has been shown to be controlled by one or more genetic loci at which many alleles may reside.

The two major genetic systems of self-incompatibility are the sporophytic and gametophytic self-incompatibility systems (East and Mangelsdorf, 1925; Heslop-Harrison, 1975). These are distinguished by the phenotype expressed by the pollen grain during pollination. In gametophytic self-incompatibility, displayed in the Solanaceae and exemplified by *Nicotiana*, the single S-allele of the haploid pollen grain determines the self-incompatibility phenotype of the pollen. In the sporophytic system, displayed in the Brassicaceae and exemplified by *Brassica*, the two parental S-alleles determine the self-incompatibility phenotype of the pollen grain.

The sporophytic and gametophytic self-incompatibility systems also differ in the site of inhibition of pollen tube growth in the pistil tissue of the flower (Sears, 1937). In *Brassica*, self-incompatible pollen fails to germinate and/or penetrate the papillar cells of the proliferated stigma epidermis. In *Nicotiana*, self-incompatible pollen germinates but pollen tube growth is arrested in the style.

The molecular genetics of self-incompatibility has been studied in the sporophytic species *Brassica oleracea* and the gametophytic species *Nicotiana alata* (for reviews, see Nasrallah and Nasrallah, 1986; Bernatsky, Anderson, and Clarke, 1988). Self-incompatibility in both species is controlled by a single, multiallelic locus. Genes have been cloned from both species that encode S-allele-associated glycoproteins and show linkage to the S-locus. The sequences of the *B. oleracea* and the *N. alata* genes are not similar at the DNA or amino acid level (Nasrallah et al., 1987; Bernatsky et al., 1988).

Cellular and developmental expression of the self-incompatibility genes have been shown to correlate with the site and timing of pollen inhibition in each system. In *B. oleracea*, the self-incompatibility genes are called SLG genes (S-locus genes; Lalonde et al., 1989) and are expressed specifically in the papillar cells of the stigma, the site of inhibition of pollen germination and pollen tube growth in incompatible pollinations (Nasrallah, Yu, and Nasrallah, 1988; Kandasamy et al., 1989). The S-associated genes of *N. alata* are expressed throughout the pathway of pollen tube growth, in the secretory cells of the stigma, the transmitting tissue of the style, and the placental epidermis of the ovary (Cornish et al., 1987; Anderson et al., 1989).

As a first step in the analysis of SLG gene regulation, we introduced a cloned *B. oleracea* SLG gene into the readily transformable species tobacco (*Nicotiana tabacum*), which is self-compatible. We were interested to determine whether the SLG gene would be expressed specifically in the stigmatic papillar cells as in *B. oleracea* or whether another pattern of expression would result in transgenic tobacco.

We report here that tobacco transformed with a *B. oleracea* SLG gene expresses the SLG gene at the RNA and protein level and that this expression is developmentally regulated. The transgenic tobacco shows tissue-specific expression that is different from that of *B. oleracea* and similar to the expression of S-associated genes in *N.*

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alata. The expression of the *B. oleracea* SLG gene does not effect self-incompatibility in transgenic tobacco.

RESULTS

Tobacco Transformed with the *Brassica* SLG Gene Is Self-Compatible

A genomic sequence described previously (Nasrallah et al., 1988) encodes the S-locus-specific glycoprotein (SLSG) of the S13 self-incompatibility allele of *B. oleracea*. An 11-kb EcoRI restriction fragment isolated from the S13 SLG genomic clone was inserted into the multiple cloning site of the binary vector pCIB10 (Rothstein et al., 1987), which carries a chimeric kanamycin resistance gene for selection of transformed plants. Figure 1A shows a partial restriction map of the resulting transformation vector pH1. The *B. oleracea* SLSG coding region is flanked by approximately 4.2 kb of upstream and 5.0 kb of downstream genomic sequence. Vector pH1 was mobilized into *Agrobacterium tumefaciens* strain pCIB542/A136 (derived from helper plasmid pEHA101; Hood et al., 1986; G. Helmer, personal communication). The resulting strain was used to transform tobacco (*N. tabacum* cv petit havana) using a modified leaf disc procedure (Horsch et al., 1985).

Twenty-three kanamycin-resistant plants were grown to maturity along with 19 nontransformed plants obtained by leaf disc culture. No morphological differences were observed between the transformed and nontransformed plants. All plants set seed when flowers were selfed by hand pollination and were judged self-compatible on this basis. Twelve kanamycin-resistant plants were selected for further study of the integration and expression of the SLG gene.

DNA purified from leaves of kanamycin-resistant plants was digested with restriction endonucleases, separated by agarose gel electrophoresis, and blotted to nylon membrane. Hybridization analysis was performed using a labeled cDNA probe, pBOS13 (Nasrallah et al., 1987), which encodes the *B. oleracea* S13 SLSG. Figures 1B and 1C show autoradiograms of gel blot hybridization of DNA from a representative sample of the kanamycin-resistant plants. No hybridization to the pBOS13 probe was detected in DNA from nontransformed tobacco under the hybridization conditions used. DNA from two kanamycin-resistant plants did not hybridize to the pBOS13 probe and did not express the SLG gene (data not shown). Ten kanamycin-resistant plants had DNA fragments that hybridized to the pBOS13 probe and, thus, were confirmed as transgenic plants. Figure 1B shows that the transgenic plants had the expected 11-kb EcoRI restriction fragment that hybridized to the pBOS13 probe, except for plants 41 and 47, which had an 8-kb and a 20-kb EcoRI restriction fragment, respectively. The size difference of these fragments indicates that some rearrangement of the SLG gene occurred in plants 41 and 47.

The number of sites of insertion of the SLG gene in the transgenic plants was determined by treating leaf DNA

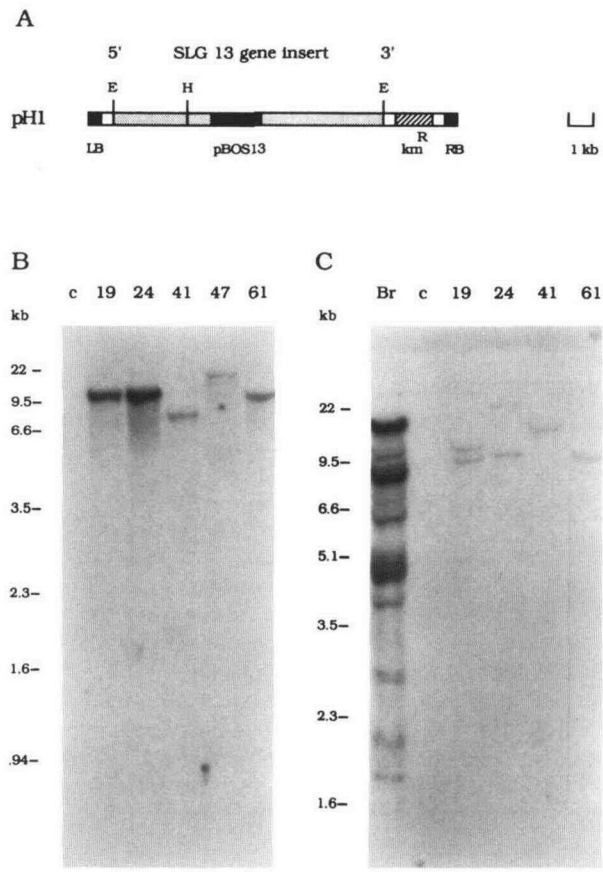

Figure 1. DNA Analysis of Tobacco Plants Transformed with the *Brassica* SLG Gene.

(A) A partial restriction map of the transformation vector pH1. The vector consists of an 11-kb EcoRI fragment, isolated from a genomic clone encoding the S13 SLG gene, inserted into the binary vector pCIB10. The map of pCIB10 outside the T-DNA borders is not shown. pCIB10 is approximately 13 kb and contains an additional km^R gene for selection in bacteria. The region corresponding to the pBOS13 cDNA insert used as the probe in hybridization analyses is indicated by the hatched region within the EcoRI fragment. E and H indicate EcoRI and HindIII restriction sites, respectively.

(B) DNA gel blot of CsCl-purified DNA from nontransformed tobacco (c) and individual transgenic plants (numbered) digested with the restriction enzyme EcoRI and probed with pBOS13.

(C) DNA gel blot of CsCl-purified DNA of S13 homozygous *B. oleracea* (Br), nontransformed tobacco (c), and transgenic plants restricted with HindIII and probed with pBOS13.

Molecular size markers in kilobases appear at left of **(B)** and **(C)**. 10 μg of DNA were loaded per lane in **(B)** and **(C)**.

with the restriction enzyme HindIII, which cuts once upstream of the SLG coding region, as shown in Figure 1A. Genomic restriction fragments yielded by HindIII digestion vary in size depending on the nature of the flanking DNA at the site of insertion in the transgenic plants. Different size genomic fragments that hybridize to the pBOS13 probe thus indicate different sites of insertion of the SLG gene. The number of SLG gene insertion sites was determined for six transgenic plants; results of a representative DNA gel blot hybridization are presented in Figure 1C. A single hybridizing band was observed in plants 41 and 61 (and plants 28 and 47, not shown), indicating that the SLG DNA inserted once in the genome of these transgenic plants. Two bands of similar hybridization intensity were observed in plants 19 and 24, indicating two different integration events. HindIII-digested DNA from S13 homozygous *B. oleracea* served as a positive control for hybridization in Figure 1C. As previously reported (Nasrallah et al., 1988), the *Brassica* sequences that hybridize to the SLG cDNA probes represent a multigene family.

The SLG gene was stably inherited, as determined by hybridization analysis, in populations of progeny plants resulting from self-pollination of the original transgenic plants (data not shown). The progeny plants were self-compatible as judged by seed set under greenhouse conditions.

The *Brassica* SLG Gene Is Expressed in Stigma and Style Tissues of Transformed Tobacco

Several regions of floral tissue were assayed for expression of the SLG gene in transgenic tobacco plants: the upper 1 mm of the pistil containing the stigmatic region, here described as the stigma; the style, which included the upper 20 mm of pistil excluding the stigma in mature buds and flowers, and the entire style excluding the stigma in immature buds; anther; petal and sepal; and ovary.

A monoclonal antibody, MAb/H8, raised against *B. oleracea* S6 SLSG cross-reacts with the *B. oleracea* S13 SLSG (Kandasamy et al., 1989) and was used to detect the S13 glycoprotein in transformed tobacco using protein gel blot analysis. The anti-SLSG monoclonal antibody reacts with protein extracts from *Brassica* stigmas and does not react with extracts from other *Brassica* tissues. A cluster of three major antigenic protein bands with apparent molecular masses in the range of 55 kD to 60 kD is recognized in *Brassica* by the anti-SLSG antibody, as shown in Figure 2, Br lane.

All of the 10 transformed plants studied expressed the SLSG in stigma and style tissue of open flowers. Figure 2 shows an antibody-stained protein gel blot of style proteins from nontransformed tobacco and from eight different transgenic plants. There was no cross-reaction of the antibody to stigma or style proteins of nontransformed tobacco. The stylar extracts of the transgenic plants showed a cluster of protein bands that were recognized by the anti-SLSG antibody and had the same apparent molecular mass as the major *B. oleracea* bands. The upper two of the major *B. oleracea* SLSG bands were present on protein gel blots of all transgenic plants. The third, lower *B. oleracea* SLSG band was detected only in the extracts of transgenic plants that stained most intensely with the anti-SLSG antibody. The difference in staining intensity among the different transgenic plants in Figure 2 indicates that the different transgenic plants expressed the SLSG at different levels. The degree of SLSG expression shows some correlation with the number of sites of insertion of the SLG gene in the genome. Plants 19 and 24, which showed two sites of insertion of the introduced gene, expressed the SLSG at higher levels than most of the other transgenic plants. However, plant 41, which showed one insertion and rearrangement of the

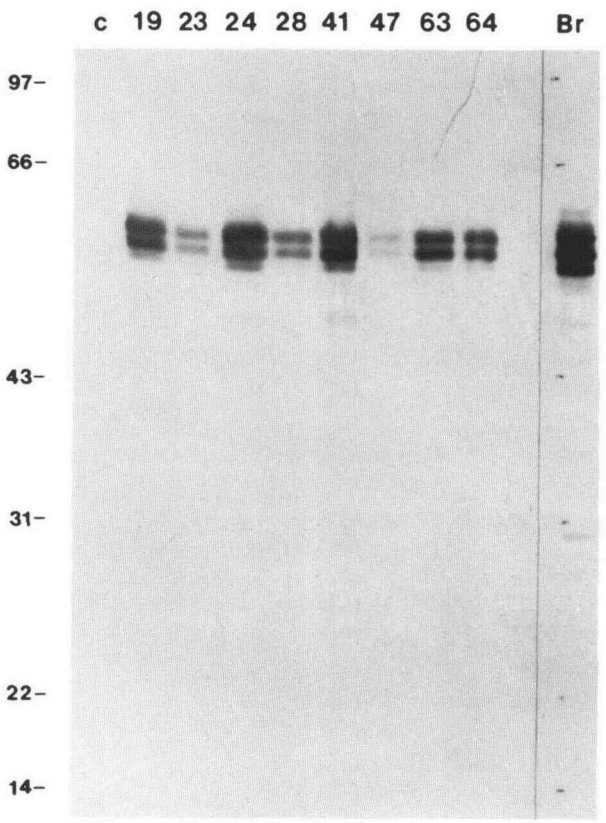

Figure 2. Expression of SLSG in Different Transgenic Plants.

Soluble protein was isolated from styles of nontransformed (c) and transgenic tobacco flowers and from stigmas of mature buds of S13 homozygous *B. oleracea* (Br). 25 μg of each sample were subjected to protein gel blot analysis using the anti-SLSG antibody. Molecular mass markers in kilodaltons are shown at left and can also be seen as ink marks on the nitrocellulose adjacent to the Br lane.

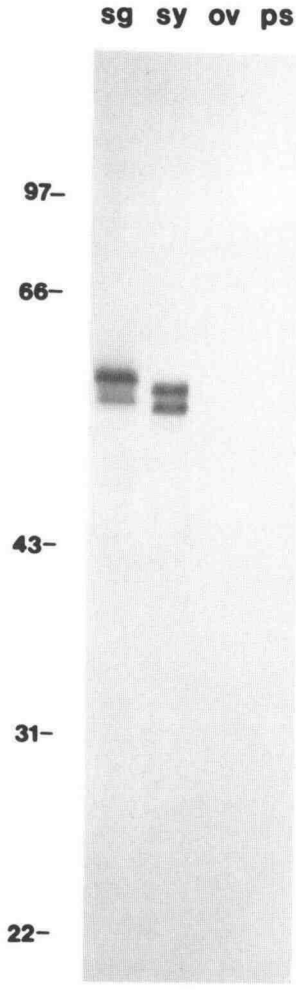

Figure 3. Tissue-Specific Expression of SLSG in Transgenic Tobacco.

Soluble protein was isolated from stigma (sg), style (sy), ovary (ov), and from petal and sepal (ps) tissue from flowers of transgenic plant 61. 25 μg protein from each sample were subjected to protein gel blot analysis. Molecular mass markers in kilodaltons are shown at left.

introduced gene, also expressed the SLSG at a high level. In contrast, plant 47, which also showed rearrangement of the SLG gene and one insertion, showed one of the lowest levels of SLSG expression among the transgenic plants.

Figure 3 shows that the SLSG was detected in protein extracts of stigma and style but not ovary or petal tissues of open flowers of transgenic plants. As will be discussed later, leaf and anther proteins also did not show reactivity with the anti-SLSG antibody. A distinct mobility shift was detected between antigenic proteins from stigma and style tissue of transgenic plants (Figure 3); the significance of this shift is unknown.

Six of the 10 transgenic plants were studied for expression of the SLG gene at the RNA level. Gel blots of total RNA from anther, style, stigma, and leaf tissue were hybridized to the pBOS13 probe. All six plants showed a single transcript of the same size that hybridized to the pBOS13 probe in RNA isolated from stigma and style but not anther or leaf tissues. Figure 4 shows that the transcript from stigmas of transgenic plants was of similar size to that from *B. oleracea* stigmas. Different levels of S-transcript were detected in different transgenic plants and correlated well with levels of SLSG expression as detected on protein blots (data not shown).

The SLG Gene Is Developmentally Regulated in Stigma and Style of Transformed Tobacco

Developmental expression of the SLG gene was studied at the protein and RNA levels. Protein gel blot analysis

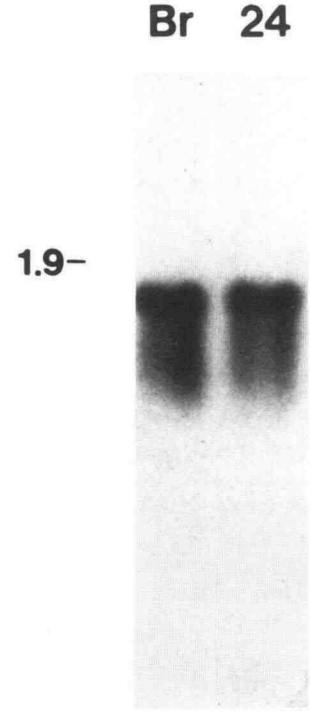

Figure 4. RNA Gel Blot Hybridization of Stigma RNA of *Brassica* and Transgenic Tobacco.

RNA was isolated from stigmas of mature buds of S13 homozygous *B. oleracea* (Br) and flowers of transgenic tobacco plant 24. 2 μg of total RNA from each sample were fractionated on agarose gels, transferred to nylon membrane, and hybridized to a ^{32}P-labeled pBOS13 probe. The migration of the transcript that hybridizes in each sample is similar. The position of the 1.9-kb rRNA band is indicated at left.

was performed using leaf tissue and stigma, style, and anther tissue from buds 5 days before flowering (−5d), 3 days before flowering (−3d), and from open flowers. Levels of antigenic proteins in different samples were quantitated using densitometry. Figure 5B shows such an analysis for transgenic plant 19. SLSG was first observed in stigma and style of −3d buds. Antigenic protein bands in stigma extracts showed a twofold to threefold increase in intensity from the −3d bud to the open flower. SLSG levels in the −3d bud were highest in the upper 1 mm of the pistil which contains the stigma. SLSG was not detected on protein blots of −5d bud tissues. Leaf and anther tissue did not react with the anti-SLSG antibody, as shown in Figure 5B.

The developmental expression of SLG transcripts was studied in three transgenic plants (plants 19, 47, and 23). For all three plants, the S-transcript was detected in stigma and style of transgenic plants as early as 5 days before flowering. A gel blot of total RNA of tissues from transgenic plant 19 is shown in Figures 5C and 5D. Differences in transcript level were quantitated by densitometric scanning of hybridization signals. A threefold increase in stigma and style transcript was observed in plant 19 from 5 days to 3 days before flowering. From 3 days before flowering to the open flower a twofold to threefold increase in stigma and style transcript was observed. Mature buds (1 or 2 days before flowering) showed an intermediate level of expression between the −3d and open flower stages (data not shown). Transgenic plants 19 and 47 showed similar patterns of temporal regulation, whereas plant 23 showed a different pattern. Plant 23 showed high levels of SLG transcript in stigma and style of −5d buds; transcript levels did not increase in −3d buds and increased only slightly in the open flower (data not shown). SLG transcript level was highest in the upper 1 mm of pistil containing the stigma in all transgenic plants and at all developmental stages examined. No hybridization was detected in total RNA isolated from leaf tissue and from anther tissue of −5d buds, −3d buds, and open flowers (Figures 5C and 5D).

Immunolocalization Shows That the SLG Gene Is Expressed in the Stylar Transmitting Tissue

The SLSG was localized in tissue sections with the anti-SLSG monoclonal antibody using the silver-enhanced colloidal gold technique for light microscopy. Paraffin sections of pistil and anther tissue of developing buds and flowers from two transgenic plants were analyzed together with similar tissue from nontransformed tobacco. Figure 6 shows the results of this staining in the stigma and upper style of mature buds of transgenic plant 19. In Figures 6A and 6B, the section was treated with the colloidal gold/silver enhancement technique in the absence of anti-SLSG antibody and did not show staining. Figures 6C and 6D show a tissue section adjacent to that in Figures 6A and

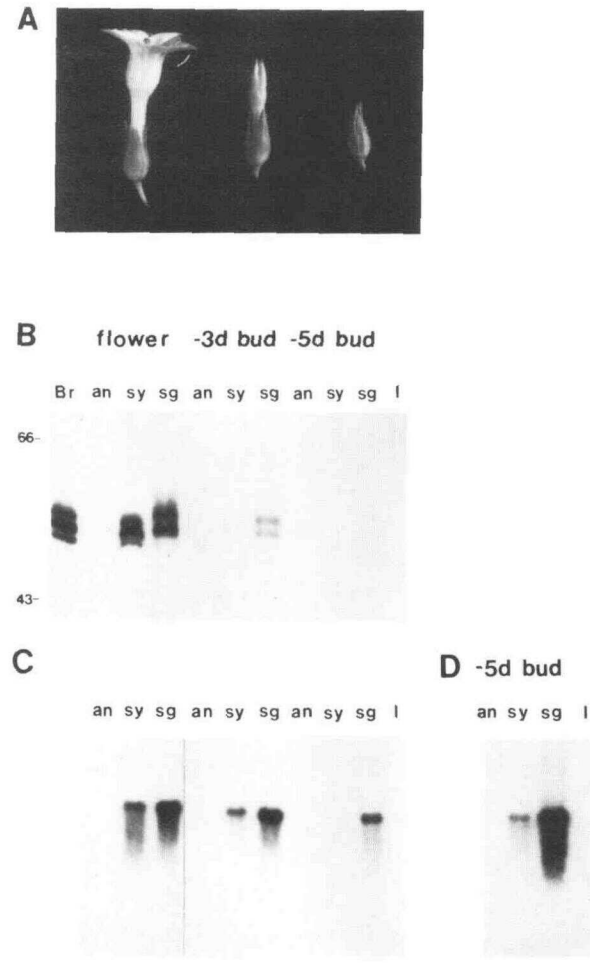

Figure 5. Developmental Expression of the SLG Gene in Transgenic Tobacco.

(A) Stages of tobacco floral development. Shown from the left are a flower, a bud 3 days before flowering (−3d bud), and a bud 5 days before flowering (−5d bud).

(B) Protein gel blot of tissues from transgenic plant 19 stained with the anti-SLSG antibody. Soluble protein was isolated from stigmas of mature buds of S13 homozygous *B. oleracea* (Br), and anther (an), style (sy), and stigma (sg) tissue from transgenic plant 19 at three stages of floral development. Soluble protein from leaf (l) tissue was also isolated. 40 μg of protein from each sample were subjected to protein gel blot analysis. Molecular mass markers in kilodaltons are shown at left.

(C) RNA gel blot of tissues from transgenic plant 19. Total RNA was isolated from the same tissues and developmental stages as in **(B)**. 2 μg of each sample were fractionated on agarose gels, transferred to nylon membrane, and hybridized to a ^{32}P-labeled pBOS13 probe. RNA from anther, style, and stigma of flowers was run on the same gel, transferred, and hybridized together with the −3d and −5d bud tissues. Portions of the same x-ray film were spliced together to make this figure.

(D) Longer film exposure of the right-most four lanes of the blot shown in **(C)** showing low transcript level in style of −5d bud and absence of hybridization in anther and leaf tissue.

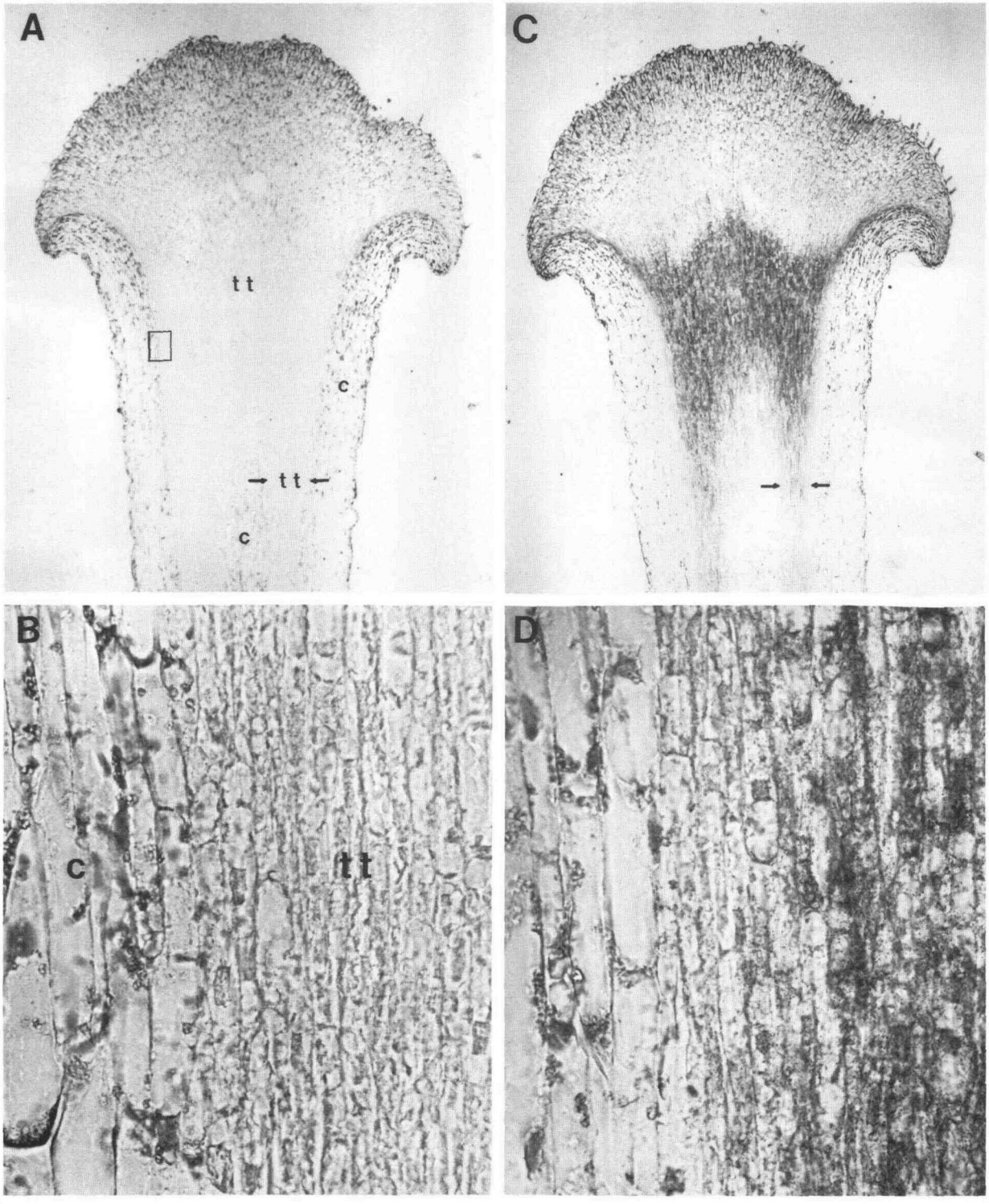

Figure 6. Immunolocalization of SLSG in Pistil Tissue of Transgenic Tobacco Plant 19.

(A) Photomicrograph of a pistil tissue section treated with the silver-enhanced immunogold staining technique in the absence of the anti-SLSG antibody. Transmitting tissue (tt) and cortex (c) of the style are identified. The plane of section is oblique.
(B) High magnification of boxed area in **(A)**, showing cortical and transmitting tissue cells.

6B that was treated with the anti-SLSG antibody. Silver grains are concentrated heavily in the transmitting tissue of the style, indicating the presence of SLSG in this tissue. Specific staining of the transmitting tissue was observed in buds 3 days before flowering, 1 or 2 days before flowering, and in open flowers. Staining was most intense in the open flower and less intense in bud tissue.

Only the cells of the transmitting tissue showed a significant level of staining in the two transgenic plants examined (plants 19 and 47). Plant 19 showed a much greater intensity of silver grains than did plant 47. It is notable that plant 47 also showed lower abundance of SLSG on protein blots (see Figure 3) and showed rearrangement of the introduced SLG sequence. The cortical tissue of the stigma and style was unstained in the two transgenic plants. In plant 19, but not in plant 47, an extremely low level of staining was observed in the epidermal cells of the stigma. Anther tissue of transgenic plants did not stain with the anti-SLSG antibody. Nontransformed tobacco did not show staining.

DISCUSSION

Transgenic tobacco plants expressed the SLG gene at the RNA and protein levels as determined by RNA gel blot hybridization and binding of an anti-SLSG monoclonal antibody to protein gel blots. Expression of the SLG gene was specific to the stigma and style tissue in flowers and buds of transgenic plants.

Independent transformed plants expressed the SLG gene at different levels, as shown in the protein blot in Figure 2 and by RNA gel blot hybridization (data not shown). Highest levels of SLSG expression were correlated to multiple sites of insertion of the SLG gene in two transgenic plants. Rearrangement of the introduced SLG gene apparently resulted in a higher level of SLSG in one transgenic plant and a lower level of expression in another. These differences in expression may result from differences in the site of insertion of the rearranged DNA in the tobacco genome and/or differences in sequence orientation within the rearranged SLG gene.

The anti-SLSG antibody recognized proteins of transgenic tobacco similar in electrophoretic mobility to the *B. oleracea* S13 SLSG. In *B. oleracea*, the three molecular mass forms of SLSG have been suggested to derive from different post-translational modification of the same primary translational product (Nasrallah and Nasrallah, 1984). The results obtained in transgenic tobacco demonstrate that the three forms are derived from a single SLG gene and that the stylar tissue of tobacco correctly processes the three forms as in *B. oleracea*. It is not clear why the lowest molecular weight *Brassica* SLSG band is expressed in transgenic tobacco at a lower level than the upper two bands, nor why protein gel blots of extracts from the stigma (upper 1 mm of the pistil) and the remaining style tissue differ in mobility of the anti-SLSG antibody-reactive bands. The latter may be due to different patterns of post-translational modification of SLSG in different regions of the pistil of transgenic plants, or to the presence in *Nicotiana* stigma extracts of components that interfere with the electrophoretic migration of SLSG.

Maximal levels of SLSG occurred in open flowers of transgenic tobacco. This is consistent with expression of the self-incompatibility phenotype in *B. oleracea* and *N. alata* and with reported expression of S-proteins in both systems. Expression of the SLG gene in transgenic tobacco differed from *B. oleracea* in that maximal levels of transcript occurred in open flowers in transgenic plants; in *B. oleracea*, maximal transcript is observed 1 day before flowering and decreases at anthesis (Nasrallah et al., 1985). SLG transcript in stigma and style of transgenic plants was present in the earliest bud stage examined, 5 days before flowering. However, protein blots of transgenic plants did not show detectable SLSG until 3 days before flowering. A similar pattern of protein versus transcript accumulation was shown in self-incompatible *N. alata*. In *N. alata*, S-associated proteins are not detected at the immature bud or "green bud" stage but are present in styles of mature buds (Anderson et al., 1986); the transcript of the cloned S-associated gene of *N. alata* is, however, present at the green bud stage (Cornish et al., 1987).

The SLG gene was expressed in the stylar transmitting tissue of transgenic tobacco, as demonstrated by silver-enhanced immunogold labeling. A similar pattern of expression was observed in tobacco transformed with another SLG gene and is described at the ultrastructural level in the companion paper by Kandasamy et al. (1990). The pattern of SLG gene expression observed in trans-

Figure 6. (continued).

(C) Adjacent section to **(A)**, treated with the silver-enhanced immunogold technique in the presence of the anti-SLSG monoclonal antibody. Dense staining occurs in the transmitting tissue of the upper style and extends into the transmitting tissue of the lower style (arrows). The cortical cells of the stigma and style are unstained.

(D) High magnification photomicrograph of **(C)**, taken from the same region of the style as **(B)**.

Specific staining was detected microscopically by the presence of very tiny silver grains and masses of silver grains giving the appearance of a smear of silver staining. Silver staining is evident throughout cells of the transmitting tissue. The cortical cells are unstained. Magnifications of **(A)** and **(C)**, ×40; of **(B)** and **(D)**, ×500.

genic tobacco is consistent with S-associated transcript localization reported for *N. alata* (Cornish et al., 1987). The transmitting tissue is the site where pollen tubes of *N. alata* and other self-incompatible Solanaceous species encounter a self-incompatibility barrier. S-associated protein in *N. alata* has recently been shown by immunofluorescence and immunogold histochemistry to localize to the walls of the stigmatic papillae and glandular tissue, the extracellular matrix of the transmitting tissue, the cell wall of the placental epidermis, and the surface of the ovule (Anderson et al., 1989). In our immunocytochemical analysis of the stigma and style of transgenic tobacco, we find only very low levels of expression of SLSG in the stigmatic epidermis and papillar cells as compared with high levels of SLSG in transmitting tissue.

Tobacco transformed with the *B. oleracea* SLG gene expressed the gene in a manner more analogous to the gametophytic self-incompatibility system represented by *N. alata* than to the sporophytic *B. oleracea* system, where the SLG gene is expressed only in the stigmatic papillar cells. This represents a new level of homology between the two different self-incompatibility systems. The protein-coding regions of the SLG genes of *B. oleracea* and the S-associated genes of *N. alata*, as previously noted, do not share significant sequence similarity. Possibly, the promoter regions of the *B. oleracea* and *N. alata* self-incompatibility genes (the sequences of which are not yet reported) share sequence motifs that interact with different spatial and temporal regulatory systems presented in the host plants. The different organization of the stigma in the two species (dense papillar cells and "dry" stigma in *Brassica*, sparse papillar cells and "wet" glandular stigma in *Nicotiana*) (Heslop-Harrison, 1975) and the different physiology of pollen tube growth in a self-incompatible response may reflect generally different gene regulatory systems in the two species. In some way that is not understood, the host tobacco plant is able to express a gene for *Brassica* self-incompatibility in the tissues appropriate to a self-incompatibility response in that host.

The transgenic plants are not self-incompatible as determined by seed set, although the SLG gene is expressed in pistil tissue. The apparent lack of expression of the SLG gene in anther tissue offers a possible explanation for the absence of a self-incompatibility response in transgenic tobacco. It is also possible that the molecular structure of *B. oleracea* SLSG, expressed at the dry stigma surface in *Brassica*, is not suitable for inhibition of pollen tubes at advanced stages of growth and/or cannot inhibit pollen tube growth when expressed in the glandular matrix of the tobacco style. Results of tobacco transformation experiments using the *N. alata* S-associated genes have not yet been reported but are of interest especially if the *N. alata* S-protein is more effective than that of *B. oleracea* at influencing compatibility in *N. tabacum*. Transformation of *Brassica* with *N. alata* S-associated genes, when available, would allow further comparison between the sites of expression of sporophytic and gametophytic self-incompatibility genes.

METHODS

Transgenic Plants

Triparental mating was performed using the pH1 binary vector plus pRK2013 (Figurski and Helinski, 1979) and pCIB542/A136. Leaf strips of axenic plantlets of *Nicotiana tabacum* cv Petit Havana were transformed using standard methods (Horsch et al., 1985; Rogers, Horsch, and Fraley, 1986). Kanamycin-resistant shoots were rooted, transplanted to soil, and grown to maturity in Environmental Growth Systems chambers at 70°F with a 16 hr/8 hr light/dark cycle. Nontransformed plants regenerated from tissue culture of untreated leaf strips were also grown in these growth chambers.

Tissues for DNA, protein, or RNA preparation were dissected from individual transgenic plants, frozen in liquid nitrogen, and stored at −70°C until use.

Stages of Floral Development

Stages of floral development in *N. tabacum* cv Petit Havana were established by collecting bud length data from 10-mm buds to flowers several days after anthesis. On average, buds 5 days before flowering were 13 mm long; buds 3 days before flowering were 25 mm long; buds 2 days before flowering were 40 mm; buds 1 day before flowering were 48 mm. Flowers were 45 mm on the first day they opened and were not collected for study after this point.

DNA and RNA Analysis

DNA was prepared from leaves of transgenic plants by a miniprep procedure (Mettler, 1987) or by nuclear DNA extraction and CsCl purification (Bingham, Levis, and Rubin, 1981). DNA was cut to completion with restriction enzymes, fractionated on 0.6 and 0.7% (w/v) agarose gels, and transferred to GeneScreen-*Plus* membrane (Du Pont-New England Nuclear) after denaturation. RNA was prepared from leaf, stigma, style, and anther tissue by SDS-proteinase K extraction (Hall et al., 1978; Beachy et al., 1979). Total RNA was denatured with glyoxal, electrophoresed on 1% (w/v) agarose gels, and transferred to GeneScreen membrane (Du Pont-New England Nuclear).

Concentration of DNA and RNA solutions was determined by spectrophotometry. Hybridization for both DNA and RNA blots was performed at 65°C using a solution containing 10% (w/v) dextran sulfate, 0.3 M sodium phosphate (pH 7.0), 5% (w/v) SDS, 10 mM EDTA, and 0.14 mg/mL denatured salmon sperm DNA. The hybridization probe was a cDNA insert from pBOS13 (Nasrallah et al., 1987) labeled with ^{32}P-dATP using a random-primer kit (Boehringer Mannheim). Levels of hybridization signals in different samples were quantitated using a Quick Scan scanning densitometer (Helena Laboratories, Beaumont, TX).

Protein Gel Blot Analysis

Soluble proteins were extracted from leaf, stigma, style, and anther tissue in 100 mM Tris-HCl buffer (pH 7.6). Protein concentrations were determined using a bicinchoninic acid/copper sulfate assay system (Pierce). Prior to fractionation on SDS-PAGE, protein was denatured at 100°C for 5 min in a solution containing 100 mM dithiothreitol, 2% (w/v) SDS, 80 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and bromphenol blue. Electrophoresis was performed at 20 ma and 2°C using gels of 10% polyacrylamide and 0.1% SDS (Dreyfuss, Adam, and Choi, 1984), and electroblotted to nitrocellulose (Towbin, Staehelin, and Gordon, 1979). Protein gel blots were stained with the anti-SLSG monoclonal antibody (Kandasamy et al., 1989) using the Protoblot Western Blot Alkaline Phosphatase system (Promega). Conditions for staining of protein blots were as described in the Protoblot technical manual except that incubation with the anti-SLSG antibody was for 1 hr at a 1:500 dilution. Levels of antigenic proteins in different samples were quantitated using a Quick Scan scanning densitometer (Helena Laboratories).

Immunolocalization

S-protein in flowers of transgenic plants was visualized in situ at the light microscope level using silver-enhanced immunogold labeling. Whole immature buds or dissected pistil and anther tissue of transgenic plants were fixed for 2 hr in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0), dehydrated, and embedded in Paraplast Plus media (Monoject Scientific, St. Louis, MO). Ten-micrometer longitudinal sections were cut and mounted using an egg albumin adhesive (Berlyn and Miksche, 1976). Sections were treated sequentially in phosphate-buffered saline [10 mM sodium phosphate (pH 7.2), 150 mM sodium chloride] containing one of the following: (1) 5% (v/v) normal goat serum (Sigma) and 5% (w/v) BSA (Serva); (2) anti-SLSG antibody, 1:100 in 0.1% (w/v) BSA; (3) gold-conjugated goat anti-mouse antisera (Sigma) in 0.1% (w/v) BSA and 5% (v/v) gelatin (Janssen Life Science Products, Piscataway, NJ). Sections were incubated in each solution for 1 hr at room temperature; rinses in phosphate-buffered saline followed the antibody incubations. Two cycles of silver enhancement were performed using IntenSE II reagents (Janssen).

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