

# Origin of Allelic Diversity in *Antirrhinum* S Locus RNases

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**In many plant species, self-incompatibility (SI) is genetically controlled by a single multiallelic S locus. Previous analysis of S alleles in the Solanaceae, in which S locus ribonucleases (S RNases) are responsible for stylar expression of SI, has demonstrated that allelic diversity predated speciation within this family. To understand how allelic diversity has evolved, we investigated the molecular basis of gametophytic SI in *Antirrhinum*, a member of the Scrophulariaceae, which is closely related to the Solanaceae. We have characterized three *Antirrhinum* cDNAs encoding polypeptides homologous to S RNases and shown that they are encoded by genes at the S locus. RNA in situ hybridization revealed that the *Antirrhinum* S RNases are primarily expressed in the stylar transmitting tissue. This expression is consistent with their proposed role in arresting the growth of self-pollen tubes. S alleles from the Scrophulariaceae form a separate group from those of the Solanaceae, indicating that new S alleles have been generated since these families separated (~40 million years). We propose that the recruitment of an ancestral RNase gene into SI occurred during an early stage of angiosperm evolution and that, since that time, new alleles subsequently have arisen at a low rate.**

## INTRODUCTION

In many angiosperm species, self-fertilization is prevented by self-incompatibility (SI) mechanisms. Genetically, many SI systems are simple, being controlled by a single S locus that exhibits a very high degree of allelic diversity. It has been estimated that more than 30 distinct alleles can be present in wild populations of SI species, and molecular analysis has revealed extensive sequence diversity between different alleles (de Nettancourt, 1977; Anderson et al., 1986; Clarke and Kao, 1991; Hinata et al., 1995). Studies of the evolution of the S locus ribonuclease (S RNase) genes within the Solanaceae family have shown that certain S alleles within a species are more closely related to the alleles of different species than to those of the same species (Ioerger et al., 1990). This indicates that some S allele lineages predate the divergence of species in the Solanaceae family, suggesting that some S alleles may have existed for as long as 30 million years (Ioerger et al., 1990). However, it is not known whether this conservation extends to species from other families. To test this, we examined the molecular basis of SI in *Antirrhinum*, a member of a closely related family, the Scrophulariaceae.

The genus *Antirrhinum* contains both self-incompatible and compatible species, and SI was first reported in studies of crosses between wild and cultivated species (Baur, 1911, 1919; Lotsy, 1911). Early studies, involving species that include *A. hispanicum*, *A. glutinosum*, *A. molle*, and *A. latifolium*, showed that SI in *Antirrhinum* is determined by a multiallelic S locus

with gametophytic control of pollen compatibility (Gruber, 1930, 1932; Sherman, 1939). In one study, more than 20 alleles were estimated to exist in a wild population of *A. glutinosum* (Gruber, 1932). Interestingly, the S locus was also shown to be linked to *cycloidea*, a gene controlling flower symmetry in *Antirrhinum* (Baur, 1919; Brieger, 1935; Tseng, 1938; Sherman, 1939).

We have characterized *Antirrhinum* cDNAs encoding polypeptides homologous to S RNases, demonstrated that they represent genes linked to the S locus, and shown that their expression patterns are consistent with a role in SI. Phylogenetic analysis of S RNases from the Scrophulariaceae and Solanaceae indicates that considerable allelic diversity has evolved during the time that these families have been separated (~40 million years) such that alleles are not shared between these groups. Further analysis of S RNases and S-like RNases from five families of the angiosperms showed that the S RNase genes may share a single common origin and that new S alleles have arisen at a very low rate.

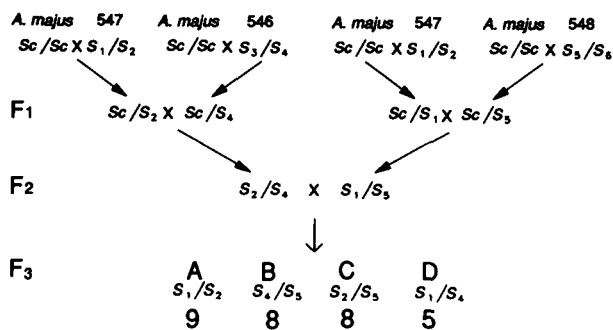
## RESULTS

### Generation of Interspecific SI Hybrids between *A. majus* and *A. hispanicum*

*A. majus* strains used in the horticulture industry and for experimental studies are self-compatible and carry nonfunctional S alleles, *Sc/Sc* (Baur, 1919; Gruber, 1930; Sherman, 1939).

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Therefore, interspecific crosses were conducted to introduce functional *S* alleles into *A. majus* lines. Three different self-incompatible lines of *A. hispanicum* (546, 547, and 548) were used for introgression of the *S* alleles, as shown in Figure 1. The *S* alleles of *A. hispanicum* were arbitrarily assigned  $S_1/S_2$  for line 547,  $S_3/S_4$  for line 546, and  $S_5/S_6$  for line 548 after each had been demonstrated to carry different alleles. Because  $F_1$  progeny from *A. majus* and *A. hispanicum* are expected to be heterozygous for *Sc* and a functional *S* allele, SI plants were generated by intercrossing  $F_1$  plants to give two  $F_2$  families of 80 plants each (Figure 1). Only 13% of the  $F_2$  plants were found to be SI rather than the expected 25%, suggesting that in addition to the *Sc* allele, other genetic modifiers promoting self-compatibility are present in the *A. majus* background. Indeed, earlier studies showed that *A. majus* carries a second gene, unlinked to the *S* locus, causing self-fertility (Brieger, 1935; Tseng, 1938; Sherman, 1939). To obtain a population of fully SI plants, two SI  $F_2$  plants were crossed to give an  $F_3$  family of 30 plants (Figure 1). All these plants were SI, and reciprocal crosses between individual plants of the  $F_3$  family identified four groups of plants (A, B, C, and D; Y. Xue, R. Carpenter, H.G. Dickinson, and E.S. Coen, manuscript in preparation). Crosses made between individuals in different groups were compatible, whereas those within groups were incompatible, suggesting that four *S* alleles were segregating in the family. The *S* genotypes of the four SI groups were inferred from DNA linkage analysis (see below) and are as follows: A,  $S_1/S_2$ ; B,  $S_4/S_5$ ; C,  $S_2/S_5$ ; and D,  $S_1/S_4$ . Further, the groups segregated in a ratio  $\sim 1:1:1:1$ , which indicated that the *S* alleles were segregating as expected (Figure 1).



**Figure 1.** Scheme for *S* Locus Introgression.

The *S* loci from *A. hispanicum* lines 546 ( $S_3/S_4$ ), 547 ( $S_1/S_2$ ), and 548 ( $S_5/S_6$ ) were introduced into *A. majus* carrying nonfunctional *S* alleles, *Sc/Sc*. SI plants were obtained in the  $F_2$  generation produced by intercrossing  $F_1$  progeny between *A. majus* and *A. hispanicum*. Two of these  $F_2$  plants were crossed to generate an  $F_3$  family in which the different *S* allele combinations segregated. A, B, C, and D are four groups of plants occurring in the  $F_3$  family. Individual plants were incompatible within a group but cross-compatible between the groups. The *S* genotypes of these four groups are indicated, and the numbers of plants obtained for each group are shown below.

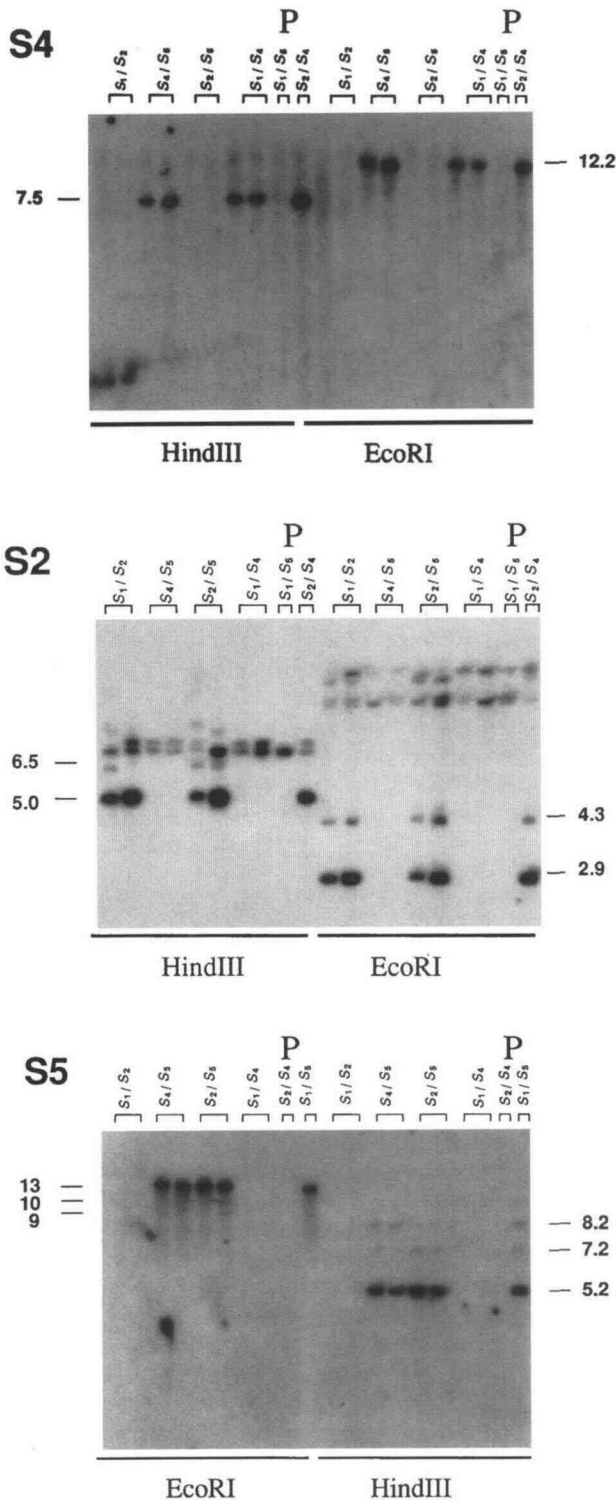
### Isolation of *S* RNase Homologs from *Antirrhinum* and Their Cosegregation with the *S* Locus

SI in *Antirrhinum* is gametophytically controlled, showing a similar behavior to SI in the Solanaceae, in which stylar *S* RNases have been demonstrated to be responsible for the identification and rejection of self-pollen tubes (McClure et al., 1989; Lee et al., 1994; Murfett et al., 1994). Using plants from the  $F_3$  family segregating for *S* alleles mentioned above, we isolated RNase homologs to determine whether they were linked to the *S* locus. Thus, if an RNase homolog from *Antirrhinum* is linked to the *S* locus, it should be expressed in two of the SI groups in the  $F_3$  family (Figure 1). For example, an RNase homolog linked to  $S_5$  should be expressed only in the B and C groups of the  $F_3$  family. Candidate *S* RNase homologs could therefore be screened by probing RNA extracted from the different SI groups and identifying those that were only expressed in two of the groups.

Putative *S* RNase cDNAs were isolated by reverse transcriptase–polymerase chain reaction (RT-PCR), using a primer based on the conserved C2 domain of the solanaceous *S* RNases (Ioerger et al., 1991). Using cDNA prepared from styles harvested 1 day before anthesis, we obtained several hundred clones from each SI group. Stylar-specific clones were selected by differential colony screening, using cDNA generated from style or leaf RNA. Additional rounds of differential colony hybridization of cDNAs were performed using labeled first-strand cDNAs from the four different SI groups. cDNA clones expressed only in two of the  $F_3$  SI groups were selected for DNA linkage analysis.

Genomic DNA gel blots from plants of each of the four SI groups and their parents were probed with three candidate cDNAs to test their linkage to the *S* locus (Figure 2). Three cDNA clones were linked to  $S_2$ ,  $S_4$ , and  $S_5$  and named *S2*, *S4*, and *S5*, respectively. These linkages were confirmed for all 30 plants in the  $F_3$  family (data not shown). Genomic DNA from the *A. hispanicum* parental lines was hybridized with the *S2*, *S4*, and *S5* cDNA probes and gave the expected results: line 547 hybridized with *S2*, line 546 with *S4*, and line 548 with *S5*. No signal was detected with genomic DNA from *A. majus* lines (data not shown).

DNA gel blots showed that although the *S4* cDNA hybridized with a single fragment in HindIII- or EcoRI-digested genomic DNAs, the *S2* and *S5* cDNAs produced more complicated hybridization patterns (Figure 2). *S2* hybridized with two *S*-linked fragments with HindIII (5.0 and 6.5 kb) or EcoRI (2.9 and 4.3 kb). These two fragments were shown to result from digestion at HindIII and EcoRI sites within the *S2* cDNA sequence. In addition, *S2* hybridized with two fragments that showed no linkage to the *S* locus. This lack of linkage indicates that these fragments are not likely to play a direct role in the expression of SI. *S5* detected a single, strongly hybridizing HindIII (5.2 kb) or EcoRI (13 kb) fragment showing *S* linkage. In addition, *S5* hybridized with at least two more HindIII (7.2 and 8.2 kb) or EcoRI (9 and 10 kb) fragments that also cosegregated with the *S* locus (Figure 2).



**Figure 2.** DNA Linkage Analysis of the Antirrhinum S RNase Genes S2, S4, and S5, Representing the S<sub>2</sub>, S<sub>4</sub>, and S<sub>5</sub> Alleles of the S Locus, Respectively.

The hybridization of S5 with these fragments could have three possible explanations: (1) the presence of several internal restriction enzyme sites within the S<sub>5</sub> gene, (2) partial digestion as a result of DNA methylation, or (3) the presence of additional sequences homologous to S5 and linked to S<sub>5</sub>. The first explanation seems unlikely because no internal HindIII or EcoRI sites were found in the S5 cDNA sequence and also because S5 appeared to have a single intron of <1.2 kb (data not shown). Although we cannot exclude the second possibility, methylation of HindIII sites is rare in plants (Gruenbaum et al., 1981). It is possible, therefore, that, in addition to S5 RNase, the S<sub>5</sub> allele contains other sequences homologous to S5 cDNA. Disappointingly, we have so far been unable to identify a cDNA showing linkage to S<sub>1</sub>. Perhaps S<sub>1</sub> is so diverged that the primer used in PCR was ineffective, or its expression level was too low to be cloned by this strategy. Currently, we are testing other methods of cloning S<sub>1</sub>-linked cDNAs.

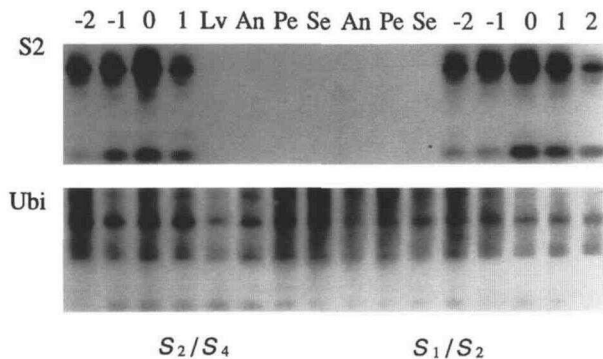
To test further the linkages of the cDNAs to particular S alleles, two SI plants with the genotypes S<sub>2</sub>/S<sub>5</sub> and S<sub>4</sub>/S<sub>5</sub> were crossed reciprocally. No S<sub>5</sub> homozygote was expected in the progeny from these crosses because pollen carrying S<sub>5</sub> is rejected in both pollinations. DNA gel blots of 29 progeny from these crosses (14 plants from S<sub>2</sub>/S<sub>5</sub> × S<sub>4</sub>/S<sub>5</sub> and 15 from S<sub>4</sub>/S<sub>5</sub> × S<sub>2</sub>/S<sub>5</sub>) showed that no S5 homozygous plants were generated and that S2 and S4 segregated in the expected 1:1 ratio (eight S2/S4; six S4/S5 and six S4/S2; nine S5/S2), confirming linkage of the isolated S RNase homologs to the S locus.

**Expression of Antirrhinum S RNases during Style Development**

To reveal the tissue specificity and temporal expression patterns of the RNase sequences, RT-PCR was conducted with RNA prepared from different tissues. The S2 RNase was expressed in styles but not in sepals, petals, anthers, or leaves (Figure 3). Expression in styles peaked during anthesis, coinciding with the acquisition of SI by the styles. Similar results were obtained for S4 and S5 (data not shown).

The spatial distribution of the S RNase transcripts was analyzed by RNA in situ hybridization. The Antirrhinum pistil consists of three major parts: stigma, style, and ovary (Figure 4A). Pollen germinates on the surface of the stigma and grows through the transmitting tissues located in the center of the style, finally reaching the ovary. By using a digoxigenin-labeled

Three micrograms of genomic DNAs from two representative plants of the four SI groups and the parents (P), with their genotypes indicated, was digested with HindIII or EcoRI and probed with S4, S2, or S5 cDNAs. The lengths of the fragments are indicated in kilobases at left and right.



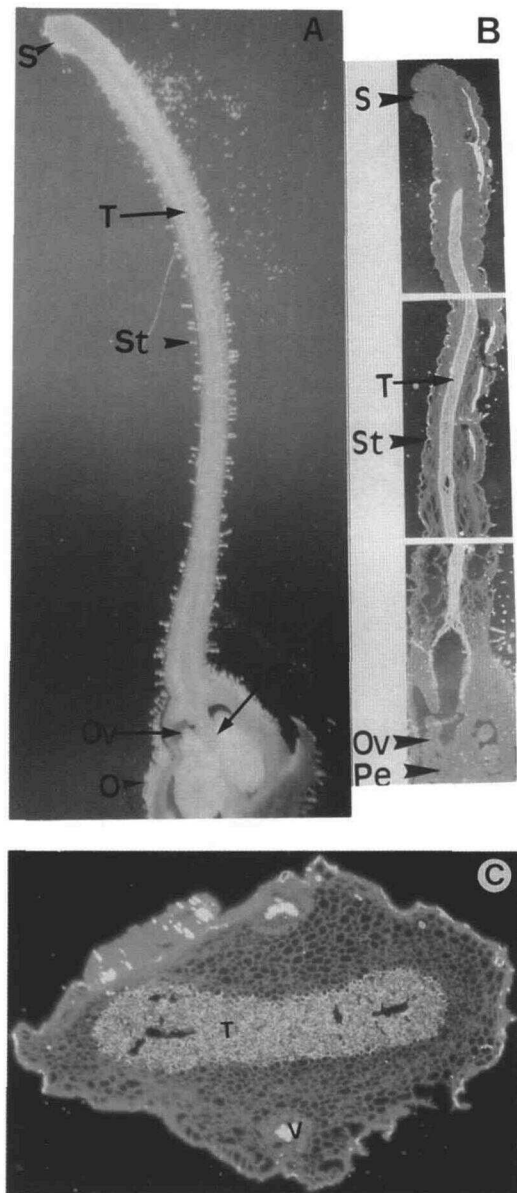
**Figure 3.** Tissue Specificity and Temporal Expression of the Antirrhinum S2 S RNases.

Gene-specific RT-PCR products from style RNAs of SI plants with  $S_2/S_4$  or  $S_1/S_2$  genotypes were separated and probed first with S2 and then with a ubiquitin (Ubi) probe. The smaller band hybridizing with the S2 cDNA probe may have been the result of internal priming by the oligo(dT) adapter. Different stages of style development are indicated by  $-1$  or  $-2$  (1 or 2 days before anthesis), 0 (anthesis), and 1 or 2 (1 or 2 days after anthesis). Lv, leaves; An, anthers; Pe, petals; Se, sepals.

antisense probe corresponding to the 3' region of S5 cDNA, transcripts were detected mainly in the stylar transmitting tissue, with no expression in either the stigma or the placental epidermis (Figures 4B and 4C). No signal was detected in controls using a sense S5 cDNA probe (data not shown). The expression patterns for S2 and S4 were similar to S5 (data not shown). Unlike the Solanaceae, S RNases from Antirrhinum show little or no expression in the stigma or placental epidermis (Cornish et al., 1987; Anderson et al., 1989). Overall, these results suggest that the Antirrhinum S RNases have expression patterns consistent with a role in SI.

#### DNA Sequence Analysis of Antirrhinum S RNase Genes

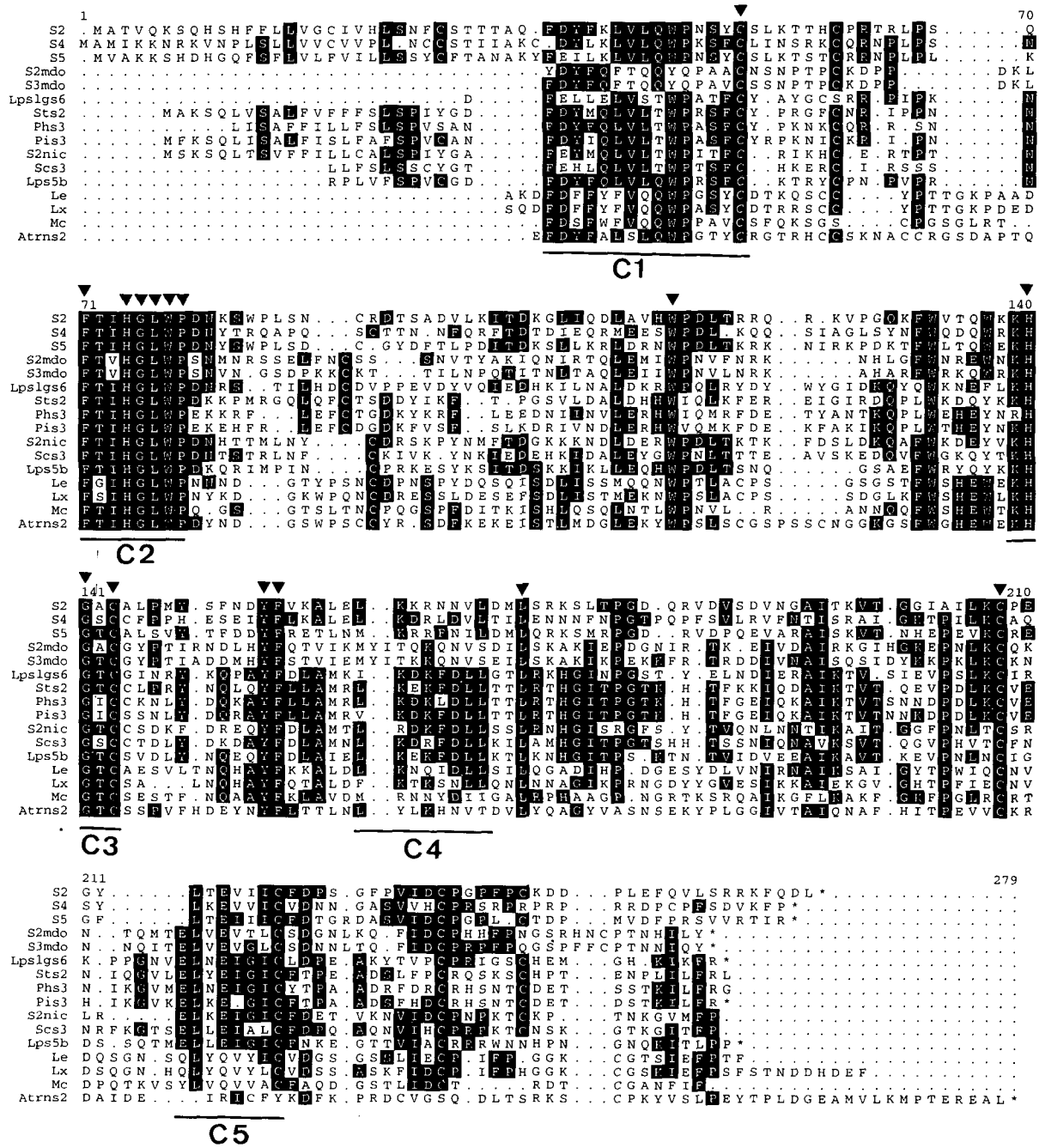
To obtain the full-length cDNA sequences, the 5' regions of S2, S4, and S5 were generated by 5' RT-PCR. Analysis of the full-length sequences revealed them to encode predicted polypeptides of 235, 233, and 233 amino acids for S2, S4, and S5, respectively. Figure 5 shows an alignment of the predicted polypeptide sequences of Antirrhinum S RNases together with representative members of the S RNases from apple and several solanaceous species. Figure 5 also includes several plant S RNase-like (S-like) genes not encoded by the S locus and therefore playing no clear role in SI (Taylor et al., 1993). The Antirrhinum sequences showed 40 to 55% identity with each other at the amino acid level and extensive identity ( $\sim 40\%$ ) with the solanaceous S RNases. Four of the domains conserved between the solanaceous S RNases (C1, C2, C3, and



**Figure 4.** Spatial Distribution of Antirrhinum S RNase Transcripts.

(A) Longitudinal section of an Antirrhinum pistil showing the stigma (S), style (St), transmitting tissue (T), ovary (O), ovules (Ov), and placental epidermis (Pe).

(B) and (C) RNA in situ hybridization of the longitudinal (B) and transverse sections (C) of the style at the stage of anthesis showing tissue specificity of S5 RNase transcripts. Sections were probed with a digoxigenin-labeled antisense probe corresponding to the 3' region of S5 and viewed by dark-field microscopy. The signal color is salmon pink. Epifluorescence was used to reveal calcofluor white-stained cell walls (light blue). V, vascular tissue.



**Figure 5.** Amino Acid Sequence Alignment of the Antirrhinum S RNases with Other Plant S RNases and S-like RNases.

The alignment was generated using the Genetics Computer Group (Madison, WI) PILEUP and PRETTY programs. C1 to C5 correspond to previously identified conserved domains among S RNases of Solanaceae species (Tsai et al., 1992). Conserved residues are indicated by black boxes, and black triangles indicate conservation among all sequences. Black dots are gaps introduced to maximize the alignment. S RNases include sequences from Antirrhinum (S2, S4, and S5), apple (S2mdo and S3mdo) (Broothaerts et al., 1995), *Lycopersicon peruvianum* (Lps1gs6 and Lps5b) (Tsai et al., 1992; Rivers et al., 1993), *Solanum tuberosum* (Sts2) (Kaufmann et al., 1991), *Petunia hybrida* (Phs3) (Clark et al., 1990), *P. inflata* (Pis3) (Ai et al., 1990), *Nicotiana glauca* (S2nic) (Anderson et al., 1986), and *S. chacoense* (Scs3) (Xu et al., 1990). S-like RNases include the sequences from *L. esculentum* (Le and Lx) (Jost et al., 1991; Löffler et al., 1993), *Momordica charantia* (Mc) (Ide et al., 1991), and *Arabidopsis* (Atrns2) (Taylor et al., 1993).

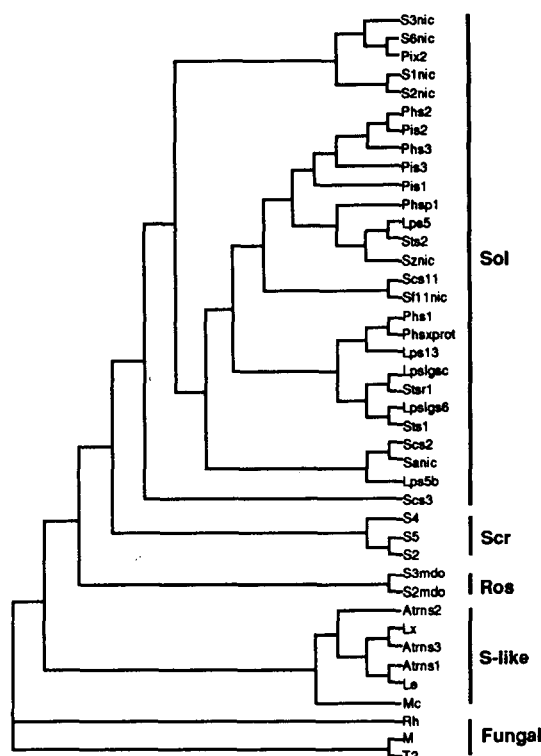
C5) were found in *Antirrhinum* and apple, with only C4 absent (Kheyr-Pour et al., 1990; Ioerger et al., 1991; Tsai et al., 1992; Broothaerts et al., 1995). Sixteen residues were conserved in the amino acid sequences of all S RNases and S-like RNases (Figure 5), including the histidine residues of the C2 and C3 domains, which have been shown to be the active sites for ribonuclease activity (Kawata et al., 1990; Huang et al., 1994; Royo et al., 1994). The conservation of three cysteine residues at positions 49, 143, and 208 suggests that these proteins may share a common backbone fold (Kurihara et al., 1992).

To investigate the origins and early diversification of S alleles, the phylogenetic relationships between different plant S RNase or S-like RNase proteins were analyzed using a neighbor-joining method from the PHYLIP program (Felsenstein, 1989). The RNase genes analyzed were from five angiosperm families: the Solanaceae, Scrophulariaceae, Rosaceae, Brassicaceae, and Cucurbitaceae. We constructed a phylogenetic tree showing the relationships between different fungal RNases and S RNase and S-like RNase genes (see Figure 6). The tree indicates that the members of the S RNase genes are organized into a monophyletic group distinct from the S-like gene group. Within the S RNases, the S alleles from the Scrophulariaceae form a different group from those of the Solanaceae. Similar results were also obtained by trees based on parsimony (data not shown).

## DISCUSSION

We have characterized three *Antirrhinum* cDNAs encoding sequences with homology to RNases. Three lines of evidence suggest that they are encoded at the S locus and derive from three functional S alleles. First, DNA gel blots probed with these cDNAs resulted in no recombinants in three segregating families comprising a total of 59 plants, indicating linkage to the S locus. Second, RT-PCR and RNA in situ hybridization analysis showed that these genes are specifically expressed in styles and that their transcript levels peak at anthesis, coinciding with the time at which the style acquires its ability to reject self-pollen. Third, the RNases showed extensive sequence polymorphism at the amino acid level (40 to 55% identity between alleles). This level of polymorphism is unusual for most genes, but it is typical for S alleles described in other species (Anderson et al., 1989; Clark et al., 1990; Kaufmann et al., 1991). Taken together, these results show that these RNases in *Antirrhinum*, a member of the Scrophulariaceae, are likely to mediate the stylar expression of SI in a way similar to that demonstrated for the Solanaceae (McClure et al., 1989; Huang et al., 1994; Lee et al., 1994; Murfett et al., 1994).

The predicted amino acid sequences of the *Antirrhinum* S RNases show extensive homology to other members of the S RNase family. Of the five domains conserved among the S RNases of the Solanaceae, four are conserved in *Antirrhinum* (Figure 5; Tsai et al., 1992). The regions corresponding to the two hypervariable regions observed in the Solanaceae



**Figure 6.** A Neighbor-Joining Phylogenetic Tree of the Plant S RNase and S-like RNase Proteins Using Fungal RNases as an Outgroup.

The different gene groups are indicated on the right. The S RNase group formed a monophyletic group and divided into three lineages, the Solanaceae (Sol), the Scrophulariaceae (Scr), and the Rosaceae (Ros). The S-like RNases are clustered into a distinct group (S-like). The S RNase sequences used are as follows: *Nicotiana glauca*, Sanic, S2nic, S1nic, S3nic, S6nic, Sznic, and Sf11nic (Anderson et al., 1986, 1989; Kheyr-Pour et al., 1990); *petunia*, Phs1, Phs2, Phs3, Phsp1, Phxprot, Pix2, Pis1, Pis2, and Pis3 (Ai et al., 1990, 1992; Clark et al., 1990; Lee et al., 1992); *potato*, Scs2, Scs3, Scs11, Sts2, Sts1, and Sts1 (Xu et al., 1990; Kaufmann et al., 1991; Saba-El-Leil et al., 1994); *tomato*, Lps5, Lps5b, Lpslgs6, Lps13, and Lpslgs6 (Tsai et al., 1992; Rivers et al., 1993; Chung et al., 1994; Royo et al., 1994); *Antirrhinum*, S2, S4, and S5; *apple*, S2mdo and S3mdo (Broothaerts et al., 1995). The S-like RNases analyzed were bitter melon (*Momordica charantia*; the Cucurbitaceae), Mc (Ide et al., 1991); *Arabidopsis* (the Brassicaceae), Atrns1, Atrns2, and Atrns3 (Taylor et al., 1993; Bariola et al., 1994); and *tomato* (the Solanaceae), Le and Lx (Jost et al., 1991; Löffler et al., 1993). The fungal sequences included RNase Rh of *Rhizopus niveus*, RNase T2 of *Aspergillus oryzae*, and RNase M of *A. saitoi* (Horiuchi et al., 1988; Kawata et al., 1988; Watanabe et al., 1990).

(Tsai et al., 1992) also show a high degree of variability in *Antirrhinum*. These regions are thought to be responsible for allele specificity (Clarke and Kao, 1991). Previous comparisons suggested that the region between the C4 and C5 domains may be required for a specialized function of the S RNase in SI

because it contains several highly conserved residues that are absent from the S-like RNases (Taylor et al., 1993). However, this region was not conserved in Antirrhinum or apple, suggesting that this interpretation requires reevaluation.

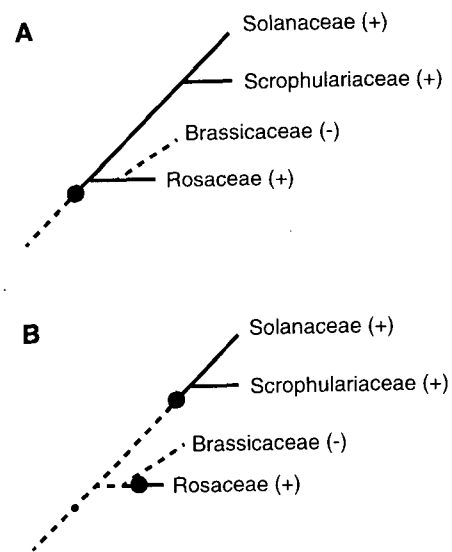
Three types of evidence indicate that the rate of mutation to new S alleles is very low. First, up to 60% amino acid sequence divergence is found between different S alleles from the same species. With a rapid rate of mutation, new alleles would be continually spreading in the population, resulting in a rapid turnover of alleles. This is because selection of S alleles is frequency dependent; a rare allele would have an advantage because pollen bearing it has less chance of landing on a stigma carrying the same allele (Wright, 1939). A very low mutation rate is therefore needed to avoid a rapid turnover of alleles, allowing sequence divergence to accumulate between alleles that survive for long periods of time. Second, comparison of S alleles from different species in the Solanaceae indicates that some alleles can persist for very long periods of time, having diverged from each other more than 20 to 30 million years ago (Loerger et al., 1990). Third, the lack of success in attempts to mutate the S locus to new specificities indicates that the generation of new alleles by natural mutation is likely to be very low (reviewed in de Nettancourt, 1977).

Despite this low rate of allelic generation, our results indicate that new alleles have been generated since the divergence of the Solanaceae and Scrophulariaceae. The phylogenetic analysis shows that S alleles from the Scrophulariaceae are not shared with those of the Solanaceae but form a separate group. This independent clustering was not altered, even when the sequence from an additional Antirrhinum S allele,  $S_3$ , was included in the analysis (Y. Xue, R. Carpenter, H.G. Dickinson, and E.S. Coen, unpublished results).

Comparisons between S alleles from these two families allowed us to make a rough estimate of the rate of mutation to new alleles. Assuming similar mutation rates in the Scrophulariaceae and the Solanaceae ribulose biphosphate carboxylase large subunit genes, these two families began to diverge  $\sim 40$  million years ago (R. Olmstead, personal communication; Crane et al., 1995). If we assume that each population maintains  $\sim 10^2$  alleles, new alleles would have to arise at a rate of approximately one per million years to account for the distinct alleles generated over 40 million years. Assuming one generation a year and an average population size of  $10^5$ , the mutation rate would be  $\sim 10^{-11}$ . Such a rate would allow alleles to survive during the divergence of species within the Solanaceae, while also accounting for the divergence between S alleles in the Scrophulariaceae and Solanaceae. This low rate may be explained if more than one event is needed to generate new functional SI specificity, an interpretation supported by genetic and molecular studies, which indicated that the S locus contains separate genes responsible for the male and female expression of SI (Lewis, 1949; Lee et al., 1994; Nasrallah et al., 1994). The low frequency of mutation is thus explicable if the production of S alleles with new specificities requires a separate mutation in two different genes.

The phylogenetic analysis also shows that S alleles of the Rosaceae, Scrophulariaceae, and Solanaceae form distinct lineages and resemble each other more closely than they resemble S-like RNases (Figure 6), suggesting that the RNase SI system was present in the common ancestor of these three families and that the recruitment of RNase into SI could have occurred as early as 70 million years ago (Figure 7A; Crane et al., 1995). Because the Rosaceae and Brassicaceae are thought to have diverged after this common ancestor (Chase et al., 1993), the S RNase SI system would also be expected to be present in the Brassicaceae. However, Brassica SI is sporophytically regulated (pollen compatibility is determined by its parental genotype), and recognition involves an S receptor kinase in the pistil. This receptor kinase shows no homology to the S RNases (Nasrallah et al., 1987; Stein et al., 1991).

The absence of an S RNase SI system in Brassica could be explained by two hypotheses. First, although the phylogenetic evidence supports the clustering of the Rosaceae S RNases with those of the Solanaceae and Scrophulariaceae, it does not rule out the possibility of separate lineages, in which case the recruitment of RNase into SI in the Rosaceae could have



**Figure 7.** Two Models to Explain the Recruitment of Ribonucleases into SI during Angiosperm Evolution.

The phylogenetic relationships between the families were derived from Chase et al. (1993). The recruitment events are indicated by black dots. The presence or absence of a ribonuclease SI system in a particular family is indicated by (+) or (-). The broken lines indicate a lineage without a ribonuclease SI system. The solid lines indicate a lineage with a ribonuclease SI system.

**(A)** An ancestral RNase gene was recruited into SI in a common progenitor, leading to the four families and lost in the Brassicaceae lineage. **(B)** Two ancestral RNase genes were independently recruited into SI in common ancestors, leading to the Solanaceae and Scrophulariaceae or the Rosaceae, respectively. SI in the Brassicaceae evolved independent of the RNase genes.

been an independent event (Figure 7B). Alternatively, the RNase system could have been lost during the evolution of the Brassicaceae, perhaps after the S locus glycoprotein and S receptor kinase were secondarily recruited into SI (Figure 7A). This view is supported by the result from phylogenetic analysis of S allele sequences showing that the age of Brassica SI is ~50 million years (Uyenoyama, 1995). Such an interpretation might also explain why the spatial pattern of Antirrhinum S RNase transcripts is similar to that directed by the promoter of the Brassica S locus glycoprotein gene when coupled to a reporter gene in transgenic tobacco (Kandasamy et al., 1990; Moore and Nasrallah, 1990). Further, genetic analysis of the sporophytic SI in the Brassicaceae suggests that a second gene, G, linked to an S gene, with gametophytic action, also controls the expression of SI (Lewis et al., 1988). It is unclear, however, whether the G gene is related to the S RNase gene.

## METHODS

### Plant Material

*Antirrhinum majus* stocks JI75 and JI522 and family K195 (all carrying *Sc/Sc*) and *A. hispanicum* lines 546 ( $S_3/S_4$ ), 547 ( $S_1/S_2$ ), and 548 ( $S_5/S_6$ ) were grown under standard greenhouse conditions (Carpenter et al., 1987). *A. hispanicum* lines originated from Spain and were obtained from the *Gatersleben* collection in Germany and maintained by vegetative propagation (line 546 was from the Botanical Garden in Barcelona; 547 was from Genilta, Spain; 548 was from Orgiva, Spain; Hammer et al., 1990). Stages of style development were determined according to Baldwin et al. (1992).

### Molecular Techniques

Antirrhinum DNA and RNA were isolated and hybridized as previously described (Coen et al., 1986, 1990). Polymerase chain reaction (PCR), cloning, and sequencing were performed using standard techniques. A primer (G2443) corresponding to the conserved C2 domain of the S locus ribonuclease (S RNase) family (5'-TT(T/C)ACYATYCA(T/C)-GGYCTYTGGCC-3', where Y is G, A, T, or C; Kheyr-Pour et al., 1990; loerger et al., 1991) was used in 3' rapid amplification of cDNA ends (reverse transcriptase [RT]-PCR), with RNAs prepared from styles harvested 1 day before anthesis (Frohman et al., 1988). Two representative plants each from the four self-incompatibility (SI) groups were individually used for RNA isolation. Two micrograms of total RNA was used for first-strand cDNA synthesis with a commercial kit (Bethesda Research Laboratories). PCR was performed using a program of 35 cycles at 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min with a 10-min final extension at 72°C. The PCR products were treated with T4 DNA polymerase before being ligated into the EcoRV-cut pKR vector (Waye et al., 1986). Styler-specific S RNase cDNA sequences were first selected by differential screening with labeled first-strand leaf and style cDNA probes. Additional differential screenings were made using labeled first-strand cDNA probes from various S genotypes.

The 5' regions encoding the S2, S4, and S5 alleles were obtained by 5' rapid amplification of cDNA ends using a commercial kit (Bethesda

Research Laboratories). The gene-specific primers (S2, 5'-CTGAGC-ATGTCTAGAAC-3'; S4, 5'-CAGTTCATGTGGTGGAAA-3'; and S5, 5'-CCGCTGAAGCATGTCTAA-3') were used to prime first-strand cDNA synthesis. For each cDNA, at least three independent clones were sequenced to avoid PCR artifacts. DNA sequence and amino acid analyses were performed using the Genetics Computer Group (Madison, WI) package. DNA sequences were submitted to the EMBL data base under the following accession numbers: S2 (X96465), S4 (X96466), and S5 (X96464).

### Phylogeny Construction

Amino acid sequences were aligned using the LOCALPILEUP of the Genetics Computer Group programs. Distance matrices based on amino acid sequence were produced with the PROTDIST program with the PHYLIP package (Felsenstein, 1989), using the Dayhoff PAM matrix. Phylogenetic trees were constructed from the results of 100 bootstrap replicates by the neighbor-joining method (Saitou and Nei, 1987), using the NEIGHBOR program of the PHYLIP package with the fungal RNases as an outgroup.

### Transcript Analysis

Expression of Antirrhinum S RNases was analyzed by RT-PCR using gene-specific primers (S2, 5'-AAAGATCACTGACAAAGG-3'; S4, 5'-CTC-AAGCAACAGTCTATC-3'; and S5, 5'-AAGAGGAAAAATATCGC-3'). As a control, the primers capable of amplifying Antirrhinum ubiquitin cDNAs (5'-CTTTGTGAAGACTCTGACC-3' and 5'-GGACTCCTCTGGAT-GTTG-3'; EMBL accession number X67957) were also included in the same reaction. After separation on 1.2% (w/v) agarose gel, the PCR products were blotted and probed with S allele-specific cDNAs and an Antirrhinum ubiquitin probe (kindly provided by C. Martin, John Innes Centre).

### RNA in Situ Hybridization

The 3' regions of S2 (551 bp), S4 (527 bp), and S5 (469 bp) cDNAs were amplified by PCR using gene-specific primers (S2, 5'-AAAGATCACTGACAAAGG-3' and 5'-GTATAAGATCGAGTGGG-3'; S4, 5'-CTC-AAGCAACAGTCTATC-3' and 5'-ACCACCCACCTCCAAATC-3'; S5, 5'-AAGAGGAAAAATATCGC-3' and 5'-TTAGAGATGTATTATAT-3') and then treated with T4 DNA polymerase and ligated into *Sma*I-cut pBluescript KS+ (Stratagene, La Jolla, CA) in sense or antisense orientations. Probe labeling using digoxigenin, tissue treatments, in situ hybridization, and signal detection were as previously described (Coen et al., 1990). No signal was detected using control sense-strand probes.

### ACKNOWLEDGMENTS

We thank Tim Robbins and Des Bradley for their critical comments on the manuscript and James Brown for his help with the phylogenetic analysis. We are grateful for support from the Biotechnology and Biological Sciences Research Council and the Gatsby Charitable Foundation. We thank Peter Walker for growing the plants and Gwyneth Ingram and Richard Waites for their work on this project during sum-



mer vacation. Y.X. also gratefully acknowledges the support of the K.C. Wong Education Foundation, Hong Kong.

Received January 25, 1996; accepted March 25, 1996.

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