Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8816–8820, October 1991
Plant Biology

Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*

(cell recognition/*S* locus)

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Communicated by André T. Jagendorf, July 1, 1991 (received for review May 6, 1991)

ABSTRACT **Self-recognition between pollen and stigma during pollination in *Brassica oleracea* is genetically controlled by the multiallelic self-incompatibility locus (*S*). We describe the *S* receptor kinase (*SRK*) gene, a previously uncharacterized gene that resides at the *S* locus. The nucleotide sequences of genomic DNA and of cDNAs corresponding to *SRK* predict a putative transmembrane receptor having serine/threonine-specific protein kinase activity. Its extracellular domain exhibits striking homology to the secreted product of the *S*-locus glycoprotein (*SLG*) gene and is connected via a single pass transmembrane domain to a protein kinase catalytic center. *SRK* alleles derived from different *S*-locus genotypes are highly polymorphic and have apparently evolved in unison with genetically linked alleles of *SLG*. *SRK* directs the synthesis of several alternative transcripts, which potentially encode different protein products, and these transcripts were detected exclusively in reproductive organs. The identification of *SRK* may provide new perspectives into the signal transduction mechanism underlying pollen recognition.**

Pollination and the subsequent invasive growth of pollen tubes into the female stigmatic and pistil tissues prior to fertilization provide an opportunity to study cell–cell interactions in flowering plants. In crucifers such as *Brassica oleracea*, self-recognition between pollen and stigma is controlled by the multiallelic self-incompatibility, or *S*, locus (1). In general, pollen germination and/or tube growth are arrested at the stigma surface if the pollen and stigma are borne by plants having identical *S*-locus genotypes. This arrest prevents self-fertilization and is termed the self-incompatibility (SI) response. Two related genes have been identified at the *S* locus by molecular methods (2–4). Of these, only one gene, the *S*-locus glycoprotein (*SLG*) gene has been characterized extensively. *SLG* encodes a secreted glycoprotein that is highly polymorphic in different *S*-locus genotypes (2) and may therefore be involved in determining the recognition specificity displayed in SI. Furthermore, *SLG* is expressed in stigmatic papillae (3) and anthers (5, 6), consistent with models for SI in which both pollen and stigma bear recognition determinants derived from the *S* locus.

In this study, we show that the second *S*-locus-linked gene (4) encodes a putative receptor protein kinase, and we have therefore designated it *SRK*, for *S* receptor kinase.† Its structure is similar to that predicted in a recently described maize root cDNA clone, *ZmPK1* (7), and is analogous to the growth factor receptor tyrosine kinases in animals. The putative ligand-binding domain is homologous to *SLG* and displays genotype-specific sequence polymorphisms that parallel those of *SLG*. *SRK* transcripts were detected only in the male and female reproductive organs, thus showing a pattern of expression similar to that of *SLG*. These findings offer foundation to the hypothesis that SI is mediated by receptor–ligand interactions between pollen and pistil and provide a possible framework for the study of signal transduction pathways that underlie cellular recognition in plants.

MATERIALS AND METHODS

Plant Material and Determination of Incompatibility Phenotype. The *B. oleracea* S_2S_2, S_6S_6, and $S_{14}S_{14}$ homozygous strains have been described (2, 4). Analysis of incompatibility phenotypes in progeny derived by selfing hybrids was as reported (4, 8).

Isolation and Analysis of Genomic and cDNA Clones. The isolation of *SLG*-related genomic clones from libraries of *B. oleracea* S_6S_6 and S_2S_2 DNA has been described (3, 4). Two SRK_6 cDNA clones, pS6-27 and pS6-32, were isolated from an S_6S_6 stigma cDNA library (2) by screening $\approx 1 \times 10^5$ plaques with the kinase-domain probe of the SRK_6 (see below). A third cDNA clone, pJS30, was isolated after amplification by PCR of S_6S_6 stigma cDNA using the mRNA GeneAmp kit (Perkin–Elmer/Cetus). The amplification was primed with an upstream oligonucleotide primer (5′-ACTTGTGGCAAAGCTTCGATT-3′) and a downstream primer (5′-CCATCCCGAATTCCGAGATCT-3′) complementary to sequences within the S and kinase domains of SRK_6, respectively. The resulting fragment was cloned in the vector pCR1000 using the TA cloning kit (Invitrogen). Because PCR can introduce artifactual base substitutions, three independent isolates of pJS30 were sequenced. Dideoxynucleotide sequencing was performed with double-stranded plasmid templates (9) prepared by cloning restriction fragments into appropriate Bluescript vectors (Stratagene) and by generating nested deletions with the Erase-a-Base kit (Promega). In addition, oligonucleotides complementary to regularly spaced sites along the SRK_6 gene were used as sequencing primers. Oligonucleotides were prepared at the Cornell Biotechnology Analytical and Synthetic Facility. Sequence analysis was performed with the IBI Pustell and GCG version 6.0 (University of Wisconsin Biotechnology Center) software packages. The GenBank and Swiss-Prot data bases were searched for protein similarity by using FASTA (10).

Nucleic Acid Isolation and Gel Blot Hybridization. The isolation and blot analyses of plant DNA were as described (8). RNA blot analysis was also as described (8) except that poly(A) RNA was isolated from plant tissues by using the FastTrack RNA purification kit (Invitrogen) and resolved on 1% (wt/vol) agarose gels in the presence of formaldehyde. An RNA ladder (Bethesda Research Laboratories) was used to

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Abbreviations: SI, self-incompatibility; aa, amino acid(s).

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M76647).

estimate the molecular weight of mRNA transcripts. DNA probes were labeled with ^{32}P using the random primer kit of Boehringer Mannheim. Three SRK_6-derived probes were used (see Fig. 2A): the S-domain probe was a 1186-base-pair (bp) *Hin*cII fragment that spanned the majority of SRK_6 exon 1; the intron probe was a 747-bp *Pst* I/*Hin*cII fragment derived from intron 1; the 2135-bp kinase-domain probe spanned exons 2–7. The actin probe was a 1.6-kilobase (kb) cDNA derived from a *B. oleracea* actin gene. For DNA blot analysis, we used an *SLG*-specific probe derived from the 3′ untranslated region of SLG_6 cDNA (3), and the SRK_6-specific intron probe described above.

Transgenic Plants. A 5.8-kb *Eco*RI fragment that contained the SRK_6 coding region with 0.45 kb of upstream and 1.42 kb of downstream flanking sequence and a chimeric hygromycin phosphotransferase gene for selection of transformed plants were inserted into the polycloning site of the binary vector pBIN19 (11). Cells of *Agrobacterium tumefaciens* strain pCIB542/A136 that harbored the resulting plasmid were used to transform flowering stem disks of *B. oleracea* as described (12). The presence of the SRK_6 transgene was confirmed in hygromycin-resistant plants by DNA blot analysis of *Eco*RI-digested DNA by using the intron probe.

RESULTS AND DISCUSSION

Isolation of the *SRK* Gene and Its Linkage to the *S* Locus. SRK_6, the *SRK* gene derived from a *B. oleracea* S_6S_6 homozygote, was isolated from a collection of *SLG* homologous genomic clones (3). Attempts to characterize clones derived from the *S* locus identified a genomic region defined by nine independent overlapping clones. Sequence analysis of DNA flanking the region of *SLG* homology in these clones revealed sequence similarity to known protein kinases and to *ZmPK1* (7) (see below). SRK_6 was shown to be genetically linked to the *S* locus by restriction fragment length polymorphism analysis of a population of F_2 plants segregating for the S_6 and S_{14} SI phenotypes (Fig. 1). As previously reported (8), an *SLG*-specific probe identified an *Eco*RI restriction fragment length polymorphism between the two parental strains that indicated which *SLG* allele(s) was present in each of the plants examined (Fig. 1A). When a DNA fragment immediately downstream of the *SLG* homologous region of the SRK_6 gene (derived from intron 1 of SRK_6; see Fig. 2A) was used as a probe, a single *Eco*RI restriction fragment was identified in the S_6 parent but not in the S_{14} parent (Fig. 1B). A perfect correlation was observed between the presence of the SLG_6 allele, the presence of the SRK_6 allele, and the S_6 incompatibility specificity as determined by pollination assays in a total of 78 F_2 plants in this population (16 S_6S_6:38 S_6S_{14}:24 $S_{14}S_{14}$), of which 9 are shown in Fig. 1. We conclude that *SLG* and *SRK* are tightly linked genetically and that they reside at the *S* locus.

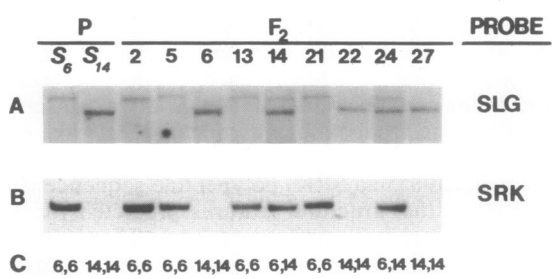
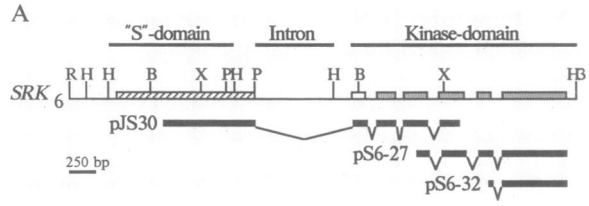


FIG. 1. Restriction fragment length polymorphism analysis of an F_2 population segregating for the S_6 and S_{14} self-incompatibility alleles. DNA isolated from parental (P) plants homozygous for either the S_6 or S_{14} allele and from nine F_2 progeny was digested with *Eco*RI and analyzed by blot hybridization. Segregation patterns observed after hybridization with probes specific for *SLG* (A) and SRK_6 (B) are shown. (C) Incompatibility phenotype of each plant as determined by pollination tests.

Sequence Analysis of *SRK*. The structure of SRK_6 was determined by sequencing both strands of a 4.8-kb genomic fragment and three overlapping S_6S_6 stigma cDNA clones, pS6-27 (1105 bp), pS6-32 (686 bp), and pJS30 (1523 bp) (Fig. 2A). Comparison of the genomic and cDNA sequences revealed 7 exons interspaced with 6 introns ranging in size from 76 bp (intron 3) to 896 bp (intron 1). The coding region (Fig. 2B) has a single open reading frame that predicts a protein of 857 amino acids (aa). Two alternative polyadenylylation sites generate a 3′ noncoding region of either 192 or 261 bp.

The predicted SRK_6 polypeptide (98,071 D) consists of several domains. Each domain is encoded by separate exons or groups of exons, consistent with the view that multidomain proteins evolved by exon shuffling (14). The first 437 aa residues, encoded by exon 1, are highly similar to SLG_6 (2), and we therefore refer to this as the *SLG* homologous domain, or S domain. The hydrophobic residues (−32 to −1) at the N terminus constitute a putative signal peptide that is 67% identical to the signal sequence of SLG_6 (Figs. 2B and 3). The rest of the S domain (residues 1–406) is 89% identical to SLG_6 and contains a cluster of 12 cysteine residues, a hallmark of the *S* multigene family (15). Exon 2 encodes a 20-aa hydrophobic stretch (residues 415–434) that is likely to form a membrane-spanning helix. As is typical of transmembrane domains (16), 6 of the 8 aa that follow this region are basic. The remaining sequence, encoded by exons 3–7, contains a putative protein kinase catalytic domain (residues 489–781) that is flanked by a 55-aa juxtamembrane domain and a 44-aa C terminus domain. A diagnostic feature of protein kinases is the presence of 11 conserved subdomains wherein lie 15 invariant or nearly invariant aa residues (13). These subdomains as well as all 15 conserved residues were identified in the predicted protein sequence of SRK_6 (Figs. 2B and 3). The substrate specificity of protein kinases, either serine/threonine or tyrosine, generally correlates with consensus sequences within subdomains VI and VIII (13). The sequences in subdomains VI (DLKVSN) and VIII (GTY-GYMSPE) strongly suggest that the SRK_6 product has serine/threonine kinase activity. A data base search with the SRK_6 polypeptide sequence revealed greatest similarity, after *SLG*, to maize *ZmPK1* (7), showing conservation with both the S domain (29% identity) and the kinase domain (36% identity) (Fig. 3). All of the remaining sequences identified in the search corresponded to the catalytic core domains of protein kinases. Among the highest scoring were members of the *raf* serine/threonine kinase family and the tyrosine kinase family, but these were more distantly related to SRK_6 than the putative catalytic domain of *ZmPK1* (data not shown).

These results suggest that SRK_6 encodes an integral membrane protein. The S domain plus 9 residues encoded by exon 2 would be located extracellularly, and the putative kinase domain and flanking regions would be oriented toward the cytoplasm. Of 10 potential N glycosylation sites [NX(S/T)], 7 would occur extracellularly (Fig. 2B), and 5 of these are conserved with the 9 sites in SLG_6. This structure is similar to receptor tyrosine kinases, which have a glycosylated extracellular domain responsible for ligand binding, a single-pass transmembrane domain, and an intracellular tyrosine kinase catalytic domain (17). The existence of receptors with possible serine/threonine kinase specificity has only recently been suggested by the description of *ZmPK1*, whose function has not been determined, and the *Caenorhabditis elegans daf-1* gene, which controls the development of a nonfeeding larval stage (18). However, sequence-based predictions of kinase substrate specificity can be misleading (19), and only direct biochemical evidence will determine whether these genes represent an additional class of receptor kinases.



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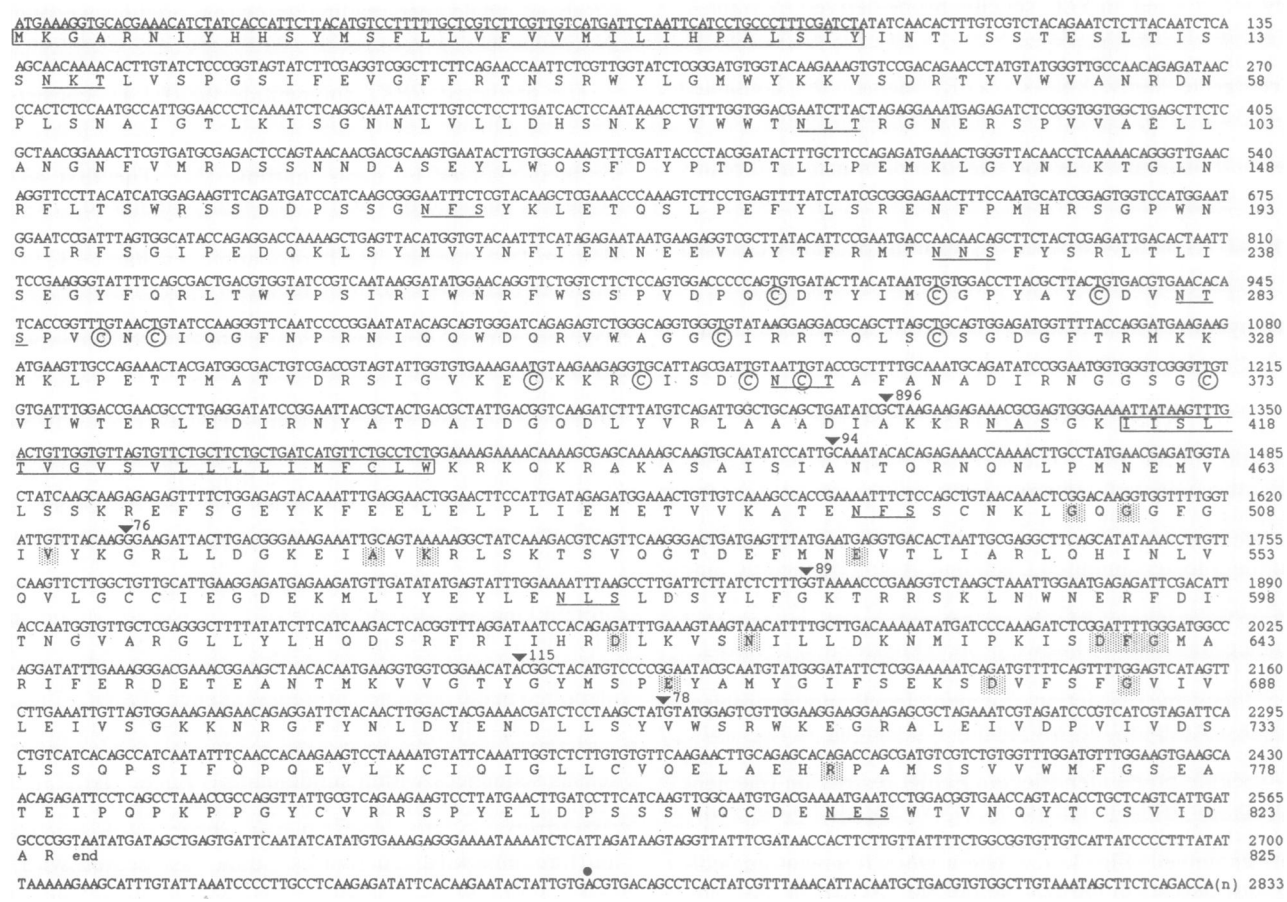

FIG. 2. Structure of the SRK_6 gene. (*A*) Partial restriction map of the sequenced genomic fragment containing SRK_6 and flanking regions. Exons are depicted as boxes; hatched box represents exon 1 and contains the S domain; open box represents exon 2 and contains the putative transmembrane domain; stippled boxes represent the remaining 5 exons that contain the putative kinase domain. Below the map, the extents of three cDNA clones are shown. Lines above the map indicate the three SRK_6-derived probes used in this paper. R, *Eco*RI; H, *Hin*cII; B, *Bcl* I; X, *Xho* I; P, *Pst* I; H3, *Hin*dIII. (*B*) Nucleotide and deduced aa sequence of the SRK_6 coding region. Positions and lengths of the introns (arrowheads) were determined by comparing the genomic sequence to the cDNA sequences of clones pJS30 (bases 479–2001), pS6-27 (bases 1723–2763), and pS6-32 (bases 2180–2833). The putative signal peptide and transmembrane domain are boxed. Circles indicate the 12 cysteine residues that are conserved in all members of the *S*-multigene family. Potential N glycosylation sites are underlined. Conserved aa residues that are diagnostic of protein kinases (13) are highlighted. The alternative polyadenylylation site observed in pS6-27 is indicated by a dot.

Sequence Divergence of *SRK* Alleles Isolated from Two Different *S* Genotypes. A pair of *S*-locus-linked genes were previously isolated from the S_2S_2 genotype and designated *SLG2A* and *SLG2B* (4). The isolation of cDNA clones corresponding to *SLG2A* had identified this gene as the S_2 homologue of SLG_6, and we therefore refer to this gene as SLG_2. We have now sequenced 7.2 kb of the *SLG2B* genomic clone and have identified the same arrangement of 7 exons and 6 introns observed in SRK_6. We therefore redesignate *SLG2B* as SRK_2, the homologue of SRK_6 in plants with the S_2 recognition genotype. Of the 7 SRK_2 exons, only the first was reported in the previous work (4). The location of the remaining 6 exons begins ≈2.2 kb downstream of the first. Strikingly, both the SRK_6 and SRK_2 genomic sequences have an in-frame stop codon (TAG) within intron 1 positioned 2 nucleotides downstream of the 5′ splice site. The placement of this stop codon relative to the S domain of *SRK* exactly matches the position of the stop codon used by *SLG* (Fig. 4*A*), suggesting that it has functional relevance. For example, the stop codon could be retained in alternative transcripts of *SRK*, causing the production of a truncated SLG-like protein consisting of only the S domain.

The predicted 855-aa SRK_2 polypeptide sequence (Fig. 3) contains all of the structural features predicted for SRK_6; however, they are only 68% conserved. The most highly conserved domains are the S (67%) and kinase (71%) domains (Fig. 4*B*). This level of divergence is surprisingly high for allelic variants, yet it parallels the polymorphism observed between alleles of *SLG*, since the SLG_6 and SLG_2 protein sequences are 68% identical. In contrast, each *SRK* S domain was more similar to its corresponding *SLG* allele (≈90% aa identity; Fig. 4*B*). Thus, *SRK* and *SLG* are structurally related gene pairs. As a unit, they have apparently evolved in concert within each *S* genotype, while diverging extensively

FIG. 3. Sequence alignment of the predicted SRK_6, SRK_2, SLG_6, and ZmPK1 proteins. Identical residues are indicated by the double line, and gaps introduced to maximize alignment are indicated by asterisks. Residues that are highly conserved in the 11 subdomains of protein kinases are highlighted.

between genotypes. The divergence in the *S* domains between *SRK* alleles is consistent with a possible role for this domain in providing the recognition specificity in different *S*-locus genotypes. Interestingly, the kinase, juxtamembrane, and C terminus domains are also highly diverged between the two *SRK* alleles. Thus, it is conceivable that these receptors, in addition to having different ligand-binding properties, also have different catalytic properties.

Expression of the *SRK* Gene. To study the expression of SRK_6, we subjected poly(A) RNA from pistils, anthers, leaves, cotyledons, and roots to gel blot analysis by using separate probes derived from the kinase domain, the S domain, and the first intron of SRK_6 (see Fig. 2*A*). The most intense hybridization signals were obtained in mature pistils where transcripts ranged in size from 1.6 to 5.9 kb (Fig. 5*A*). Some of these transcript species were assigned to genes other than SRK_6. In particular, the highly abundant S homologous 1.6-kb transcript was previously shown to be derived from the *SLG* gene in experiments with transgenic tobacco (20). Only the transcript species of 3.0 (arrowhead), 4.1 (asterisk), and 2.3 (circle) kb were unambiguously assigned to the SRK_6 gene, and their characterization is described in detail.

The 3.0-kb transcript in pistils and a slightly smaller transcript in anthers were recognized by both the kinase and S domain probes, but not the intron probe. These transcript sizes were consistent with the predicted size (2832 bp) of a fully spliced SRK_6 transcript. A 3.0-kb transcript was also detected by the kinase domain probe in leaves and in cotyledons after very long (>1 week) film exposures (data not shown), but not in seedling roots. This leaf transcript did not hybridize to the S domain probe except under low-stringency wash conditions (data not shown) and was thus derived, not from the SRK_6 gene, but from any of several *SRK*-related genes detected in the *Brassica* genome (unpublished observations). The 4.1-kb transcript hybridized to the intron probe as well as the kinase and S domain probes and was of a size consistent with that predicted for an unspliced SRK_6 transcript (4180 bp). The 2.3-kb transcript hybridized to the kinase domain and intron probes but not to the S domain probe and was possibly the result of alternative splicing or the action of an alternative promoter.

The levels of fully spliced and unspliced SRK_6 transcripts were regulated in anthers during the rapid succession of pollen developmental stages (Fig. 5*B*). Maximal expression of SRK_6 was observed in anthers at the binucleate microspore stage. At the uninucleate stage there were low levels of transcript, and by the trinucleate stage no expression was detected. This regulation of message quantity cannot be attributed to general variation of RNA levels since RNA from all stages contained equal amounts of actin transcript (Fig. 5*B*). To verify the origin of these transcripts, we introduced the SRK_6 gene into a recipient *B. oleracea* strain that carried the S_2 allele (12). Because the *SRK* genes derived from the S_2 and S_6 alleles are highly diverged, the *SRK* transcripts endogenous to this strain were not recognized by the SRK_6-derived probes at the stringency used. In 10 transgenic plants analyzed, several showed detectable levels of the spliced and unspliced transcripts (Fig. 5*C*). These transcripts were ob-

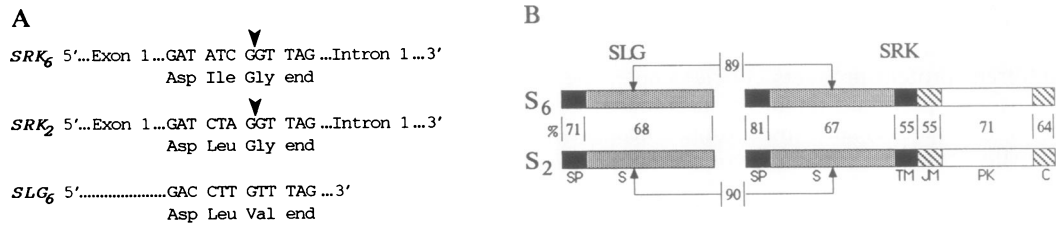

FIG. 4. (*A*) DNA sequence at the first exon/intron junction in SRK_6 and SRK_2 and the corresponding sequence in SLG_6. Arrowheads indicate splice sites. Dots represent nucleotides omitted for diagrammatic clarity. *SRK* genes have an in-frame stop codon located 2 nucleotides downstream of the 5′ splice site of intron 1, which is removed in fully spliced transcripts. Transcripts of *SLG* are not spliced and therefore retain the stop codon at this position. (*B*) Concerted evolution of the *SLG*/*SRK* gene pair within *S* genotypes and their divergence between genotypes. The predicted SLG and SRK proteins encoded by the S_6 and S_2 genotypes are represented schematically. Numbers indicate percentage identity between putative domains. SP, signal peptide; S, S domain; TM, transmembrane domain; JM, juxtamembrane domain; PK, protein kinase domain; C, C terminus domain.

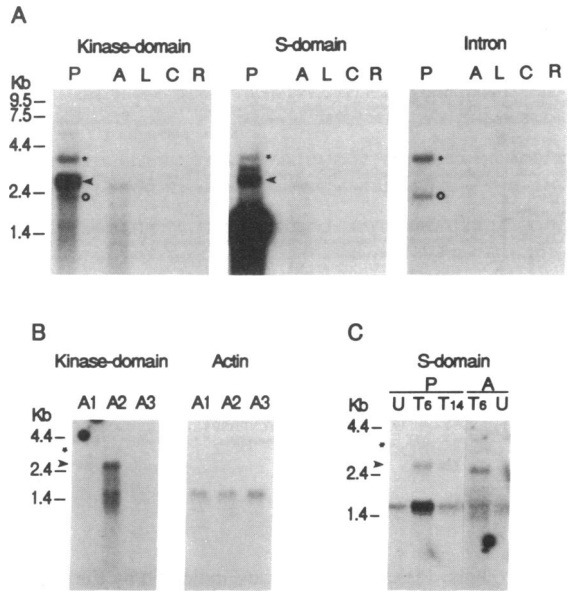

FIG. 5. Expression of the SRK_6 gene. (*A*) SRK_6 homologous transcripts in pistils (lanes P), anthers (lanes A), leaves (lanes L), cotyledons (lanes C), and roots (lanes R) of *B. oleracea* S_6S_6. Each lane contained ≈2 μg of poly(A) RNA. The blot was hybridized sequentially with a ^{32}P-labeled probe derived from the kinase domain, the first intron, and the S domain of SRK_6. Subsequent hybridization with the actin probe showed comparable amounts of RNA in each lane (data not shown). Arrowhead, asterisk, and circle denote the *SRK*-encoded transcripts described in the text. Migration of RNA molecular weight markers is shown on the left. (*B*) SRK_6 homologous transcripts in developing *Brassica* anthers. Each lane contained ≈5 μg of poly(A) RNA isolated from anthers that contained uninucleate (lanes A1), binucleate (lanes A2), and trinucleate (lanes A3) microspores. Stages were determined by 4′,6-diamidino-2-phenylindole staining of nuclei (6). The blot was hybridized first with a ^{32}P-labeled probe derived from the kinase domain of SRK_6 and subsequently with an actin probe to show equivalent amounts of RNA in all three lanes. (*C*) Expression of SRK_6 in transgenic *Brassica*. Poly(A) RNA from transgenic plants (lanes T6 and T14) and a control untransformed plant (lanes U) was hybridized with the SRK_6 S domain probe. Approximately 10 times more anther (lanes A) than pistil (lanes P) RNA was analyzed. The differences in the intensities of the endogenous pistil 1.6-kb transcripts between plants reflect the differences in the amounts of RNA loaded in each lane.

served exclusively in pistil and anther tissue and were not detected in untransformed controls. Transcripts of 1.6 kb were detected in both transformed and untransformed plants and therefore represented endogenous messages not encoded by the SRK_6 transgene.

Thus, like *SLG* (5, 6), the *SRK* gene is specifically transcribed in pistils and anthers. However, *SRK* transcripts are substantially less abundant in mature pistils than *SLG* transcripts, since they produced a 140- to 180-fold weaker hybridization signal with the S-domain probe. In addition, *SRK* directs the synthesis of several mRNA species that potentially encode different protein products. For example, the unspliced 4.1-kb transcript would retain the in-frame stop codon in intron 1 (see above) and thus would encode a secreted C-terminally truncated protein encompassing only the S domain. In contrast, the 2.3-kb transcript, which lacks most or all of exon 1, possibly encodes an N-terminally truncated protein. Instances in which receptor tyrosine kinase genes encode N-terminally truncated (21) as well as C-terminally truncated forms (22) have been identified.

***SRK* and Pollen Recognition.** Several lines of evidence imply that *SRK* functions in the mechanism of SI. First, *SRK* is located at the *S* locus, as was independently demonstrated for two different *SRK* alleles (this report; see also ref. 4). Second, the predicted products of these *SRK* alleles show the high degree of polymorphism expected for proteins responsible for cellular recognition specificity. Finally, *SRK* transcription is active in the male and female reproductive organs, from which are derived the two interacting cell types that mediate recognition. We propose that *SRK*, acting in combination with *SLG*, produces cellular recognition molecules that are responsible for the allelic specificity of SI. A possible model for this recognition might involve the allele-specific binding of a putative ligand that might also be encoded at the *S* locus. Alternatively, *SRK* and *SLG* might display allele-specific homophilic binding. The concerted evolution of the *SLG/SRK* gene pair suggests that their products interact functionally, perhaps as components of a ligand binding complex or as unassociated competitors for this binding. Moreover, by phosphorylating intracellular substrates, *SRK* might couple these initial molecular recognition events to the signal transduction chain that leads to pollen acceptance or rejection. Attempts have been made to alter the SI phenotype when alleles of *SLG* and *SRK* were individually transformed into recipient plants having a different *S*-locus genotype, with results that could not conclusively demonstrate that these genes are responsible for recognition specificity. A test of the hypothesis that both of these genes are required for recognition specificity can be made when an *SLG/SRK* gene pair is introduced into a recipient plant.

We wish to thank M. K. Kandasamy for his help with plant transformation, J. Acciai for constructing nested deletions, J. Andersland for his help designing figures, and R. Wu for providing the rice actin clone used to isolate the *Brassica* actin cDNA. This work was supported by U.S. Department of Agriculture Grant 89-3721-4458 and Department of Energy Grant DE-FG02-88ER 13909.

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