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The S-Locus Receptor Kinase Gene in a Self-Incompatible *Brassica napus* Line Encodes a Functional Serine/Threonine Kinase

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An S-receptor kinase (SRK) cDNA, SRK-910, from the active S-locus in a self-incompatible *Brassica napus* W1 line has been isolated and characterized. The SRK-910 gene is predominantly expressed in pistils and segregates with the W1 self-incompatibility phenotype in an F_2 population derived from a cross between the self-incompatible W1 line and a self-compatible Westar line. Analysis of the predicted amino acid sequence demonstrated that the extracellular receptor domain is highly homologous to S-locus glycoproteins, whereas the cytoplasmic kinase domain contains conserved amino acids present in serine/threonine kinases. An SRK-910 kinase protein fusion was produced in *Escherichia coli* and found to contain kinase activity. Phosphoamino acid analysis confirmed that only serine and threonine residues were phosphorylated. Thus, the SRK-910 gene encodes a functional serine/threonine receptor kinase.

INTRODUCTION

Signal transduction by receptor kinases occurs in many aspects of cell growth, development, and differentiation (Cadena and Gill, 1992; Karin, 1992). The majority of receptor kinases characterized to date have been found to specifically phosphorylate tyrosine residues (Ullrich and Schlessinger, 1990). Mutations in these types of receptors have also been implicated in oncogenesis (Aaronson, 1991; Cantley et al., 1991). Recently, there have been a few reports of other receptor kinases with homologies to serine/threonine cytoplasmic kinases. One of these receptor kinases has been shown to possess serine/threonine phosphorylation activity (Lin et al., 1992), whereas another displays serine, threonine, and tyrosine kinase activity (Douville et al., 1992). In plants, very little is known about the role of receptor kinases in signal transduction. There have been three reports on the isolation of putative plant receptor kinases (Walker and Zhang, 1990; Stein et al., 1991; Tobias et al., 1992). Based on sequence homology only, these genes appear to encode serine/threonine kinases. One of these receptor kinases has been implicated in the self-incompatibility system of *Brassica oleracea* (Stein et al., 1991).

Self-incompatibility in Brassica is controlled by a single dominant genetic locus called the S-locus (Bateman, 1955). The sporophytic nature of this incompatibility system results in the pollen phenotype being derived from the genotype of the diploid pollen parent and not from the haploid pollen genotype. This is hypothesized to occur by the deposition of an S-factor in the exine (outer coat) of the pollen grain by the anther tapetum (parental tissue) during pollen development (de Nettancourt, 1977). When a pollen grain lands on the stigma surface, the action of the S-locus results in a block in fertilization if the same S-allele is present in the pollen parent and the pistil. The response is very rapid and for the stronger alleles leads to a block in pollen hydration and germination and an inability to penetrate the stigma barrier (Zuberi and Dickinson, 1985; Gaude and Dumas, 1987). There are multiple alleles at the S-locus, and it has been estimated that in *B. oleracea*, there are nearly 50 different alleles (Ockendon, 1974, 1982). In heterozygous plants, the majority of *B. oleracea* S-alleles have been found to be dominant, codominant, or recessive to the second allele in a nonlinear arrangement dependent on the allele combinations. A few alleles, called pollen recessive alleles, have been shown to be always recessive to other S-alleles in the pollen (Thompson and Taylor, 1966). Both of the diploid Brassica species, *B. campestris* and *B. oleracea*, possess this self-incompatibility system, whereas *B. napus*, an allotetraploid composed of the *B. campestris* and *B. oleracea* genomes, generally occurs as a self-compatible plant (Downey and Rakow, 1987). There are a few naturally occurring self-incompatible *B. napus* lines (Olsson, 1960; Gowers, 1981), and self-incompatible lines have also been generated by introgressing an S-locus from *B. campestris* (Mackay, 1977).

Initial studies on the Brassica self-incompatibility system have shown that there is an abundant soluble glycoprotein present in the cell wall of the stigma papillae cells associated with this response (Nasrallah et al., 1970; Hinata and Nishio, 1978; Kandasamy et al., 1989). Several genes for these S-locus glycoproteins (SLGs) have been cloned and characterized

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(Nasrallah et al., 1987; Trick and Flavell, 1989; Dwyer et al., 1991; Goring et al., 1992a, 1992b). Among the alleles associated with a strong incompatibility phenotype, there is greater than 80% homology at the DNA level (Dwyer et al., 1991). The weak pollen recessive alleles are also highly homologous to each other but only about 70% homologous to the first group of phenotypically strong alleles (Scutt and Croy, 1992). Transformation of a self-compatible *B. napus* line with these SLG alleles does not produce a self-incompatibility phenotype (Nishio et al., 1992). Recently, a second gene at the S-locus has been cloned from *B. oleracea.* This gene, the S-locus receptor kinase (SRK) gene, shows sequence homologies at the N-terminal end to SLG genes and at the C-terminal end to serine/threonine kinases (Stein et al., 1991).

We have been working with a canola line (*B. napus* ssp oleifera) called W1, which was produced by introgressing a *B. campestris* S-locus into the self-compatible Westar canola cultivar. The S-locus in this line gives a very strong self-incompatibility response and provides a potentially useful line and S-allele for producing hybrids. The SLG allele (SLG-910) at the W1 S-locus has been characterized and shown to be very similar to many of the *B. oleracea* phenotypically strong SLG alleles (Goring et al., 1992a). We have now isolated a cDNA for the corresponding SRK-910 allele and have addressed the question of whether this cDNA encodes a functional kinase, as suggested by the translated amino acid sequence. In this paper, we show that the SRK-910 gene associated with the S-locus in the W1 line does specify a protein with serine/threonine kinase activity.

RESULTS

Sequence Analysis of the SRK-910 cDNA

The W1 line, which has been described previously, is a self-incompatible *B. napus* ssp oleifera (canola) cultivar derived from the introgression of a *B. campestris* S-locus into the self-compatible canola Westar line (Goring et al., 1992a). DNA blot analysis of W1 genomic DNA with the SLG-A14 allele isolated from another canola line (Goring et al., 1992b) revealed two cross-hybridizing HindIII bands of 3.6 kb and 6.5 kb, respectively. The 6.5-kb band was shown to encode a highly expressed SLG-910 allele, which segregated with W1 self-incompatibility (Goring et al., 1992a). Characterization of the 3.6-kb band led to the cloning and sequencing of a cDNA representing an SRK allele designated as SRK-910. This allele was also found to segregate with the W1 self-incompatibility phenotype (data not shown).

The SRK-910 DNA sequence has an open reading frame of 2574 bp for a predicted protein sequence of 858 amino acids, as shown in Figure 1. There are features in this sequence that are representative of SLG alleles, such as the 12 cysteine residues conserved in all SLG sequences (Figure 1, stars). In addition, there are seven potential N-glycosylation sites (Figure 1, dashed lines) in keeping with the fact that the SLG proteins are glycosylated (Takayama et al., 1986, 1989). A hydropathy plot of the predicted amino acid sequence in Figure 2 shows a putative signal peptide at the N-terminal end and a putative transmembrane domain separating the SLG homologous N terminus with the rest of the coding region. Homology comparisons of the SRK-910 SLG domain to other SLG alleles indicated that the SRK-910 allele is most closely related to its SLG counterpart at the same locus, the SLG-910 allele. At the DNA level, there is 90% homology between the two genes and 84% similarity at the amino acid level, as shown in Figure 2. Amino acid homologies to other phenotypically strong SLG alleles range from 72 to 79% (data not shown).

The predicted amino acid sequence of the 3′ end of the gene, after the transmembrane domain, contains conserved amino acids found in serine/threonine protein kinases (Hanks et al., 1988). In plants, there have been three other reports of receptor kinases and all have contained the serine/threonine protein kinase consensus sequences (Walker and Zheng, 1990; Stein et al., 1991; Tobias et al., 1992). As shown in Figure 2, alignment of the SRK-910 sequence to those other receptor kinases indicated that it is most similar to the SRK-6 gene isolated from *B. oleracea.* Because comparisons of SLG alleles from *B. oleracea* and *B. campestris* have shown that these alleles are equally similar across species as they are within species (Dwyer et al., 1991; Goring et al., 1992a), the high level of

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MKGVRKTYDSSYTLSFLLVFFVMFLFHPALSIHINTLSSTESLTISNNRT    50
LVSPGNVFELGFFRTTSSSRWYLGIWYKNLPYKTYVWVANRDNPLSDSIG   100
TLKISNMNLVLLDHSNKSVWSTNLTRGNERSPVVAELLENGNFVIRYSNN   150
NNASGFLWQSFDFPTDTLLPEMKLGYDRKKGLNRFLTAWRNSDDPSSGEI   200
SYQLDTQRGMPEFYLLKNGVRGYRSGPWNGVRFNGIPEDQKLSYMVYNFT   250
DNSEEAAYTFRMTDKSIYSRLIISNDEYLARLTFTPTSWEWNLFWTSPEE   300
PECDVYKTCGSYAYCDVNTSPVCNCIQGFKPFNMQQWELRVWAGGCIRRT   350
RLSCNGDGFTRMKNMKLPETTMAIVDRSIGRKECKKRCLSDCNCTAFANA   400
DIRNGGSGCVIWTGELEDIRNYFDDGQDLYVRLAAADLVKKRNANGKTIA   450
LIVGVCVLLLMIMFCLWKRKQKRAKTTATSIVNRQRNQDLLMNGMILSSK   500
RQLPIENKTEELELPLIELEAVVKATENFSNCNKLGQGGFGIVYKGRLLD   550
GQEIAVKRLSKTSVQGTGEFMNEVRLIARLQHINLVRILGCCIEADEKML   600
VYEYLENLSLDSYLFGNKRSSTLNWKDRFNITNGVARGLLYLHQDSRFRI   650
IHRDMKVSNILLDKNMTPKISDFGMARIFARDETEANTRKVVGTYGYMSP   700
EYAMDGVFSEKSDVFSFGVIVLEIVSGKRNRGFYNLNHENNLLSYVWSHW   750
TEGRALEIVDPVIVDSLSSLPATFQPKEVLKCIQIGLLCVQERAEHRPTM   800
SSVVWMLGSEATEIPQPTPPGYSLGRSPYENNPSSSRHCDDDESWTVNQY   850
TCSDIDAR                                              858
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Figure 1. The Predicted Amino Acid Sequence of the SRK-910 cDNA.

The underlined sections represent the signal peptide and transmembrane domain, respectively. Conserved cysteine residues are marked by stars above the amino acid residues. Potential N-glycosylation sites are represented by dashed lines. The nucleotide sequence has been submitted to GenBank (accession number M97667).

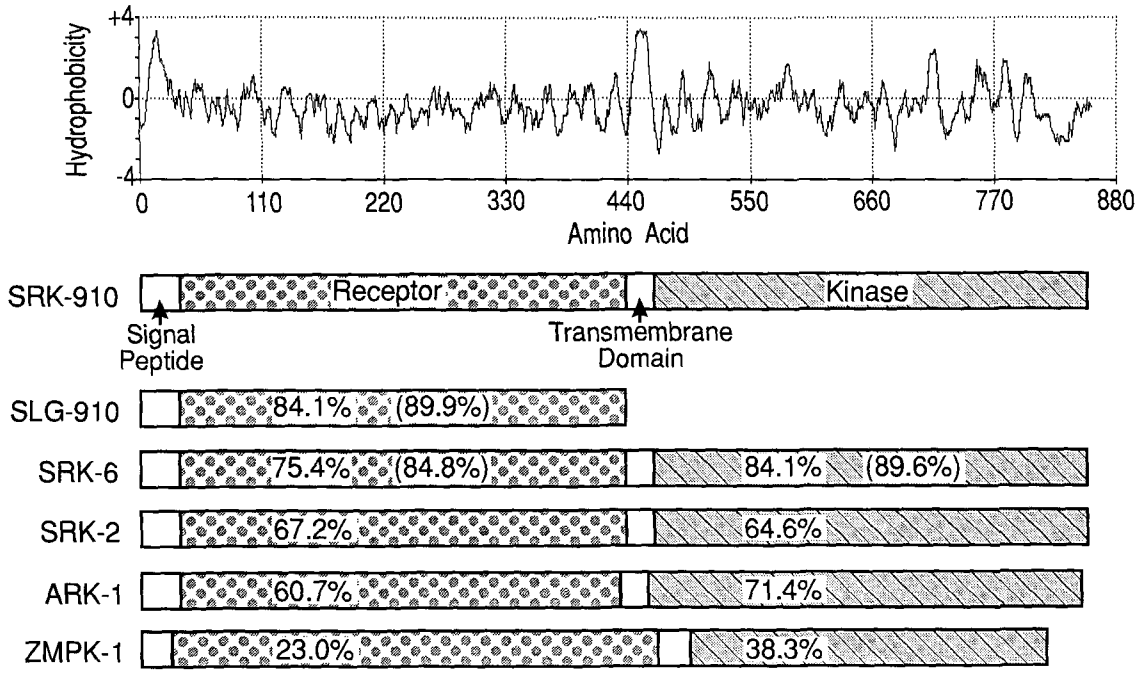

Figure 2. Analysis of the SRK-910 Sequence.

A Kyte hydropathy plot (Kyte and Doolittle, 1982) of the predicted amino acid sequence generated by PROSIS software (using a window value of 10) is shown at the top. Increased hydrophobicity is indicated by positive values. Underneath, the domains of the SRK-910 protein are illustrated. Amino acid homology comparisons are shown between the SRK-910 receptor and its SLG-910 counterpart (Goring et al., 1992a), and comparisons of the SRK-910 receptor and kinase domains to SRK-6 and SRK-2 from *B. oleracea* (Stein et al., 1991), ARK-1 from Arabidopsis (Tobias et al., 1992), and ZmPK-1 (ZMPK-1) from corn (Walker and Zhang, 1990). DNA homologies for the SLG-910 and SRK-6 are shown in parentheses.

similarity between SRK-910 (*B. campestris* origin) and SRK-6 (*B. oleracea* origin) is not surprising. However, a comparison between these two genes of the SLG domain and kinase domain separately showed an interesting feature. In the kinase domain, the homology between the SRK-910 and SRK-6 DNA sequences is 89.6% and the amino acid similarity is 84.1% with a difference of 5.5%. In the receptor domain, the DNA homology is 84.8%; however, the amino acid similarity decreases to 75.4% with a difference of 9.4%. Because it is likely that the extracellular receptor domain determines the specificity of each allele, there appears to have been a greater selection for base substitutions in this region that alter the amino acid sequence. There is a significant, but lower, level of homology to the *B. oleracea* pollen recessive SRK-2 gene and the Arabidopsis ARK-1 gene. The ARK-1 gene is not an S-locus gene because Arapidopsis, despite being closely related to the Brassica family, does not possess a self-incompatibility system. The corn ZmPK-1 gene is most distantly related to the SRK-910 gene with higher levels of homology detected in the kinase domain.

Hanks et al. (1988) have shown in an alignment of other eukaryotic protein kinases that within 11 domains there are several absolutely conserved amino acids and several conserved amino acid groups. An alignment of the 11 domains within the kinase region of the five plant receptor kinases is shown in Figure 3 with the consensus amino acids indicated on the top line. All of the absolutely conserved amino acids (Figure 3, bold type) are present. The conserved amino acid groups (Figure 3, regular type) are also present. The two underlined regions represent consensus sequences differentiating between serine/threonine kinases and tyrosine kinases. Whereas the sequence of the corn ZmPK-1 protein most closely represents the two consensus regions, the SRK-910 is most divergent, especially in the first consensus region. The second consensus region in the SRK-910 is closer to the serine/threonine kinase consensus sequence than is found for tyrosine kinases (P-I/V-K/R-W-T/M-A-P-E). Recently, a number of protein kinases have been isolated that contain the consensus serine/threonine sequences but demonstrate serine/threonine and tyrosine activity when tested. Seger et al. (1991) noted some sequence homologies specific to these serine/threonine and tyrosine kinases in domain XI. A search for these consensus sequences in domain XI of the plant kinases did not reveal any similarities.

Figure 3. Alignment of Kinase Domains from Plant Receptor Kinases.

The amino acid sequence of the SRK-910 kinase domain is compared to SRK-6 and SRK-2 from *B. oleracea* (Stein et al., 1991), ARK-1 from Arabidopsis (Tobias et al., 1992), and ZmPK-1 (ZMPK-1) from corn (Walker and Zhang, 1990). Capital letters indicate amino acids that are the same as the SRK-910 protein, and differences are denoted by small letters. As defined by Hanks et al. (1988), the kinase sequences have been divided into 11 domains, and amino acids that are conserved in protein kinases are shown in the top line. Boldface type represents amino acids that are absolutely conserved and lightface type represents conserved amino acid groups, as defined by Hanks et al. (1988). The two underlined regions represent consensus sequences found in serine/threonine kinases.

Kinase Activity of the SRK-910 Protein

To show directly that SRK-910 encodes a protein with kinase activity and to determine the specificity of the kinase activity, fusion proteins were synthesized in *Escherichia coli* and assayed for kinase activity. To demonstrate that phosphorylation of the fusion proteins was not the result of bacterial kinase activity, a mutant SRK-910 kinase was also constructed by substituting an alanine residue for the invariant lysine in domain II shown in Figure 3. This lysine is thought to be involved in proton transfer, and in other kinases, substitution mutations involving this amino acid led to a loss of kinase activity (Hanks et al., 1988). The SRK-910 wild-type and mutant kinase domains were placed in two different glutathione S-transferase vectors producing fusion proteins of 72 kD (pGEX + SRK) and 83 kD (pAGEX + SRK) in size. In Figure 4A, a Coomassie blue stain of the protein gel showed that both wild-type and mutant fusion proteins of the expected sizes could be detected (lanes 4 to 7, marked by dots), and that they were absent in the control lanes of HB101 (lane 1), pGEX-3X (lane 2), and pAGEX-2T (lane 3). The smaller proteins in lanes 4 to 7 are either *E. coli* proteins carried through the purification or degradation products from the fusion proteins. In Figure 4B, an autoradiogram of the protein gel showed that only the wild-type fusion proteins demonstrated autophosphorylation activity in the presence of γ-^{32}P-ATP (lanes 4 and 6, marked by dots). In Figure 4B, lanes 5 and 7, the mutant SRK kinase fusion proteins do not show any detectable kinase activity. Thus, the SRK-910 gene does encode an active kinase, and mutation of the invariant lysine to alanine resulted in loss of activity.

To determine the amino acid specificity of the SRK-910 kinase, the phosphorylated fusion proteins were extracted from the protein gel and subjected to phosphoamino acid analysis. For the 83-kD AGEX-kinase fusion protein, only serine and threonine residues were phosphorylated, as shown in Figure 4C. Similar results were also detected for the 72-kD GEX-kinase protein (data not shown). Phosphorylation of tyrosine residues could not be detected even after a long exposure of the

autoradiogram (data not shown). Thus, the SRK-910 protein is a functional serine/threonine kinase.

Expression of the SRK-910 Gene

Poly(A)+ RNA samples extracted from various tissues were subjected to RNA gel blot analysis to determine the expression patterns of the SRK-910 gene. The results given in Figure 5A showed that SRK-910 mRNA transcripts were present predominantly in the pistil at all three stages sampled. This is a pattern of expression similar to the SLG-910 gene (Goring et al., 1992a). However, the SRK-910 transcripts are present at considerably lower levels in comparison to the SLG-910 transcripts (data not shown). As a result of the sequence similarity between the SRK-910 and SLG-910 genes and the high abundance of the SLG-910 message, some cross-hybridization was detected in the RNA blot analysis, as seen by the presence of the lower band in Figure 5A. Using specific primers, SRK-910 gene expression was also examined by polymerase chain reaction (PCR) amplification of first strand cDNA synthesized from total RNA. As shown in Figure 5B, DNA gel blot analysis of the PCR samples revealed PCR products hybridizing to the SRK-910 probe in the anther samples at a much lower level than seen for the stigma samples. Hybridizing PCR products were not present in the petal or leaf samples. Thus, there is also weak expression of the SRK-910 gene in the anther.

DISCUSSION

From the W1 line, we have now isolated a second cDNA, representing an SRK-910 allele, which segregates with the self-

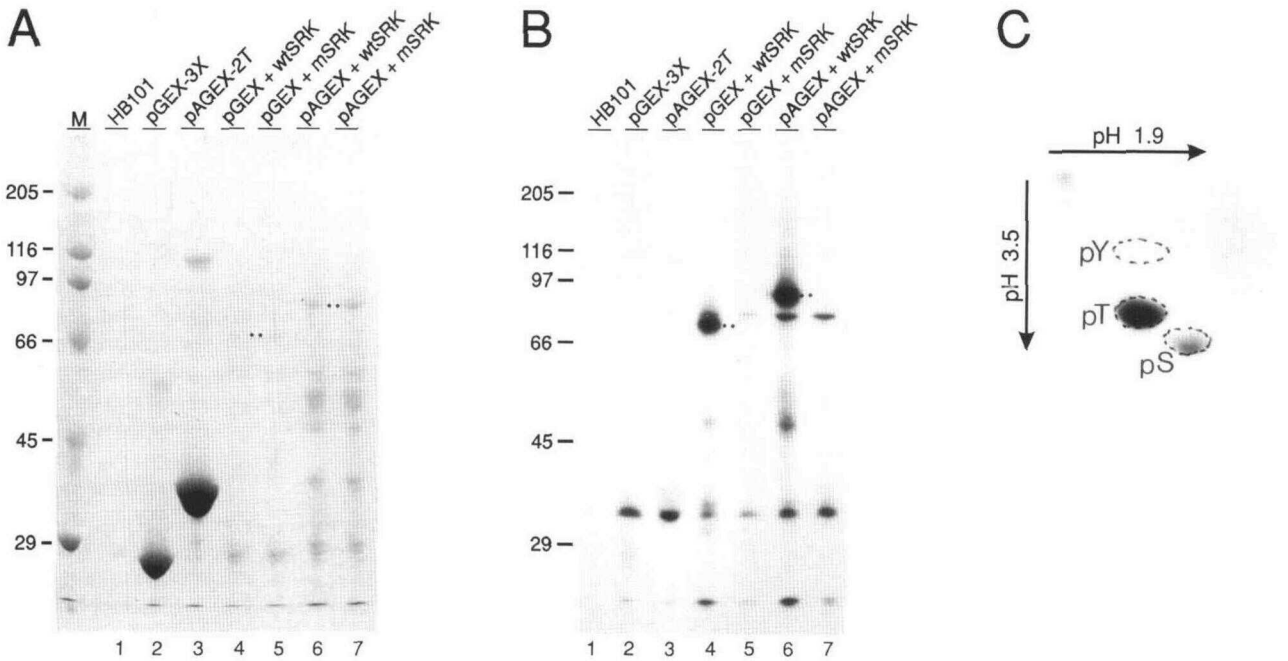

Figure 4. Analysis of SRK-910 Kinase Activity in *E. coli.*

(A) Coomassie blue stain of an SDS-PAGE gel containing glutathione S-transferase fusion proteins extracted with glutathione agarose beads and tested for kinase activity (autophosphorylation) by the addition of γ-^{32}P-ATP. Sigma SDS molecular weight markers are shown on the left. Lane 1, HB101 extract with no plasmids; lanes 2 and 3, control plasmids without an SRK-910 insert; lanes 4 and 6, wild-type SRK-910 kinase domain fused to the two different vectors; and lanes 5 and 7, SRK-910 kinase domain carrying a mutated lysine fused to the two different vectors. The full-length fusion proteins are marked by dots.

(B) Autoradiogram of the SDS-PAGE gel shown in **(A)** to detect phosphorylated proteins. Sigma SDS molecular weight markers are shown on the left. Lane 1, HB101 extract with no plasmids; lanes 2 and 3, control plasmids without an SRK-910 insert; lanes 4 and 6, wild-type SRK-910 kinase domain fused to the two different vectors; and lanes 5 and 7, SRK-910 kinase domain carrying a mutated lysine fused to the two different vectors. The positions of the full-length fusion proteins are marked by dots.

(C) Phosphoamino acid analysis of the AGEX::kinase fusion protein. Hydrolysed amino acids were separated by two-dimensional thin-layer electrophoresis. The positions of the control phosphoamino acids visualized by ninhydrin are marked by the dotted circles. pY, phosphotyrosine; pT, phosphothreonine; pS, phosphoserine.

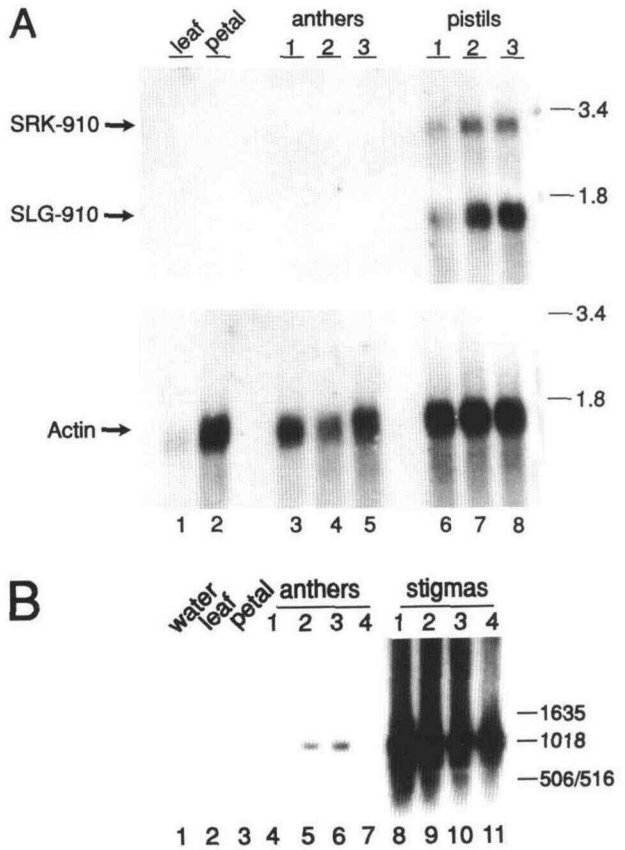

Figure 5. Expression of the SRK-910 Gene.

(A) RNA gel blot analysis of the SRK-910 transcripts in poly(A)$^+$ RNA extracted from different tissues. The anther and pistil samples were extracted from different bud sizes with (1) = 2 to 3 mm; (2) = 4 to 5 mm; and (3) = 6 to 7 mm in length. After hybridization with the SRK-910 probe, the RNA blot was reprobed with an *Arabidopsis actin* clone to show that RNA was present in all lanes. The presence of some 18S (1.8 kb) and 25S (3.4 kb) ribosomal RNA in the poly(A)$^+$ RNA preparations allowed for their positions to be marked (on the right).

(B) PCR analysis of SRK-910 transcripts. First strand cDNA synthesized from total RNA samples were amplified for 25 cycles with SRK-910 specific primers and hybridized to the SRK-910 probe. The anther and stigma (plus style) samples (lanes 1 to 4) were extracted from different bud sizes ranging from ~4 to 7 mm in length. The 1-kb ladder (Bethesda Research Laboratories) was used as the molecular weight markers.

incompatibility phenotype. Although this S-allele originated from a self-incompatible *B. campestris* plant, the SRK-910 gene is similar in sequence to the SRK-6 gene isolated from the S6 *B. oleracea* line by Stein et al. (1991). In addition, the two genes show comparable patterns of expression in the floral tissues, the 5′ ends of the coding regions are highly homologous to their SLG counterparts, and the C-terminal ends of the predicted protein sequences contain conserved amino acids found in serine/threonine kinases. Other putative plant receptor kinases have also shown sequence similarities to serine/threonine kinases (Walker and Zhang, 1990; Tobias et al., 1992). However, we have demonstrated here that SRK-910 is a functional serine/threonine kinase. The carboxy half of the SRK-910 protein was found to phosphorylate only serine and threonine residues and did not appear to phosphorylate tyrosine residues, as demonstrated for serine/threonine/tyrosine protein kinases (Seger et al., 1991). When the kinase domains from putative plant receptor kinases were aligned, in addition to the serine/threonine consensus sequences, they contained all of the conserved amino acids that have been found in protein kinases isolated from other eukaryotes. Some of these conserved amino acids have been implicated in ATP binding or proton transfer, and thus are important for the enzyme activity (Hanks et al., 1988). In the case of the invariant lysine in domain II, we have demonstrated that altering this amino acid will also abolish kinase activity in the SRK-910 protein.

Although it has not been shown directly through plant transformation that the SLG-910 and SRK-910 genes are involved in W1 self-incompatibility, tight linkage between these genes and the W1 self-incompatibility phenotype suggests that they play a role in mediating this reaction. If the proteins encoded by the 910 locus are involved, it is still not known what type of protein interactions occur during the recognition of self-pollen. It is possible that self-recognition is mediated by direct associations between the SLG and SRK proteins, although there are no known examples of this type of interaction. Alternatively, there may be an additional gene at the S-locus that codes for a protein ligand recognized by the receptor kinase. In animal cells, there are examples where both a receptor kinase and a truncated receptor are generated by alternate splicing of mRNA transcribed from the same gene. Consequently, the two protein products are identical or nearly identical in sequence (Johnson et al., 1990; Petch et al., 1990). Whereas the precise role of these truncated receptors in signal transduction is not known, in the case of the epidermal growth factor receptor, there is a differential expression of the truncated and full-length receptor mRNAs leading to the proposition that the truncated receptors may represent another level of regulation to modulate ligand responsiveness by the transmembrane receptor (Petch et al., 1990). Because plants have a thick cell wall surrounding the cell membrane, the S-locus glycoproteins present in the cell wall of the stigma papillae cells (Kandasamy et al., 1989) may serve to recruit ligand molecules for the S-locus receptor serine/threonine kinases predicted to be localized to the cell membrane. In this case, the amino acid sequence of the receptor domain of the SRK and the homologous region in the SLG may be important for differentiating between allele-specific ligand molecules. Alignment of several SLG alleles has shown domains of conserved and variable regions (Dwyer et al., 1991). Because the variable regions are likely to be responsible for the specificity of each allele, these regions were examined for conserved amino acids between the SLG-910 and SRK-910 sequences, but obvious conserved stretches were not observed. However, single amino acids that

would be brought together when the proteins are folded correctly would not be easily detected.

The demonstration that the SRK-910 gene encodes a functional serine/threonine kinase suggests what type of cellular events may occur following self-pollination. For some of the well characterized animal receptor tyrosine kinases, multiple signal transduction pathways have been observed upon receptor activation, and much is known about the target proteins phosphorylated by activated receptor tyrosine kinases, some of these being cytoplasmic serine/threonine kinases (Cantley et al., 1991). Although nothing is known about the nature of the proteins phosphorylated by receptor serine/threonine kinases, they may share some of the second messengers activated by the receptor tyrosine kinases. In the self-incompatibility reaction, one of the rapid responses that has been clearly documented is the deposition of (1,3)-β-glucan (callose) in the stigma papillae cell in contact with the self-incompatible pollen (Heslop-Harrison et al., 1974). Thus, activation of (1,3)-β-glucan synthase may be one of the targets in the signal pathway initiated by the S-receptor kinase. At this time, there is no evidence to support this hypothesis, and further work is required to determine the exact nature of the cellular processes mediating self-incompatibility.

METHODS

Cloning of the SRK910 Gene

The polymerase chain reaction (PCR) was used to amplify an internal 800-bp genomic fragment from the 3.6-kb HindIII band using primers to conserved regions in S-locus glycoprotein (SLG) alleles as previously described (Goring et al., 1992a). Although sequence analysis of the 800-bp genomic PCR clone (no introns) showed high levels of homology to the SLG-910 coding region (89%), specific primers from this region could be designed to isolate the remainder of the coding region. The 3′ end of the S-receptor kinase (SRK)-910 cDNA was isolated by amplification of pistil cDNA using the rapid amplification of cDNA ends (RACE) procedure (Frohman et al., 1988). The cDNA synthesis, the dT_{17}-adaptor and adaptor primers, and PCR amplification were as described by Goring et al. (1992a) except that ~400 ng of poly(A)$^+$ RNA was used for the cDNA synthesis. After the first round of amplification with an SRK-910–specific primer, a specific band was not detected. The resulting products (faint smears) were fractionated on a 1% low melting point agarose gel, and agarose plugs were removed with Pasteur pipettes (Zintz and Beebe, 1991) in the range of 1.5 to 2.5 kb. The DNA-containing agarose plugs were melted at 70°C for 10 min and subjected to a second round of PCR amplification using 200 nM each of the adaptor and a second, internal SRK-910–specific primer for 30 cycles. The resulting 1.5-kb band started at the 3′ end of the SLG homology region and contained the kinase domain.

The 5′ end of the SRK-910 coding region was amplified using the inverse genomic PCR technique (Ochman et al., 1988). One-hundred nanograms of size fractionated (3.6 to 3.9 kb), HindIII-digested W1 genomic DNA was ligated under dilute conditions promoting circularization (Ochman et al., 1988). The ligated DNA was amplified using conditions suggested by Maga and Richardson (1991). After 40 cycles, the PCR reaction was precipitated with ethanol and size fractionated on a low melting point agarose gel. A faint band could be detected at ~3 kb in size, and agarose plugs were removed as described above and amplified for 21 cycles. The resulting band contained 1 kb of the coding region with no introns (SLG homology region) overlapping with the original 800-bp genomic PCR clone, another 1.8 kb upstream of the initiation codon, and 59 bp at the 3′ end of the SLG homology region followed by a partial intron separating the SLG homology domain from the kinase domain. All PCR products were cloned into pBluescript KS+ (Stratagene) and sequenced as described previously (Goring et al., 1992a). Two to three different clones from separate PCR reactions were sequenced for each section to solve any discrepancies in the SRK-910 sequence. The sequence of the SRK-910 coding region was derived from the three overlapping clones. DNA and protein sequence analyses were carried out using the DNASIS and PROSIS software (Hitachi America Ltd., San Bruno, CA).

Fusion Proteins and Kinase Assays

Mutation of the invariant lysine to alanine was carried out using PCR mutagenesis. Two overlapping regions (nucleotide 1256 to 1681; nucleotide 1378 to 1779) were amplified with one of the inside primers (nucleotide 1660 to 1681) introducing the AAA-GCA change. The two separate PCR fragments (~400 bp in length) were mixed together and reamplified with the outside primers (nucleotide 1256 to 1779) to produce a 523-bp fragment, which was then cloned and sequenced. With this strategy, half of the clones carried the introduced mutation. A 400-bp BclI/EcoRI fragment (nucleotide 1383 to 1761) containing the mutation was then cloned into the kinase domain to replace the wild-type sequence. The wild-type and mutant kinase domains, starting near the end of the transmembrane domain (nucleotide 1383 to 2749), were placed in pGEX-3X (Smith and Johnson, 1988), which creates protein fusions with glutathione S-transferase, and in pAGEX-2T (D. B. Smith, L. C. Berger, and A. G. Wildeman, manuscript submitted), which contains two IgG binding domains from *Staphylococcus aureas* protein A in front of the glutathione S-transferase protein.

For the kinase assays, 50 mL HB101 cultures carrying the various fusion constructs were grown at 37°C to an OD_{600} of 0.6 (faster growing cultures were diluted during growth). Isopropyl β-thiogalactoside was then added to a final concentration of 1 mM, and the cultures were incubated at 37°C for 1 hr. Purification of the fusion proteins on glutathione agarose beads was carried out essentially as described by Smith and Johnson (1988), except that instead of PBS, the extraction buffer of Douville et al. (1992) was used for resuspension and washes. In addition, the protein extracts were mixed with the glutathione agarose beads for 30 min at room temperature. After the washes, the agarose beads containing the fusion proteins were washed an additional two times with the kinase buffer (30 mM Tris, pH 7.5, 20 mM Hepes, pH 7.1, 10 mM $MgCl_2$, 2 mM $MnCl_2$; Douville et al., 1992) and resuspended in a final volume of 50 uL kinase buffer. Twenty-five microcuries of γ-^{32}P-ATP (6000 Ci/mmol) was added to each sample and left at room temperature for 30 min. The beads were spun down, resuspended in 20 uL of 2 × sample buffer, boiled for 5 min, and electrophoresed through an 8.5% SDS-PAGE gel (Laemmli, 1970). Subsequently, the SDS-PAGE gel was stained with Coomassie blue, dried down, and exposed overnight to x-ray film at −70°C. The fusion proteins, which could be detected by the Coomassie blue stain, were excised and extracted from the gel and subjected to phosphoamino acid analysis, as described in Cooper et al. (1983) and Boyle et al. (1991).

RNA and DNA Gel Blot Analysis and PCR Expression Analysis

The poly(A)$^+$ RNA samples for the RNA gel blot analysis were extracted using the Micro-FastTrack mRNA isolation kits (Invitrogen, San Diego, CA). The procedures for gel electrophoresis and blot hybridization were as described previously (Goring et al., 1992b). After hybridization, the blots were washed twice in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS for 30 min. The washing temperatures were 67°C for the SRK-910 probe and 50°C for the Arabidopsis actin probe.

To examine the expression of the SRK-910 gene using PCR, total RNA samples were extracted using the method of Jones et al. (1985). Ten micrograms of total RNA was used for first strand cDNA synthesis using random hexamers and the procedure of Harvey and Darlison (1991). The first strand cDNA was amplified with SRK-910 primers spanning the kinase region that contains several introns. One-third of each cDNA sample was amplified for 25 cycles, and then subjected to gel electrophoresis and DNA gel blot analysis.

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