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1) SRK has serine/threonine protein kinase activity:

During the first year of the funding period, we had determined the structure of the *SRK* gene and its protein product (Stein et al. 1991). The SRK protein was predicted to be similar to the growth factor receptor tyrosine kinases in animals. We had noted however (Stein et al. 1991) that the sequence of the predicted catalytic domain of SRK is more similar to that of the catalytic domains of protein serine/threonine kinases than to protein tyrosine kinases. In fact, we have shown that the SRK protein has intrinsic serine/threonine kinase activity. A paper that describes these results (Stein & Nasrallah, 1993) is in press (pre-print appended). The following is a summary of this work.

We subcloned the protein kinase-homologous domain of the *SRK6* cDNA into the bacterial expression vector pGEX-3X. In addition, we constructed a second plasmid identical to the first except that it carried a conservative mutation that substituted Arg for the Lys^{524} codon of *SRK6*. This lysine corresponds to the ATP-binding site, is essential in protein kinases, and is a common target for site-directed mutagenesis as a means to obtain kinase-defective proteins. When induced with IPTG, cultures bearing the wild-type and mutant SRK catalytic domains each produced an approximately 64 kD protein that reacted with anti-SRK6 antibodies. Following pulse-labeling of the two bacterial strains with ^{32}P and affinity purification of the recombinant wild-type and mutant SRK6 proteins, we found that the wild-type SRK6 protein but not the mutant form was detectably phosphorylated. Phosphoamino acid analysis of the affinity purified ^{32}P-labeled GST-SRK6 fusion protein demonstrated that SRK was phosphorylated predominantly on serine and to a lesser extent on threonine, but not on tyrosine. Thus, SRK6 is a functional serine/threonine protein kinase.

2) Expression of the *SRK* gene:

We had performed RNA gel blot analysis of untransformed Brassica and of transgenic Brassica plants expressing an *SRK* transgene to show that this gene is expressed specifically in pistils and anthers (Stein et al. 1991), consistent with models of SI in which both pollen and stigma bear determinants of recognition derived from the S locus. Having established that the *SRK* gene is expressed in pistils and anthers, we set out to identify the specific cell types responsible for this expression. If our hypothesis that SRK acts as a receptor in both stigma and pollen in the interaction of self-incompatibility, we would expect the *SRK* gene to be expressed in the surface cells of the stigma that capture and interact with pollen and in the pollen grain itself. To investigate this question, we constructed a plant transformation vector carrying a chimeric gene in which the promoter of the *SRK* gene was fused to the reporter ß-glucuronidase (GUS). This construct has been introduced into Arabidopsis, Brassica and Nicotiana. *SRK* promoter activity was detected in the papillar cells of Arabidopsis and Brassica and in the style of Nicotiana, in a manner similar to the activity of the *SLG* promoter. The expression of *SRK* in the papillar cells of the stigma is consistent with the hypothesis that SRK functions as a receptor for pollen recognition. We are currently examining the expression of the reporter gene in anthers.

3) Identification of SRK protein kinase activity in plant tissues:

A. Immunological and biochemical approaches:

As with other receptors, the SRK protein is a relatively low-abundance protein. To study SRK receptor activity, our approach is to apply immunoprecipitation methods. We have generated two rabbit polyclonal antibodies against the kinase domain of SRK. One antibody was raised against a synthetic peptide that corresponds to the predicted residues 790 to 803 within the C-terminal region of SRK_6 (see attached preprint). The second antibody was generated against a bacterial fusion protein containing the kinase catalytic domain of SRK and described in section (1) above. Both antibodies cross-react with bacterial fusion proteins containing the kinase catalytic domain of SRK.

In addition, we have available to us monoclonal and polyclonal antibodies that were produced against the S-locus glycoprotein SLG and against glycoproteins encoded by S-locus related genes. We have shown that three of these antisera cross-react with bacterial fusion proteins that contain

the *S* domain of SRK.

In preparation for identifying SRK protein in plant tissues and characterizing its activity in response to pollination, we have adapted methods for preparing microsomal fractions from small amounts of tissue and for immunoprecipitating membrane proteins. Using these methods, we have shown that immunoreactive protein is enriched in microsomal fractions from stigmas. In addition, we have recovered, by immunoprecipitation of stigma extracts, protein that exhibits *in vitro* protein kinase activity. The application of these methods should allow us to test our working hypothesis that the SRK protein kinase is activated by contact between a papillar cell and self-pollen.

B. Over-expression of SRK:

While not outlined in the original proposal, we have constructed a gene fusion to over-express SRK in vegetative plant tissues. We have placed the *SRK* transcriptional region downstream of the CaMV 35S promoter, and are currently generating transgenic tobacco plants that express the gene fusion. We have been successful at over-expressing the secreted glycoprotein products of the *S-Locus Glycoprotein* (*SLG*) gene and the *S-Locus Related* (*SLR1* and *SLR2*) genes in tobacco vegetative cells. Based on their electrophoretic properties, these glycoproteins appear to be correctly modified in tobacco cells. Our long-term objective is to establish a plant cell culture system that can be utilized in receptor activation studies and in the identification of a ligand.

4) The molecular basis of *S* haplotype polymorphism:

We have extended our analysis of *SLG/SRK* gene pairs isolated from several different *S* haplotypes. In addition to the two *Brassica oleracea S* haplotypes that we initially reported (Stein et al. 1991), we have isolated and sequenced *SLG/SRK* gene pairs from a third *B.oleracea* haplotype and from a *B.campestris* haplotype. These studies have confirmed the highly polymorphic nature of *SRK*. Furthermore, the initial observation that *SLG* and the *S* domain of *SRK* share approximately 90% amino-acid sequence identity within a haplotype has been confirmed. These observations support our hypothesis that the two genes co-evolve. A detailed

analysis of the available sequences is currently being performed. We will attempt to identify within these genes, any regions that are highly polymorphic between different haplotypes, but that are identical or very similar within a haplotype. We will also search the sequences for evidence of homogenizing gene conversion events similar to those postulated to explain the coevolution of genes in other systems. We hope that this analysis will point to domains within the *SLG* and *SRK* genes that are functionally relevant to self-pollen recognition.

5) The role of *S*-locus genes in self-incompatibility:
Transformation of Brassica and analysis of self-compatible mutants.

A long-term goal of our work on the *S*-locus genes of Brassica is to reconstitute a self-incompatibility phenotype in transgenic plants. More than one gene is encoded at the complex and polymorphic *S* locus, leading us to abandon the classical designation of "*S* alleles" for the more appropriate designation of "*S* haplotype". Because the physical distance separating the *S*-locus genes is estimated to be on the order of 200 kilobases of DNA, any attempt at phenotype modification cannot be straight-forward. Since technologies for transforming plants with large fragments of DNA are not feasible at this time, it is necessary to transform Brassica plants individually with *S*-locus genes isolated from the same *S* haplotype, followed by crosses designed to assemble the desired genotype in hybrid transgenic progeny. This latter approach is being carried out in two recipient species.

A genetic approach to investigating the function of *S*-locus genes is through the analysis of self-compatible mutant strains. We have identified and analyzed a recessive mutation of *B. campestris* that leads to self-compatibility. Our results are reported in the appended paper (Nasrallah, Kandasamy, and Nasrallah, 1992, The Plant Journal, 2, 497-506). We have shown that the mutation is stigma specific, is unlinked to the *S* locus, and results in the down-regulation at the RNA level of the *SLG* gene and two other related genes, *SLR1* and *SLR2*. but not *SRK*. Our hypothesis is that the mutation affects a locus that potentially encodes a *trans*-acting factor responsible for the high-level expression of the *SLG, SLR1* and *SLR2* gene in the stigma.

CONTINUATION REQUEST

We request continued funding to pursue the further characterization of the SRK protein. Our goals relative to the immunological studies of the SRK protein, its subcellular localization and the analysis of its kinase activity in Brassica tissues remain as outlined in the original application.

Characterization of a Putative Receptor Protein Kinase and its role in Self-Incompatibility

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Plant reproduction is dependent on cellular interactions that occur in the flower between the pollen grain and the stigma surface. In self-incompatible crucifers, active inhibition of self-pollen tube development occurs at the stigma surface and is controlled by a highly polymorphic and complex *S* locus. Some sixty *S*-locus variants or *S* haplotypes exist in natural populations of *Brassica*. Each haplotype carries two genes that are expressed specifically in reproductive tissues. Of these, the *SRK* gene encodes a transmembrane protein kinase, while the *SLG* gene encodes a secreted glycoprotein highly homologous to the extracellular domain of its sister *SRK* gene. The structure of *SRK* indicates that pollen-stigma signaling in crucifers is similar in basic outline to animal signaling systems that are mediated by ligand-activated receptor tyrosine kinases. Our hypothesis is that contact with self-pollen activates the SRK protein which, by phosphorylating intracellular substrates, couples the initial molecular recognition events at the stigma-pollen interface to the signal transduction chain that leads to pollen rejection. Our research is aimed at understanding the role of SRK in specific pollen recognition. We have shown that SRK has intrinsic serine/threonine protein kinase activity. We have determined the sequence of SRK genes isolated from different *S* haplotypes with the goal of identifying domains that may be important in self recognition. Future experiments will aim at identifying the cell types of the stigma and anther in which the SRK protein is expressed, its subcellular localization, and the analysis of its kinase activity in *Brassica* tissues.

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