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**"Characterization of a Putative S-Locus Encoded Receptor Protein Kinase
and its Role in Self-Incompatibility"**

The objectives of the proposal were to characterize the *S*-Receptor Kinase (*SRK*) gene by:

- 1) analyzing the structure of the *SRK* gene in different *S* homozygotes
- 2) characterizing the pattern of *SRK* expression in stigma and anther tissue
- 3) identifying and purifying the *SRK* protein, and determining its subcellular localization
- 4) characterizing the protein kinase activity of the *SRK* protein
- 5) analyzing the regulation of this protein and its activity in relation to self-incompatibility.

As detailed in the following pages, we have accomplished objectives # 1, 2, and 4, and we have developed the tools that will allow us to address objectives # 3 and 5.

Publications resulting from DOE-funded research:

- Stein, J.C., Howlett, B., Boyes, D.C., Nasrallah, M.E., and Nasrallah, J.B. (1991). Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. *Proc. Natl. Acad. Sci. USA* **88**, 8816-8820.
- Nasrallah, M.E., Kandasamy, M.K., and Nasrallah, J.B. (1992). A genetically defined trans-acting locus regulates S-locus function in Brassica. *The Plant J.* **2**, 497-506.
- Stein, J.C., and Nasrallah, J.B. (1993). A plant receptor-like gene, the *S*-locus receptor kinase of *Brassica oleracea* L., encodes a functional serine/threonine kinase. *Plant Physiol.* **101**, 1103-1106.
- Tantikanjana, T., Nasrallah, M.E., Stein, J.C., Chen, C.H., and Nasrallah, J.B. (1993). An alternative transcript of the *S* locus glycoprotein gene in a class II pollen-recessive self-incompatibility haplotype of *Brassica oleracea* encodes a membrane-anchored protein. *Plant Cell* **5** (in press).
- Dzelzkalns, V.A., Nasrallah, J.B., and Nasrallah, M.E. (1992). Cell-cell communication in plants: Self-incompatibility in flower development. *Dev. Biol.* **153**, 70-82. [Review]
- Nasrallah, J.B., and Nasrallah, M.E. (1993). Pollen-stigma signalling in the self-incompatibility response of Brassica. *Plant Cell* **5** (in press) [Review]

The major results of our research effort include the determination of the *SRK* gene structure, the demonstration of *S*-haplotype-associated *SRK* polymorphisms and possible co-evolution of *SRK* and *SLG*, the characterization of the temporal and spatial expression patterns of *SRK*, and the demonstration that *SRK* has intrinsic serine/threonine kinase activity. Our results have indicated that *SLG* originated from an *SRK*-like gene by a gene duplication event and suggested a possible molecular basis for leaky *S* haplotypes. The data have allowed us to develop a model of self-incompatibility based on the interaction of *SRK* and *SLG* and the activation of *SRK* in response to self-pollination. More generally, the information that we have obtained is potentially relevant to understanding mechanisms of signalling in plants. Thus, the interaction of membrane-based receptor protein kinases with secreted forms of their extracellular domains may represent a generalized mechanism by which receptors signal across the plant cell wall.

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Objective #1: Structural characterization of the *SRK* gene:

Publications: Stein et al. (1991); Tantikanjana et al. (1993).

A. *SRK* gene structure:

We have shown that the *SRK* gene encodes a 3.0-kb transcript and consists of six exons separated by seven introns (Stein et al., 1991). Like many other eukaryotic genes, the *SRK* gene appears to have originated by shuffling of exons that represent distinct functional domains. Thus, exon 1 encodes the extracellular "S" domain, exon 2 encodes the transmembrane and juxtamembrane domain, and exons 3 through 7 encode the cytoplasmic kinase domain. The nature of exon 1 as a distinct structural entity is further underscored by its physical separation from the remaining exons by the longest intron, which varies from 896 base pairs to over 5.5 kilobases (Stein et al., 1991; Tantikanjana et al., 1993; J.B. Nasrallah, unpublished data).

B. *S*-haplotype associated *SRK* polymorphisms:

We have analyzed *SLG*/*SRK* gene pairs isolated from several different *S* haplotypes. In addition to the two *Brassica oleracea* *S* haplotypes that we reported in 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 shown that *SLG* and *SRK* genes derived from different *S* haplotypes exhibit a high degree of sequence polymorphism as expected for genes involved in specific pollen recognition. Within a haplotype however, the two genes are approximately 90% identical. These observations suggest that the two genes have evolved in concert, perhaps by a mechanism involving gene conversion, and imply a functional relationship between the protein products of the two genes.

A detailed analysis of the available sequences is currently being performed. Our aim is 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 co-evolution of genes in other systems. This analysis may point to domains within the *SLG* and *SRK* genes that are functionally relevant to self-pollen recognition.

C. A structural variant of an *S*-locus gene:

The characterization of the *SRK*₂ gene revealed that the sequence similarity between *SLG*₂ and *SRK*₂ extended beyond the S domains into the 3' regions of the genes. Further analysis of the structure of the *SLG*₂ gene showed that this gene has an unusual structure (Tantikanjana et al., 1993). We showed that *SLG*₂ produces two transcript forms: the expected 1.6-kb transcript that predicts a secreted glycoprotein, and an alternative 1.8-kb transcript that predicts a membrane-anchored protein. Stigmas of the *S*₂ haplotype and pistils of transgenic tobacco plants transformed with the *SLG*₂ gene produce a membrane-associated 62-kD protein as well as soluble 57- and 58-kD glycoforms.

The analysis of the *SLG*₂ gene has been informative in two respects. Firstly, it has provided important information relating to the evolutionary relationship between the two *S*-locus linked

genes, and has suggested that *SLG* genes originated from an ancestral *SRK*-like gene by a gene duplication event. Secondly, the structure of the *SLG*₂ gene and the occurrence of a membrane-anchored form of *SLG*₂ has important functional implications. Because of the sequence similarity between *SLG*₂ and the extracellular domain of *SRK*₂, the membrane-anchored form of *SLG*₂ may be viewed as a naturally occurring truncated form of the receptor that lacks the kinase catalytic domain. In animal systems, such truncated forms of receptor protein kinases have been engineered in vitro, reintroduced into appropriate cells, and shown to interfere with the function of the wild-type receptor, presumably by interacting with the wild-type receptor through their extracellular domain, creating unproductive receptor heterodimers, and suppressing signal transduction. It is interesting to note that the *S*₂ haplotype from which the *SLG*₂ gene is derived belongs to the class II group of *S* haplotypes that exhibit a weak incompatibility phenotype in the stigma and are pollen recessive. The leakiness and/or recessive nature of the *S*₂ haplotype may be associated with the production of the anomalous membrane-bound form of *SLG*. This membrane-anchored *SLG* would simulate a truncated kinase-negative form of *SRK* and interfere with receptor function, resulting in a leaky self-incompatibility phenotype. Such a partially active receptor system could also account for the pollen-recessive nature of the *S*₂ haplotype in heterozygotes, if competitive interactions between the proteins encoded by different *S* haplotypes is assumed to occur in pollen-stigma recognition. Thus, the unique properties of the *SLG*₂ gene that we have described provide us with a hypothesis to explain the differences in the activities and genetic properties of *S* haplotypes. We are currently testing this hypothesis by a transgenic plant approach.

Objective #2: Expression of the *SRK* gene:

Publication: Stein, J.C. et al. (1991).

In preparation: Stein, J.C. and Nasrallah, J.B. Developmental and cell-type specific regulation of the Brassica *SRK* gene.

The question of where the *SRK* gene is expressed is important for formulating models of receptor action in the self-incompatibility response. If *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.

We showed by RNA gel blot analysis of untransformed Brassica and of transgenic Brassica plants expressing an *SRK* transgene that *SRK* 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. 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 β -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 in papillar cells as a receptor for pollen recognition. In transgenic tobacco anthers, the *SRK* promoter is active, like the *SLG*

promoter, in mature pollen grains. The activity of the two promoters differs in anthers of transgenic *Arabidopsis* however: the *SRK* promoter is active in developing microspores, whereas the *SLG* promoter is active in the tapetum. We are currently examining the activity of the *SRK* promoter in *Brassica* anthers.

The expression pattern of *SRK* in pistils and anthers is similar to that previously reported for *SLG* (Sato et al., 1991) and is consistent with models of SI in which pollen and stigma bear determinants of recognition derived from the *S* locus. At least in stigmas, where the analysis of promoter activity has been characterized for the two genes, both genes are expressed in papillar cells, suggesting that the products of the two genes may interact.

Objective #4: The protein kinase activity of SRK:

Publication: Stein and Nasrallah (1993).

Our sequence analysis of the *SRK* gene predicted a protein that was similar to the growth factor receptor protein kinases in animals (Stein et al. 1991). 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 kinase-homologous domain of SRK has intrinsic serine/threonine kinase activity (Stein & Nasrallah, 1993). 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 Lys⁵²⁴ 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 ³²P 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 phosphorylated. Phosphoamino acid analysis of the affinity purified ³²P-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.

Objective #3: Identification and immunolocalization of the SRK protein:

A. Immunological and biochemical approaches:

As with other receptors, the SRK protein is a relatively low-abundance protein. To identify the SRK protein and to analyze SRK receptor activity in plant tissues (Objective #5), our approach is to apply immunochemical 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 residues 790 to 803 within the C-terminal region of SRK₆ (Stein & Nasrallah, 1993). The second antibody was generated against a bacterial fusion protein that contains a larger C-terminal region of the kinase catalytic domain of SRK₆. Both antibodies detect SRK₆ kinase-domain fusion

proteins on protein immunoblots.

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 in buffers that contain inhibitors of proteases and protein phosphatases, protein that exhibits *in vitro* protein kinase activity towards exogenous substrates. 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*:

We have constructed two gene fusions designed to over-express *SRK* in plant tissues. These experiments were not outlined in the original proposal, however we believe that over-expression of *SRK* will be useful for the identification and characterization of the *SRK* protein.

In one construct, we placed the *SRK* transcriptional region downstream of the CaMV 35S promoter and we have generated transgenic tobacco plants that carry the gene fusion. Future analysis of these plants will determine if over-expression of the *SRK* protein has been achieved. In other work, 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 functional assays for a ligand.

In the second construct, we placed the *SRK* transcriptional region downstream of the *SLG* promoter. This construct is designed to over-express *SRK* in Brassica stigmatic papillar cells.

Objective #5: The *SRK* gene and self-incompatibility:

Analysis of self-compatible mutants:

Publication: Nasrallah et al. (1992).

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 (Nasrallah et al., 1992). This mutation is unlinked to the *S* locus and results in drastically reduced levels of *SLG* expression in the stigma but not in anthers, and in a concomitant loss of the incompatibility response in the stigma but not in pollen. It 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.

We have recently identified two other self-compatible mutant strains in which self-compatibility co-segregates with the *S* locus. These mutant strains contain a functional *SLG* gene since they produce SLG glycoprotein in the stigma at levels comparable to those in self-incompatible strains. They have defective *SRK* genes however, since no *SRK* transcripts are detected in stigmas and anthers of these strains. Together, the mutant strains analyzed to date indicate that both *SLG* and *SRK* are required for self-pollen recognition. Further, because the mutant strains are highly fertile in self- and cross-pollinations, we conclude that *SLG* and *SRK* function exclusively in the control of the SI response and are not involved in compatible pollen-stigma interactions.

Other results from experiments not outlined in the original proposal:

A. Transcriptional analysis of the Arabidopsis *ARK1* gene:

The conclusion that SRK and SLG act together as components of a recognition complex has far-reaching implications, not only for understanding self-incompatibility, but also more generally for deciphering mechanisms of cell-cell signalling and receptor action in plants. Does the presence of the plant cell wall predicate that signalling by membrane-based receptors requires a secreted form of receptor extracellular domains?

Because our laboratory had isolated the Arabidopsis *ARK1* gene (Tobias et al., 1992), we were interested in determining if the ARK1 protein, which is related to SRK but which, being expressed in vegetative tissues, presumably has a different biological function, would also potentially function in conjunction with a secreted form of its extracellular S domain. Since we had found no evidence for the physical linkage of *ARK1* to a secreted glycoprotein-encoding gene similar to *SLG*, we reasoned that *ARK1* itself could potentially encode both a receptor-like protein and a secreted glycoprotein that corresponds to the extracellular S domain of the receptor. Transcriptional analysis of *ARK1* using 3' RACE has demonstrated that two transcript species are encoded by the gene. The major transcript is a 3.0 kb transcript that predicts the full-length receptor. An alternative *ARK1* transcript is a 1.6 kb transcript. Because of the presence of an in-frame stop codon 2 bp downstream of the first intron splice junction (Tobias et al., 1992), this short transcript predicts a secreted glycoprotein that would correspond to the putative extracellular domain of the receptor. Antibodies raised to a bacterial fusion protein consisting of GST fused to the S domain of ARK1 have identified antigenic proteins in Arabidopsis tissues. The ongoing analysis of total leaf extracts, microsomal fractions and cell wall enriched fractions has provided evidence that the 1.6 kb transcripts are in fact translated to produce a cell-wall associated protein.

The observation that, among members of the *S* gene family, two different mechanisms have evolved to generate secreted forms of receptor extracellular domains argues that such secreted forms are important for receptor function. The *SLG/SRK* gene pair would represent an extreme case of a more general scenario. It is possible that the duplication of the extracellular domain of *SRK* in the form of the *SLG* gene occurred as a result of unique requirements in pollen-stigma signalling. A large excess of the secreted form of the extracellular domain relative to the transmembrane receptor may be required for the efficient functioning of the self-incompatibility system. Such a requirement would have been met by the evolution of the *SLG* gene with its highly active promoter and the consequent "amplification" of the secreted form of the extracellular

domain. A requirement for high levels of this molecular form could also provide the selective pressure to maintain the tight linkage of *SLG* to *SRK* and the extensive sequence similarity between the two genes.

B. Signalling cascades in pollination:

Submitted manuscript: Rundle, S.J., Nasrallah, M.E. and Nasrallah, J.B. Effects of inhibitors of protein serine/threonine phosphatases on pollination in Brassica.

Our analysis of *SRK* has indicated that a phosphorylation cascade is involved in the inhibition of pollen tube development in self-incompatibility. To determine if the phosphorylation state of papillar cell proteins is also important for successful pollen tube development, we treated *Brassica* flowers or developing floral buds with inhibitors of serine/threonine phosphatases. Inhibitor-treated flowers were pollinated with untreated pollen and examined for pollen tube growth by fluorescence microscopy. Our results show that type 1 or type 2A serine/threonine phosphatases play a crucial role in the growth of pollen tubes in flowers during cross-pollination. Pollen tube growth on pistils treated with 2 μ M okadaic acid or 4 μ M microcystin-LR was inhibited at the surface of the papillar cells of the stigma in crosses between self-incompatible *B. oleracea* strains of different self-incompatibility genotypes, in an inter-specific cross between *B. oleracea* and *B. campestris*, and in self-pollinations of a self-fertile *B. napus* cultivar. With four different self-incompatibility genotypes, treatment of one or two-day old flowers with protein phosphatase inhibitors did not result in a breakdown of self-incompatibility. In contrast, treatment of flower buds just prior to the anthesis stage allowed pollen tube invasion of papillar cells. However the magnitude of this effect was genotype dependent, being most pronounced in the *S*₂₂ homozygous genotype. The data support the conclusion that pollinations in *Brassica* are most likely controlled in part by the presence of phosphorylated proteins in the papillar cells of the stigma, and that the amount or the level of phosphorylation of these proteins changes during stigma development. These results hint at the operation of a developmentally regulated signalling system that acts in parallel to, and independently of, the *SRK*-based system to allow compatible pollen tube growth. Whether the two pathways converge is not known.

Plans for the third year of the DOE funding period:

Our efforts during the third year of the DOE funding period will be focused in large part on the characterization of the *SRK* protein. Identification of the *SRK* protein will be accomplished by immunoblot analysis of whole stigma extracts and of membrane-enriched fractions, and by immunoprecipitation from extracts of stigmas labeled *in vivo* with ³⁵S-methionine.

During the course of our work on the *SRK* gene, we identified in *Brassica* stigmas transcripts that are encoded by two other genes and that, like *SRK*, consist of an *S* domain joined to a kinase-homologous domain. The expression of *SRK*-related genes, while interesting, has complicated our ability to identify unambiguously the *SRK* protein in stigmas. However, we believe that we have developed a collection of tools that will allow us to attain this goal. As

detailed in the previous sections, we have in hand several antisera that react with *SRK*-derived bacterial fusion proteins and we have developed the appropriate methodologies to tackle the problem. In addition, our laboratory maintains a collection of *S*-haplotype homozygotes and plant populations that segregate for different *S* haplotypes. The availability of this plant material will allow us to take advantage of the polymorphic nature of the *SRK* gene. In particular, we predict that some of the antibodies will distinguish between *SRK*₆ and *SRK*₂ due to the high level of sequence divergence between these two proteins (Stein et al., 1991). The analysis of F2 populations that segregate for the *S*₆ and *S*₂ haplotypes will allow the unambiguous identification of the *SRK* protein. Finally, the self-compatible *SRK* null mutations that we recently identified and any transgenic plants that might over-express *SRK* in vegetative cells or in papillar cells will be very useful in this regard. We anticipate that during the third year of funding we will identify the native *SRK* protein in stigmas, characterize its properties, and determine its subcellular localization.

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ATTACHMENTS:

* Reprints:

- Stein, J.C., Howlett, B., Boyes, D.C., Nasrallah, M.E., and Nasrallah, J.B. (1991). Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc. Natl. Acad. Sci. USA 88, 8816-8820.
- Nasrallah, M.E., Kandasamy, M.K., and Nasrallah, J.B. (1992). A genetically defined trans-acting locus regulates S-locus function in Brassica. The Plant J. 2, 497-506.
- Stein, J.C., and Nasrallah, J.B. (1993). A plant receptor-like gene, the S-locus receptor kinase of *Brassica oleracea* L., encodes a functional serine/threonine kinase. Plant Physiol. 101, 1103-1106.
- Tantikanjana, T., Nasrallah, M.E., Stein, J.C., Chen, C.H., and Nasrallah, J.B. (1993). An alternative transcript of the S locus glycoprotein gene in a class II pollen-recessive self-incompatibility haplotype of *Brassica oleracea* encodes a membrane-anchored protein. Plant Cell 5 (in press).

* Preprint:

- Rundle, S.J., Nasrallah, M.E. and Nasrallah, J.B. Effects of inhibitors of protein serine/threonine phosphatases on pollination in Brassica.

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