GRANT DE-FG02-88ER13909
SIGNAL TRANSDUCTION IN THE POLLEN-STIGMA INTERACTIONS OF BRASSICA

FINAL TECHNICAL REPORT

The goal of our research is to analyze the self-incompatibility response of Brassica, a genetically controlled self-recognition system that prevents self-fertilization by arresting the development of genetically related pollen grains. The specificity of the response is controlled by the *S* locus and is based on the activity of two S-locus encoded proteins, the S-locus receptor kinase SRK, and the cell-wall localized soluble "receptor" SLG (S-locus glycoprotein) which shares a high degree of sequence similarity with the predicted extracellular domain of SRK.

The objectives of this grant were primarily to investigate the role of SRK in self-incompatibility. We proposed to define the biochemical properties of SRK and investigate its activity in relation to the self-incompatibility response; to investigate the functional relationship between SRK and SLG; to identify molecules that interact with the extracellular domain of SRK; and to identify potential targets of SRK activation. A secondary objective was to investigate the signal transduction chains in pollen-pistil interactions by imaging pollination-induced changes in cytosolic free calcium in the stigmatic epidermal cells of transgenic plants that express recombinant aequorin, and by using a pharmacological approach in an attempt to modify pollination responses with inhibitors or activators of known signalling intermediates.

SUMMARY OF ACCOMPLISHMENTS

Characterization of SRK as a papillar-cell specific and plasma membrane-localized protein:

We have obtained definitive biochemical evidence that SRK functions in the self-incompatibility response as a cell surface receptor of stigmatic epidermal (papillar) cells.

a. *Immunochemical identification of SRK:*

We used an antibody directed against the extracellular domain of the receptor and another directed against the kinase domain to identify SRK on protein blots. SRK was identified as a 108-kDa protein in stigma extracts prepared from an F2 population that segregates for two functional *S* haplotypes (S6 and S2) and another F2 population that segregates for a functional (S13) and a non-functional (Sf1) *S* haplotype. In contrast to SLG, SRK protein was found to be a low-abundance protein: it was detected only when chemiluminescent substrates were used for the development of protein immunoblots.

b. *The SRK gene is expressed predominantly in the papillar cells of the stigma*

We found that, like the *SLG* gene, *SRK* is expressed predominantly in the papillar cells of the stigma. The two genes are coordinately regulated during stigma maturation as expected for genes that interact genetically. The specific expression of SRK in stigma papillar cells was determined by histochemical GUS assays of transgenic

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tobacco and Arabidopsis plants that express an SRK::uidA chimeric gene consisting of the SRK promoter fused to the reporter ß-glucuronidase.

c. *SRK and SLG are papillar cell-specific proteins*

Previously, we had detected *SLG* transcripts, not only in stigmas, but also in anthers and microspores. Our analysis of the *SRK*::uidA transgenic plants has demonstrated that the *SRK* promoter is active at low levels in pollen. However, we were unable to identify SRK protein in anthers or pollen even when we used several different extraction procedures, loaded large amounts of protein on polyacrylamide gels, and developed the immunoblots with chemiluminescence substrates. Similarly, we were unable to detect SLG in anther or pollen extracts, even though we had previously detected *SLG* transcripts in anthers and shown that the *SLG* promoter is active in the anther tapetum and microspores. These results are significant because they indicate that *SRK* and *SLG* function exclusively in the papillar cells of the stigma, and imply that the product of an S-locus gene distinct from *SLG* and *SRK* functions as the pollen determinant of specificity in the self-incompatibility response. This conclusion is further supported by transgenic experiments performed in our laboratory under another project which showed that downregulation of *SLG* and *SRK* transcripts resulted in the loss of the self-incompatibility response in stigmas but not in pollen.

d. *SRK is an integral protein of the plasma membrane*

Biochemical characterization of SRK in stigma extracts has demonstrated that it has characteristics of a membrane protein. Furthermore, subcellular fractionation of tobacco leaf extracts that express SRK under the control of the CaMV 35S promoter allowed us to purify plasma membrane from endomembrane fractions and to demonstrate the co-localization of SRK with the plasma membrane. Extraction of these fractions with various compounds showed that SRK is an integral component of the plasma membrane. We have succeeded at isolating plasma membrane fractions from Brassica stigmas and have shown that SRK is highly enriched in these fractions (R. Dixit, J.B. Nasrallah, and M.E. Nasrallah, submitted).

e. *Topology of the SRK protein*

The sequence of the SRK protein predicts that its SLG-like (or S) domain is oriented to the outside of the cell and therefore that it represents the ligand-binding domain of the receptor. In animal systems, receptor topology has been investigated using immunofluorescence with antibodies directed against either the predicted extracellular or cytoplasmic domains or by treating intact cells with protease followed by protein gel blot analysis or immunoprecipitation. These types of experiments are not practical using stigmas for two reasons: first, they are complicated by the presence of SLG which also cross-reacts with our S-domain antibodies; second, it is very difficult to obtain protoplasts from stigmatic papillar cells. We therefore attempted to perform immunofluorescence studies using protoplasts prepared from tobacco leaf cells that express SRK under the control of the CaMV 35S promoter. However, these experiments were not successful, probably because of the low level of SRK produced by these cells (Stein et al., 1996). In addition, the enzyme treatment required for protoplast isolation may have contained contaminating proteases that might have degraded SRK.

Production of SLG and SRK in insect cells:

To overcome these problems and also (primarily) to generate glycosylated proteins for investigating the interactions of the S domain of SRK with SLG and other molecules (see below), we decided to express SLG and SRK in a baculovirus system. We have produced insect cell lines that express substantial amounts of SRK. We have also expressed SLG and the S domain of SRK (as a soluble secreted protein) from two haplotypes in insect cells. The SRK-expressing insect cells have been used in immunofluorescence and cell-elisa studies to determine the topology of SRK. These studies have demonstrated that the S domain of SRK is indeed exposed to the outside of the cell (Letham et al., in press).

We have also used SRK-expressing insect cells to develop protocols for the solubilization and immunoprecipitation of SRK. These protocols are currently being adapted for the immunoprecipitation of SRK from membrane fractions prepared from stigmas. Immunoprecipitation in conjunction with in vivo labeling of stigmas with 32P should now allow us to determine if native SRK exists as a phosphoprotein and if its phosphorylation state is affected by self- or cross-pollination.

Identification of molecules that interact with the extracellular domain of SRK:
Interaction of SLG and SRK:

An important issue in the functional analysis of SRK is to understand its relationship to SLG. Genetic data clearly demonstrate that SLG and SRK are both required for the self-incompatibility response. In addition, analysis of several S haplotypes has shown that SLG and the S domain of SRK share a higher degree of sequence similarity within a haplotype than between haplotypes, suggesting that the two genes might coevolve and that their protein products might interact functionally.

In the course of analyzing stigma extracts from self-compatible mutant plants, we observed that SRK does not accumulate in stigmas that do not produce SLG. We have identified two independent mutants that do not express SLG. One mutant carries a deletion of the SLG gene but has an intact SRK gene that produces wild type levels of transcripts. The other mutant is homozygous for the recessive *scf1* mutation, a mutation that we had previously described: *scf1* is unlinked to the *S* locus, it downregulates *SLG* at the RNA level, but does not affect *SRK* transcripts. In both of these mutants, we detected no SRK protein. Furthermore, our *SRK6* -expressing tobacco cells produced only very low levels of SRK protein despite the fact that *SRK* expression in these cells was driven by the highly active double CaMV 35S promoter and that high steady-state levels of *SRK* transcripts are detected in these cells (Stein et al., 1996). In contrast, the same promoter allows for high levels of SLG expression in tobacco cells. Based on these results, we propose that SLG is required for the accumulation of SRK.

We have tested this hypothesis by generating transgenic tobacco plants or cell lines that express both SRK and SLG. We have re-transformed an SRK6-expressing tobacco strain with a CaMV 35S::*SLG6* chimeric gene. We have also re-transformed this strain with a construct containing *SLR1*, a member of the *S* gene family that is highly expressed in stigmatic papillar cells (like SLG) but is unlinked to the *S* locus. Several independent transformants have been generated with each construct. Analysis of these transformants has demonstrated that SRK forms aberrant high-molecular mass aggregates

when expressed alone in transgenic tobacco plants, and that this aggregation is prevented in tobacco plants that co-express SRK and SLG, but not in tobacco plants that co-express SRK and SLR1. The data provide experimental support for a specific SLG-SRK association, and indicate that one function of SLG is allowing SRK to assume its proper conformation and thus to attain physiologically relevant levels in *Brassica* stigmas.

Identification of novel molecules that might interact with the extracellular S domain of SRK:

We used interaction cloning approaches to identify pollen proteins that interact with the extracellular domain of SRK. Our first attempts involved the use of bacterially expressed SLG and SRK S domains in blot overlay assays or in the screening of anther cDNA expression libraries. To date, we have not been able to identify interacting proteins by this approach. This problem was anticipated in our original proposal, and as an alternative, we had proposed the use of proteins expressed in eukaryotic systems or isolated directly from stigmas. As described above, we have expressed SLG, SRK, and the S domain of SRK in insect cells. We have also developed methods for the efficient isolation and labeling of SLG from stigmas. These reagents will be used in immnoprecipitation and immunoblot assays to obtain direct evidence for an SLG-SRK interaction and to identify other interacting molecules in stigma and pollen.

Effectors of SRK-mediated signalling:

To identify proteins that interact with the kinase domain of SRK, we used the yeast 2-hybrid system. We constructed a plasmid containing the SRK kinase domain fused to the DNA-binding domain of GAL4 and a stigma cDNA library in a GAL4 activation domain vector. The screening of approximately 106 recombinant clones has failed to identify clones that interact specifically with the wild type kinase domain but not with a kinase-negative mutant, a criterion usually suggestive of the biological relevance and specificity of the interaction.

We have taken another approach to identify effectors of SRK-mediated signalling. Because the analysis of spontaneous and induced mutations to self-compatibility have identified loci unlinked to the *S* locus that are required for self-incompatibility in the stigma, we reasoned that potential candidate genes for proteins of the SRK signalling pathway may be represented among these suppressors of self-incompatibility. The molecular cloning of these genes would represent a direct approach to elucidating the events downstream of receptor activation. We have developed methods to target these genes for molecular cloning, and have strong genetic evidence that we have indeed cloned one such gene, the MOD gene.

Isolation of an effector of the SRK-mediated signal transduction pathway

We focused initially on a mutation, designated *mod*, which was described in the C624 strain of *B. campestris* as a recessive suppressor of SI that is unlinked to the *S* locus. The *mod* mutation is epistatic to the *S* locus, it inactivates S locus function in the stigma but not in pollen irrespective of *S* haplotype, and it does not affect the expression of the known *S* locus genes. As described in Ikeda et al. (1997), we established a large F2 population derived from a cross between the self-compatible C624 strain and a self-incompatible plant homozygous for the S8 haplotype. We used the method of differential

display to compare two RNA pools, one isolated from the stigmas of several self-incompatible (*MOD/MOD* or *MOD/mod*) F2 plants and another isolated from the stigmas of several self-compatible (*mod/mod*) F2 plants. This analysis led to the isolation of a sequence that, based on restriction fragment length polymorphism (RFLP) analysis in F2, F3, and F4 plants, is genetically linked to the *MOD* locus. Independent evidence for the linkage of this sequence to the *MOD* locus was also provided by the observation that eight self-compatible mutant plants isolated following g-irradiation of pollen from a self-incompatible plant were missing the *MOD* -associated RFLP.

The *MOD* -associated gene is expressed in all *MOD* tissues examined but is expressed only at very low levels, if at all, in *mod/mod* tissues, and its transcripts are not induced by self- or cross-pollination. The gene encodes a protein with a high degree of sequence similarity to the MIP (Major Intrinsic Protein) superfamily of membrane proteins from plants, mammals, yeasts, and bacteria. Proteins in the MIP family are channel-forming proteins characterized by the presence of six membrane-spanning domains and are thought to facilitate the transport of water and other small molecules across membranes. The predicted amino acid sequence of the *MOD* -associated gene clearly places this gene within the PIP1 subgroup of plant genes that encode plama membrane intrinsic proteins, and suggests that its product (hereafter designated AQP-SI) is a channel-forming protein that facilitates passage of water across the plasma membrane.

We hypothesize that AQP-SI functions to regulate water availability at the stigma surface. Because AQP-SI is expressed in a variety of plant tissues, yet the only obvious effect of the mod mutation is the breakdown of the SI response specifically in the stigma, we must assume either that its expression is biologically irrelevant outside the stigma or that its function is redundant with that of other PIP genes in non-stigmatic cells. The stigma specificity of MOD action would then be a function of the specific expression of the S-locus encoded SRK and SLG in the epidermal cells of the stigma.

The experiments outlined in the renewal application are aimed at deciphering the role of AQP-SI in the self-incompatibility response.

<u>Signal transduction and the potential role of calcium in pollen-pistil interactions:</u>

We attempted to modify pollination responses with inhibitors or activators of known signalling intermediates. We proposed these experiments on the basis that similar experiments have been informative in some cases, e.g. in the analysis of the phytochrome-mediated response. In addition, we had previously shown an effect of protein phosphatase inhibitors on the pollination response of stigmas. Our hope was that this pharmacological approach would provide some clues regarding the nature of the signal transduction pathways that operate in the compatible and incompatible pollination responses of crucifers. We developed a method for applying solutions to the stigma surface without interfering with normal pollination responses. However, none of the compounds we used resulted in the growth of pollen tubes in self-pollinations or in the arrest of pollen tubes in cross-pollinations. It is possible that pollination signal transduction pathways do not involve any of the intermediates that would have been affected by the inhibitors we tested. Alternatively, it is possible that at least some of the compounds did not gain access to the papillar cell cytoplasm. We cannot distinguish between these possibilities.

To investigate the potential role of calcium in pollination responses, we collaborated with Dr. Robert Silver at Woods Hole. We assayed transgenic Brassica plants that express apoaequorin in their papillar cells, demonstrated the uptake of the cofactor coelenterazine into papillar cells, and showed that active aequorin can be reconstituted in these cells. The level of apoaequorin expressed in papillar cells appears to be adequate for single-cell imaging, since we were able to visualize a response by impaling a single papillar cell with a needle. However, we did not observe any differences in calcium fluxes in response to pollination with self and cross pollen.

Publications and Manuscripts Acknowledging DOE Support:

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