Investigating the Molecular Mechanism of the Self-incompatibility Response in Brassica

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The self-incompatibility response has been defined as the inability of a fertile hermaphrodite seed-plant to produce zygotes after self-pollination. Many members of the genus Brassica exhibit sporophytic self-incompatibility, rejection of self-pollen occurring on the stigma surface. Over the last 15 years a number of genes have been implicated in the self-incompatibility response in Brassica. These include both genes at the S locus, which are potentially involved in the recognition of self-pollen, and genes at unlinked loci, which are though to be involved in processes downstream of the recognition event such as signal transduction and self-pollen rejection. Here we review data from recent studies that have focused on determining the function of these genes, and their respective gene products, in the self-incompatibility response.

Key words: Brassica oleracea, cell recognition, flower, kale, pollination, receptor protein kinase, self-incompatibility, signal transduction, S locus glycoprotein.

INTRODUCTION

The phenomenon of self-incompatibility (SI) in flowering plants has fascinated biologists for over two centuries. Although a great deal has been learnt about SI since Charles Darwin described it as ‘one of the most surprising facts I have ever observed’ (quoted in Matton et al., 1994), a relatively large number of questions still remain unanswered. The history of the investigation of SI has many of the qualities of a good detective story, with painstaking analysis of clues, clever insights and a few startling twists in the plot. In the last 15 years, the identification of S locus genes from a number of different systems has opened up the possibility of understanding these systems at a molecular level (for recent reviews see McCubbin and Kao, 1996; Nasrallah, 1997; McCormick and Gaude, 1999). However, these advances have also added new complexities and raised new questions. If anything, in recent years, work on SI, and particularly on SI in Brassica, has come to resemble more and more the complex story of a police thriller with many twists to the plot, some exciting climaxes and, of course, some characters who turn out not to be what they seem. The aim of this paper is to describe recent work investigating the molecular mechanism of SI in Brassica and to attempt to clarify the roles of the genes identified by these studies.

POLLEN REJECTION ON THE STIGMA SURFACE: THE SCENE OF THE CRIME

In compatible cross-pollinations, when a pollen grain arrives on the stigma surface, it adheres, hydrates and emits a pollen tube. The pollen tubes grow through the stigma, carrying the male gametes to the ovary where they will fertilize an ovule. These processes are blocked when a self-pollen grain arrives on the stigma of a self-incompatible Brassica plant. The rapidity with which the pollination process is blocked depends both on genetic and environmental factors so that the self-pollen grain may either fail to hydrate completely, may hydrate but fail to germinate or, in some cases, germination may occur but the pollen tube will usually fail to penetrate the stigma surface. Although the above analogy between research in recent years into the mechanism of self-incompatibility in Brassica and the plot of a detective novel is apt in many respects, there is one important difference that adds an additional twist to the plot. This is that, although the arrest of pollen on a self-incompatible stigma may resemble ‘murder most foul’, all is not what it seems and the arrested pollen grain, in fact, remains viable for at least 24 h and can continue its development if SI breakdown is induced by treatment with cycloheximide (Sarker et al., 1988). Therefore, in Brassica, and in contrast to other SI systems that have been studied, SI appears to act by a cytostatic rather than cytotoxic mechanism and models of the molecular mechanism of the response need to take this fact into account.

In Brassica recognition of self-pollen by the SI system is controlled by a single, highly polymorphic genetic locus, the
S locus. Alleles of the S locus are referred to as haplotypes because of the complex nature of this locus (see below). Dominance interactions occur between S haplotypes in a heterozygous state and haplotypes can be ordered in a non-linear dominance hierarchy (Thompson and Taylor, 1966). Two major groups can be distinguished in the dominance hierarchy: class I haplotypes tend to be dominant and confer a strong SI phenotype whereas class II haplotypes tend to confer a weaker phenotype and to be recessive to the class I haplotypes.

**S LOCUS GENES EXPRESSED IN STIGMAS: RINGLEADERS AND OTHER SUSPICIOUS CHARACTERS**

A major first step in the molecular analysis of the S locus was the identification of the S locus glycoprotein (SLG) gene (Nasrallah et al., 1985). Analysis of proteins extracted from the stigmas of plants carrying different S alleles had revealed the presence of abundant, polymorphic glycoproteins characteristic of each S allele. Using this information, an SLG cDNA was identified using a differential screening approach. Subsequent analysis of SLG alleles from different haplotypes has shown that SLG is highly polymorphic (Nasrallah et al., 1987; Kusaba et al., 1997). Based on sequence data, SLG alleles can be grouped into two classes which correspond to the class I and II groups defined on the basis of SI phenotype. Stigmas acquire the ability to reject self-pollen as they mature. SLG has been shown to be expressed specifically in pistils and the accumulation of SLG protein coincides with the acquisition of the SI phenotype. The majority of the SLG protein accumulates in the stigma although SLG can also be detected in the transmitting tissue of the style and ovary (Kleman-Mariac et al., 1995). The only other organ in which SLG transcripts have been detected is anthers but SLG protein does not accumulate to a detectable level (Delorme et al., 1995).

The identification of SLG was important for several reasons. In addition to encoding a potential component of the SI response, it was the first molecular marker to be identified at the S locus and it was also the first member of a large gene family in plants, the S gene family. Both of these latter characteristics were important in the identification of a second S locus gene, the S locus receptor kinase (SRK) gene. SRK is an S-locus linked gene that shares sequence similarity with SLG and which encodes a plasma-membrane anchored glycoprotein that resembles animal receptor kinases (Stein et al., 1991). The domain with similarity to SLG (which has been designated the S domain and is present in all members of the S gene family) is predicted to be extracellular and to be separated from a cytosolic kinase domain by a single, membrane-spanning alpha helix. Analysis of a number of different alleles of SRK have shown that, like SLG, this gene is highly polymorphic and that SRK alleles can be grouped into two classes that correspond to the class I and class II groups defined on the basis of SI phenotype (Stein et al., 1991; Kusaba et al., 1997; Cabrillac et al., 1999). SRK protein is only detected in stigmas. Low levels of SRK transcripts can be detected in anthers but no SRK protein has been detected in this organ. SRK, therefore, shares many characteristics with SLG. When SLG and SRK were first identified, the many similarities between these two genes (map position, expression pattern, subcellular localization of their gene products) indicated strongly that both of their protein products functioned in the stigma to recognize self-pollen. This hypothesis was supported by the fact that, in some S haplotypes, SLG and SRK were shown to have evolved convergently so that the S domains of the two genes are more similar to each other than they are to those of other SLG and SRK alleles.

Recent analysis has shown that both SLG and SRK are complex genes and that certain alleles of both genes encode more than one protein product. For example, in the class I S haplotypes, SRK1 has been shown to encode at least seven different transcripts including transcripts from both strands of the gene (Delorme et al., 1995; Cock et al., 1997). Several of the sense transcripts retain all or part of the first intron and, because there is a termination codon just after the S' end of the intron, are predicted to encode a soluble, truncated form of SRK that corresponds to the predicted extracellular domain (Giranton et al., 1995). This truncated form of SRK, which has been called eSRK for extracellular SRK, resembles SLG. The function of eSRK is unknown but it is interesting that no eSRK protein was detected in stigmas of plants homozygous for the class II haplotype S14 (Cabrillac et al., 1999). Analysis of SRK alleles from other haplotypes will be necessary to determine whether eSRK is associated exclusively with class I S haplotypes.

Tantikanjana et al. (1993) showed that the SLG allele of the class II S haplotype possesses two exons and that alternative transcripts of this allele can encode both a secreted form of SLG and a membrane-anchored form designated mSLG. The SLG allele of the class I S6 haplotype, on the other hand, was shown to possess only one exon and to encode only a secreted form of SLG. Based on these observations it was suggested that the presence or absence of mSLG may determine whether a haplotype is dominant or recessive, respectively.

A more recent study has shown that the information obtained from the S6 haplotype cannot be extrapolated to all class II haplotypes (Cabrillac et al., 1999). The class II S15 haplotype was shown to include two different SLG genes, SLGA and SLGB (Fig. 1A). Both of these genes possess two exons interrupted by a single intron but only SLGA possesses a second exon that encodes a membrane-spanning domain. SLGA, therefore, is predicted to encode a secreted SLGA protein and a membrane-anchored mSLGA protein whereas SLGB only encodes a secreted SLGB protein. This prediction was confirmed by an analysis of the proteins present in S15 stigmas. Comparison of S15 with two other class II haplotypes, S1 and S14, indicated that these two haplotypes lacked the SLGA and SLGB genes, respectively (Cabrillac et al., 1999; Fig. 1A). This was consistent with the absence of an mSLG protein in stigmas of the S1 homozygous line. These observations indicate that mSLG need not necessarily be present for a haplotype to show a class II phenotype. The role of the mSLG protein, therefore, remains unclear at present.
Another conclusion from this study stems from the fact that the $S_5$ and $S_1$ haplotypes lack $SLGB$ and $SLGA$ respectively (Fig. 1A). This suggests that either (1) these genes are redundant or (2) neither is required for the SI response. Moreover, the fact that the $SLGA$ and $SLGB$ alleles of the $S_1$ haplotype are more similar to $SLG_2$ and $SLG_5$, respectively, than they are to each other argues against redundancy (Fig. 1B). The conclusions from this study conflict with those of previous studies that have proposed a key role for $SLG$ in the SI response. In the following paragraphs we will review the data available concerning the function of $SLG$ in an attempt to resolve this contradiction.

Several pieces of circumstantial evidence imply a role for $SLG$ in the SI response. These include its location at the $S$ locus, the fact that $SLG$ protein accumulates specifically in pistils (and particularly in the stigma), the highly polymorphic nature of $SLG$ and evidence for convergent evolution of $SLG$ and $SRK$ in some $S$ haplotypes. However, although several groups have attempted to demonstrate directly a role for $SLG$ in the SI response by transgenic or genetic approaches, the results of these studies have been ambiguous (Toriyama et al., 1991; Nasrallah et al., 1992; Nishio et al., 1992; Shiba et al., 1995; Conner et al., 1997).

A correlation has been reported between loss of $SLG$ expression and the acquisition of a self-compatible phenotype using both approaches, but the interpretation of these results is often complicated by the fact that $SRK$ expression may also have been affected. Simultaneous effects on the two genes are likely because of their shared sequence similarity, and coexpression of $SLG$ and $SRK$ has been described (Conner et al., 1997; Stahl et al., 1998). In addition, recent work described by June Nasrallah (Cornell University, NY, USA) at the Pollen–Stigma Interactions Meeting indicates that $SLG$ is required for post-transcriptional expression of $SRK$, at least in some genetic backgrounds. As a result of these phenomena it may be difficult to distinguish between direct effects of $SLG$ and indirect effects via $SRK$. For example, in one study using self-compatible plants homozygous for a mutant allele of the suppressor gene $SCFl$ the abundance of $SLG$ transcripts in stigmas was shown to be significantly reduced and no $SLG$ protein was detectable whilst $SRK$ transcripts accumulated to a normal level (Nasrallah et al., 1992). However, the abundance of $SRK$ protein in stigmas of this line was not determined and the new data presented by the Cornell University group suggests that the self-compatible phenotype may have been due to disruption of post-transcriptional expression of $SRK$, rather than a direct result of the absence of $SLG$.

Arguments against a role for $SLG$ in the SI response include evidence from the analysis of class II lines described above and a study by Gaude et al. (1995) which showed that the level of expression of $SLG$ is not correlated with the strength of the SI response. $SLG$ was abundant in stigmas of a self-compatible line homozygous for the $S_1$ allele 

![Fig. 1. Comparison of the structures and sequences of $SLG$ and $SRK$ alleles of three class II $S$ haplotypes. A. Schematic representation of $SLG$ and $SRK$ alleles of three class II $S$ haplotypes. Two different $SLG$ genes, $SLGA$ and $SLGB$, have been identified in the class II $S_5$ haplotype (Cabrillac et al., 1999). The $S_1$ haplotype includes an allele of $SLG_A$ but not $SLG_B$ whereas the $S_5$ haplotype includes an allele of $SLG_B$ but not $SLG_A$. $SLG_A$ encodes both secreted and membrane-anchored proteins ($SLGA$ and $mSLGA$, respectively) whereas $SLG_B$ encodes only secreted $SLG$ proteins ($SLGB$). Transcribed regions and coding regions are indicated by narrow boxes and thick boxes, respectively. The exon/intron structure of the kinase domains of SRK and $SLG$ has not been determined and these regions are therefore represented by dotted lines. Membrane-spanning domains (TM) are indicated by black boxes. Arrows indicate stop codons near the $S'$ ends of introns that potentially allow the production of truncated proteins from alternatively spliced transcripts that retain all or part of the intron sequences. S dom, S domain; kin, kinase domain. B. Percentage amino acid similarity between alleles of $SLGA$ and $SLGB$.](https://academic.oup.com/aob/article-abstract/85/suppl_1/147/102819/149)
pollen adhesion (Luu et al., 1999). Treatment of stigmas with an anti-SLG antibody also reduced the strength of pollen adhesion (Luu et al., 1999). In both cases the force of pollen adhesion was only partially reduced indicating that several factors, including the proteins SLR1 and SLG, contribute in an additive manner to pollen adhesion. Luu et al. (1997) have shown that the strength of pollen adhesion is not influenced by the self-incompatibility response. Therefore, a function for SLG in pollen adhesion would be independent of the SI system.

If SLG does not play a direct role in the recognition of self-pollen it becomes necessary to explain why the SLG and SRK alleles of some haplotypes exhibit convergent evolution. One possible explanation for this phenomenon may be that it is unrelated to gene function and is simply due to a combination of reduced recombination between different haplotypes of the S locus and the tendency of linked genes to exchange sequence information via gene conversion (see Cabrillac et al., 1999).

If the evidence is now against a direct involvement of SLG in haplotype-specific recognition of self-pollen, what about SRK? The circumstantial evidence implicating SLG in the SI response is also valid for SRK. Like SLG, SRK is located at the S locus, is expressed specifically (at the protein level) in stigmas and exhibits a high level of DNA polymorphism. Moreover, the resemblance between SRK and animal receptor kinases is consistent with a role in a cell–cell recognition system. More direct evidence for an involvement of SRK in SI has come from the analysis of self-compatible Brassica lines. Two examples have been reported of non-functional SRK alleles in such lines indicating a correlation between loss of SRK function and acquisition of a self-compatible phenotype (Goring et al., 1993; Nasrallah et al., 1994). Importantly, however, in neither case was the self-compatible phenotype shown to be a direct result of the SRK mutation and it is possible that other genes are mutated in these lines. Efforts to express novel SRK alleles or dominant negative forms of SRK in transgenic plants have run into problems due to insufficient expression and cosuppression (Stein et al., 1991; Conner et al., 1997; Gaude and Cock, unpubl. res.). The most convincing evidence that SRK is involved in the SI response has been obtained by Stahl et al. (1998) who observed haplotype-specific breakdown of SI in a B. napus plant transformed with a kinase-defective SRK gene. However, the breakdown of SI in these experiments was partial and the observations were made on a single transgenic line. It will be important to demonstrate a similar phenotype in additional independent transformants.

In summary, the evidence ‘convicting’ SRK of involvement in the recognition of self-pollen is now very convincing. On the other hand, and despite a considerable amount of circumstantial evidence implicating SLG in the same process, new evidence suggests that this gene is innocent of the accusations that were originally made against it and is unlikely to be involved directly in the recognition of self-pollen. SLG may, however, be an accessory to the ‘crime’, acting in a supporting role to SRK.
1995). Firstly, it was shown to be located at the S locus; SLA was first identified 500 bp downstream of SLG in the \(S_3\) haplotype. Secondarily, it was shown to be expressed specifically in anthers; two transcripts that are antisense with respect to each other accumulate specifically in anthers at different stages of development. Thirdly, one of the SLA transcripts was shown to include two short open reading frames that could encode short peptides. Fourthly, SLA seemed to be highly polymorphic in as far as an SLA probe only hybridized to DNA from a small number of haplotypes. Finally, and probably most convincingly, a self-compatible \(B. napus\) line was shown to carry an SLA allele interrupted by a large insertion.

A more recent study carried out in our laboratory, however, indicates that SLA is, in fact, only present in a limited number of \(S\) haplotypes and that it exhibits a very low level of polymorphism (Pastuglia et al., 1997). Moreover, a number of self-incompatible cauliflower lines were identified that carried an allele of SLA which was interrupted by a retrotransposon (similar to the allele found in \(B. napus\)). These data indicate that SLA is not required for the SI response and, unfortunately, it is hence unlikely that it encodes the male component.

**THE MODUS OPERANDI**

The previous sections described the genes that have been identified at the \(S\) locus and the experiments that have been carried out to determine whether they are implicated in the SI response. However, to use the detective story analogy again, these investigations may have identified some interesting suspects but do not tell us how the 'crime' was actually committed. To answer this we need not only to identify the molecules involved in the recognition process but to have a deeper understanding of how these molecules function. A relatively crude model for the molecular mechanism of self-pollen recognition can be proposed based on the structural resemblance of SRK to animal receptor kinases. In this model, the extracellular (S) domain of SRK would act as a receptor and recognize a pollen-borne ligand. Ligand binding would activate the cytosolic kinase domain leading to initiation of the SI response via a signalling cascade. The data that are currently available for SRK are consistent with this model in as much as the kinase domain of SRK has been shown to possess a serine/threonine protein kinase activity when it is expressed in *Escherichia coli* (Goring and Rothstein, 1992) and SRK has been shown to encode a glycoprotein that is localized in the plasma-membrane in stigmas (Delorme et al., 1995; Stein et al., 1996; Cabrillac et al., 1999). Recently, work carried out in our laboratory, involving expression of kinase-active and kinase-inactive recombinant forms of SRK fused to different epitope tags in a baculovirus/insect cell system, has shown that one molecule of SRK can phosphorylate another SRK molecule in a membrane environment (Giranton, Cock, Dumas and Gaude, unpubl. res.). In addition, evidence has also been obtained, using both cross-linking and velocity sedimentation methodology, showing that SRK is part of a complex in planta (Giranton et al., unpubl. res.). These are important observations because the role of oligomerization and trans-phosphorylation in the activation of animal receptor kinases is well established (Heldin, 1995). These experiments, therefore, provide the first biochemical support for models in which SRK functions in a manner analogous to animal receptor kinases.

**FROM SELF-POLLEN RECOGNITION TO SELF-POLLEN REJECTION: MIDDLEMEN AND TRAFFICKERS**

To date, the majority of the work on molecular aspects of SI in *Brassica* has concentrated on the molecules involved in pollen-pistil recognition; very little is known about how the rejection of self-pollen is mediated. In one approach to identify components acting downstream of SRK in the stigma, Daphne Goring’s group (York University, Ontario, Canada) have been using the two hybrid system to identify proteins that interact with the cytosolic kinase domain of SRK (Bower et al., 1996; Gu et al., 1998). Three interacting proteins have been identified: two thioredoxin-h-like proteins (THL-1 and THL-2) and arm repeat containing protein 1 (ARC 1) which contains arm repeats similar to those found in the *Drosophila* armadillo protein and \(\beta\)-catenin. ARC1 is perhaps the most interesting of these proteins for a number of reasons: (1) it is expressed specifically in stigmas; (2) it only associates with the phosphorylated form of the SRK kinase domain; and (3) it is specifically phosphorylated by the kinase domain of SRK *in vitro*. Recent work, involving the analysis of transgenic plants carrying antisense ARC1 constructs, was described by the York University group at the Pollen–Stigma Interactions Meeting. A partial breakdown of the SI response was observed in these plants indicating that ARC1 is indeed a component of the SI response.

Based on genetic analysis, the genes directly involved in the recognition step of the SI response are predicted to be located at the \(S\) locus but genes involved in other steps of the response such as self-pollen rejection need not necessarily be genetically linked to the \(S\) locus. Indeed, the self-incompatibility response is known to be influenced by a number of unlinked modifier and suppressor loci in addition to the \(S\) locus (Nasrallah, 1989; Nasrallah et al., 1992). Analysis of these loci provides an alternative approach to identify genes involved in other stages of the SI response.

Using differential display reverse transcription-polymerase chain reaction (DDRT-PCR) analysis, Ikeda et al.
(1997) identified an aquaporin-like gene which is expressed in stigmas of wild-type plants but not in self-compatible plants homozygous for a mutant allele of the modifier gene MOD (Hinata et al., 1983). The gene encoding the aquaporin-like protein was shown to be very closely linked to the MOD locus. Ikeda et al. (1997) proposed that the aquaporin-like gene is MOD and suggested that its gene product may be involved in controlling the hydration of self-pollen.

This is a very exciting result in that it potentially identifies a novel component of the SI system. However, the implications of the involvement of this protein in the SI response are complex because it is necessary to account for the fact that loss of the protein results in self-compatibility. If a lesson has been learnt from the analyses of the SLG and SLA genes it is that one should be prudent in assigning a role in the SI response. For this reason, and because of the complexity of the models that explain how the aquaporin-like gene could be implicated in the SI response, it will be important to test these models experimentally and to confirm that the aquaporin-like gene is identical to MOD.

**FUTURE DIRECTIONS: THE PLOT THICKENS**

Despite a number of setbacks and encounters with unexpected complexities a picture is starting to emerge of how rejection of self-pollen is mediated at the molecular level, at least on the female side. However, several questions remain to be answered, the most important being the nature of the male component of the SI response. At the Pollen-Stigma Interactions Meeting, several groups described their ongoing efforts to identify the male component using approaches ranging from differential screening, to mutagenic approaches and chromosome walking at the S locus, to biochemical approaches and bioassays. Considering the effort that is going into this work, it is likely that the male component will be identified in the near future.

The nature of the male component is not the only question that is currently being addressed. One important aspect of the SI response, about which very little is known, is the mechanism of self-pollen rejection following recognition by the stigma. Some recent advances in this area have been described above. Work in this area may help explain why some S haplotypes are early acting, causing rapid arrest of pollination, whilst others are late acting. Are these differences due to the SI system creating several 'barriers' to pollination at different stages of the pollination process (hydration, germination, pollen tube penetration, etc.) or do the differences in the stage at which arrest occurs reflect a delayed effect of the SI system acting more or less 'strongly', early in the pollination process?

Many other questions will become easier to address as we learn more about how SI works at the molecular level. An obvious example is the molecular basis of dominance between S haplotypes. The evidence that SRK molecules are able to associate with each other in an oligomeric complex suggests a possible mechanism for dominance in stigmas. Other questions of this type concern the mechanism of the breakdown of SI under certain environmental conditions, the molecular basis of S haplotype specificity and the evolutionary origin of the SI system.

Current research aimed at understanding the molecular mechanism of SI builds on genetic and physiological studies that have been carried out over the past few decades. It is fitting that the Pollen-Stigma Interactions Meeting, which provided such an exciting forum to discuss the recent developments in this area, should have been dedicated to the memory of Jack Heslop-Harrison who made such an important contribution to the laying down of this foundation.

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