A transgenic self-incompatible Arabidopsis thaliana model for evolutionary and mechanistic studies of crucifer self-incompatibility

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Received 27 October 2009; Revised 16 December 2009; Accepted 21 December 2009

Abstract

Molecular genetic studies of self-incompatibility (SI) can be difficult to perform in non-model self-incompatible species. Recently, an Arabidopsis thaliana transgenic model was developed for analysis of the SI system that operates in the Brassicaceae by inter-species transfer of genes encoding the S-locus receptor kinase (SRK) and its ligand, the S-locus cysteine-rich (SCR) protein, which are the determinants of SI specificity in the stigma and pollen, respectively. This article reviews the various ways in which the many advantages of A. thaliana and the extensive tools and resources available in this model species have allowed the use of transgenic self-incompatible SRK–SCR plants to address long-standing issues related to the mechanism and evolution of SI in the Brassicaceae. It also presents the unexpected results of a candidate gene approach aimed at determining if genes related to genes previously reported to be involved in the SI response of Brassica and genes required for disease resistance, which exhibits many similarities to the SI response, are required for SI in A. thaliana. These various studies have provided a novel insight into the basis of specificity in the SRK–SCR interaction, the nature of the signalling cascade that culminates in the inhibition of ‘self’ pollen, and the physiological and morphological changes that are associated with transitions between the outbreeding and inbreeding modes of mating in the Brassicaceae.

Key words: Arabidopsis thaliana, Brassicaceae, self-incompatibility, transgenic model.

Introduction

The large arsenal of genetic and molecular resources available in Arabidopsis thaliana have made this species the model of choice for analysis of a large variety of physiological, developmental, and evolutionary processes. However, there are limits to the usefulness of A. thaliana, because some biological phenomena do not occur in this model species. One phenomenon for which the A. thaliana model seems at first glance inappropriate is self-incompatibility (SI). Although operative in several species of the genus Arabidopsis, SI has not been reported in A. thaliana, and all of the hundreds of its accessions collected from various geographical locations are highly self-fertile. Indeed, all A. thaliana accessions analysed to date contain non-functional versions of one or both of the two S-locus-encoded proteins whose allele-specific interaction determines SI specificity in the Brassicaceae (crucifers): the S-locus receptor kinase (SRK), which is displayed at the stigma surface, and the S-locus cysteine-rich (SCR) protein (also designated SP11; Takayama et al., 2001), which is located in the pollen coat and is the ligand of the SRK receptor (reviewed in Rea and Nasrallah, 2008). However, transgenic inter-specific and inter-generic complementation experiments demonstrated the transfer of the SI trait into A. thaliana by transformation with SRK–SCR gene pairs isolated from the self-incompatible crucifers A. lyrata or Capsella grandiflora (Fig. 1) (Nasrallah et al., 2002, 2004;
Boggs et al., 2009a, b). This transgenic self-incompatible A. thaliana model, which has taken several years to develop, is now beginning to fulfil its promise and yield important results related to the evolution and mechanism of the self-incompatibility response of the Brassicaceae.

Investigating evolutionary switches to self-fertility and diversification of the SI recognition repertoire

The switch to self-fertility in A. thaliana

One set of studies used the self-incompatible transgenic SRK–SCR A. thaliana model in conjunction with the large numbers of A. thaliana geographical accessions available through stock centres to investigate issues related to mating system evolution in the Brassicaceae. Transformation of several of these accessions with A. lyrata SRK–SCR genes uncovered substantial cryptic natural variation in expression of SI (Fig. 2) (Nasrallah et al., 2002, 2004; Boggs et al., 2009a). SRK–SCR transformants of some accessions expressed a robust and developmentally stable SI phenotype similar to that observed in naturally self-incompatible species, and these accessions are inferred to have become self-fertile as a direct result of inactivation of the S locus. In contrast, SRK–SCR transformants of other accessions expressed transient SI (i.e. stigmas of young floral buds expressed strong SI, which broke down in older flowers) similar to many naturally occurring pseudo-self-compatible plants, and still other accessions expressed only weak SI or no SI (Fig. 2). The latter accessions are inferred to carry inactive alleles of SI modifier genes (i.e. genes that are required for SI but are located outside the S locus proper) in addition to having a non-functional S locus, and it is impossible at present to determine if the initial loss of SI in these accessions was caused by a mutation at the S locus or at a modifier locus. In any case, the observation that A. thaliana harbours polymorphisms at the S locus and at SI modifier loci (Sherman-Broyles et al., 2007; Boggs et al., 2007; Boggs et al., 2009a) strongly suggests that the species transitioned to self-fertility multiple times, and that loss of SI was caused by independent mutations in different geographical accessions.

Crosses between accessions that differed in expression of SI demonstrated the segregation of recessive ‘self-fertility’ alleles at several loci (Liu et al., 2007; Boggs et al., 2009a). Analysis of an inter-accession cross between C24, which expresses developmentally stable SI, and RLD, which expresses transient SI, showed that transient SI is caused by a hypomorphic allele of PUB8, a gene located at one boundary of the S locus that encodes a previously uncharacterized Arm-repeat- and U-box-containing protein that regulates SRK transcript levels (Liu et al., 2007). This result underscores the conclusion that disruption or down-regulation of the S-locus recognition genes, particularly of SRK, was a major mechanism for the switch to self-fertility in A. thaliana.

Identification of amino acid residues that determine SRK specificity

Another series of experiments that used the A. thaliana SRK–SCR model investigated the basis of SRK specificity
and diversification of the SI recognition repertoire. It had been known for several years that specificity in the SI response was based on allele-specific interactions between the highly polymorphic SRK and SCR proteins (Kachroo et al., 2001; Takayama et al., 2001), whereby an SCR protein can bind and activate the SRK protein encoded in the same S-locus haplotype, but not SRK proteins encoded in other S haplotypes. A major unanswered question in SI research is how the large numbers of extant SRK and SCR variants have co-evolved to maintain their highly specific interaction and, more generally, how new SI specificities were generated. To gain an insight into this question, it is important to identify the amino acid residues that determine specificity in the SRK–SCR interaction. This issue was partially addressed for SCR by analysis in Brassica, which identified the few residues that are responsible for specificity in some SCR variants (Chookajorn et al., 2004; Sato et al., 2004). In the case of SRK, hypervariable regions (Fig. 3) and amino acid residues bearing signals of positive selection had been identified within the extracellular ligand-binding domain and inferred to function as specificity determinants (Miege et al., 2001; Schierup et al., 2001; Charlesworth et al., 2003; Sainudiin et al., 2005). However, no studies had demonstrated empirically how many and which of these residues are responsible for SI specificity, largely due to technical difficulties in implementing the necessary experiments. Identification of specificity-determining residues requires in planta analysis of large numbers of receptor variants generated by domain swapping or site-directed mutagenesis of individual residues that differ between pairs of SRK variants. Such analyses are impractical to perform in naturally self-incompatible species such as Brassica species and A. lyrata, due to the laborious or inefficient transformation methods available for these species. By contrast, A. thaliana is easily and efficiently transformed by the Agrobacterium-mediated floral dip method, and is ideally suited for these studies.

Identifying residues important for SRK specificity in the A. thaliana self-incompatible model requires expression of different SI specificities in this species. Accordingly, several SRK–SCR gene pairs derived from A. lyrata or C. grandiflora were isolated and tested for functionality in transgenic A. thaliana (Boggs et al., 2009c). Out of five different functional SRK–SCR gene pairs (Fig. 1), two pairs were subjected to detailed site-directed mutagenesis of polymorphic residues within the SRK extracellular domain. In planta analysis of these SRK sequence variants showed that the majority of SRK residues previously reported to show signals of positive selection are not essential for specificity in the SRK–SCR interaction (Boggs et al., 2009c). Indeed, out of the approximately 100 polymorphic amino acid residues that differed between the extracellular domains of the two pairs of SRK variants analysed, only six or seven residues were found to be required for ligand-specific activation of the SI response (Boggs et al., 2009c). As shown in Fig. 3, these residues are clustered within two non-contiguous regions located at equivalent positions in the two variants tested, and these sites were also found to be highly polymorphic in other SRK proteins. These results suggest that for the majority, if not all, of SRK variants, specificity is determined primarily by short stretches of amino acid residues located in these non-contiguous regions. High-resolution structural analysis of the SRK protein in its ligand-bound and unbound forms is required to determine if these regions are surface-exposed and are brought into close proximity in the three-dimensional SRK structure to form part of an SCR-binding pocket.

Fig. 3. Structure of SRK and amino acid residues required for SI in the SRK extracellular domain. (A) Structure of the SRK protein (top), consisting of the N-terminal signal peptide (SP), the extracellular (‘S’) domain (detailed at the bottom), the transmembrane domain (TM), and the intracellular kinase domain. The location within the S domain of the hypervariable regions (hvl, hvII, hvIII, and the C-terminal variable region) that are enriched for variable amino acid residues is shown. (B) Amino acid residues required for SI specificity. Residues essential for SI were identified by in planta analysis of site-directed SRK mutants (see Boggs et al., 2009c, for details). Circles and asterisks correspond to the approximate locations of residues essential for SRK7 and SRK25 function, respectively. Note that these residues are located in overlapping segments of the hvl and hvII regions in the two SRK variants analysed. Diagrams are not drawn to scale.
**Mutational analysis of SI**

*Arabidopsis thaliana* SRK–SCR transplants, specifically those expressing transient SI such as the Columbia (Col) accession, are particularly useful for mutational analysis of SI in search of genes required for full expression of this trait. Because Col SRK–SCR plants set abundant seed, they may be subjected to chemical mutagenesis using the standard protocols typically used in *A. thaliana*. Ethyl methane sulphonate mutagenesis of these plants did, indeed, produce mutations that affect the SI response, some of which caused the loss of SI in young floral bud stigmas, while others caused enhancement of SI in older flower stigmas. Positional cloning of an enhancer mutation identified the RNA-dependent RNA polymerase RDR6, which functions in trans-acting siRNA (ta-siRNA) production, as a negative regulator of SI (Tantikanjana et al., 2009). Interestingly, this recessive enhancer mutation has pleiotropic effects: it simultaneously enhances SI and causes stigma exsertion, without exhibiting associated increases in SRK transcript levels. Although rdr6 mutants had been shown previously to exhibit stochastic stigma exsertion (Peragine et al., 2004), analysis of the SRK–SCR rdr6 plants demonstrated that SRK further enhances pistil elongation and stigma exsertion in this mutant background. Interestingly, this enhancement requires SRK catalytic activity and its extent is positively correlated with SRK transcript levels (Tantikanjana et al., 2009). Thus, the rdr6 mutation revealed that, in addition to functioning in SI, SRK plays a previously unsuspected role in pistil development. It has been observed that changes in floral architecture, including stigma exsertion, often accompany transitions between the out-crossing and selfing modes of mating (Barrett, 2002; Goodwillie et al., 2010). The dual role of SRK in SI and pistil development revealed by the rdr6 mutation provides a molecular explanation for this poorly understood co-ordinate evolution of physiological and morphological traits. The data further suggest that positive regulators or effectors of SI and pistil development are regulated by ta-siRNA(s). Identifying the targets of these ta-siRNAs is expected to illuminate the mechanism of SI and the molecular overlaps between signalling at the stigma surface and the pistil developmental pathway.

**Exploring SRK-mediated signalling using a candidate gene approach**

**Analysis of positive effectors of SI: the case of ARC1 and MLPK**

Two proteins have been proposed to function as positive effectors of SRK-mediated signalling. The Arm-Repeat-Containing 1 (ARC1) protein has been implicated in SI because antisense down-regulation of *ARC1* in transgenic *B. napus* was associated with the partial breakdown of SI (Stone et al., 1999). *ARC1*, which was identified as a protein that interacts with, and is phosphorylated by, the SRK kinase domain, is an E3 ubiquitin ligase that co-localizes with the proteasome and COP9 signalosome in an SRK-dependent manner (Gu et al., 1998; Stone et al., 2003), and it also interacts with Exo70A1, a putative component of the exocyst complex (Samuel et al., 2009). These results suggested the hypothesis that ARC1-mediated protein degradation of factors required for compatible pollen–stigma interactions is required for the SI response to be manifested (Samuel et al., 2009). The M-locus Protein Kinase (MLPK) was isolated by positional cloning and is thought to correspond to a mutation that causes complete loss of SI in the *B. rapa* variety Yellow Sarson (Murase et al., 2004). Two MLPK isoforms have been shown to localize to the plasma membrane and interact with SRK, and are thus thought to function in SRK-mediated signalling (Kakita et al., 2007).

The dependence of SI in *A. thaliana* was tested on the *A. thaliana* genes that exhibit the highest degree of sequence similarity to *Brassica ARC1* and MLPK. Strains carrying T-DNA insertions in At1g29340 (*APK1b*) and in At2g28930 (*APK1b*) were obtained, and the inactivation of these genes was confirmed by our failure to detect the corresponding transcripts in insertion homozygotes (Fig. 4A). To determine if At1g29340 and At2g28930 are required for SI, the T-DNA mutants were crossed to Col plants transformed with *SRKb* and *SCRb* driven by their native promoters (Nasrallah et al., 2002). It was expected that if these genes were required for the SI phenotype of *A. thaliana* SRK–SCR plants, loss-of-function mutations in these genes would cause either partial or complete loss of SI. However, the SI response of *SRKb–SCRb* plants homozygous for the T-DNA insertions was identical to that of *SRKb–SCRb* plants lacking the T-DNA insertions (Fig. 4A). This result indicates either that At1g29340 and At2g28930 are not required for SI or that their role in SI is masked by the redundant activities of other genes.

Surprisingly, our analysis of a hypomorphic *ARC1* allele in *B. rapa* also suggested that *ARC1* was dispensable for SI. This hypomorphic allele was fortuitously identified in a population of *B. rapa*. As shown by the DNA gel blot in Fig. 4B, *ARC1* is a single-copy gene in *B. rapa* and it exhibited a restriction fragment length polymorphism in the analysed population. To determine if the *ARC1* alleles represented by each of the two *ARC1*-positive restriction fragments differed in the amounts of transcripts they produced, poly-A RNA was isolated from the stigmas of the corresponding homozygotes and subjected to gel blot analysis. This analysis demonstrated that plants homozygous for the smaller restriction fragment exhibited a 10-fold reduction in *ARC1* transcripts relative to the stigmas of plants homozygous for the larger restriction fragment (Fig. 4B). This drastic reduction in *ARC1* transcripts is equivalent to that observed in the *ARC1* antisense experiments that were reported to cause partial breakdown of SI in *B. napus* (Stone et al., 1999). Nevertheless, manual pollination assays showed that *B. rapa* plants homozygous for the hypomorphic *arc1* allele were highly self-incompatible, and the strength and developmental stability of their SI response was indistinguishable from that of stigmas that accumulated 10-fold higher levels of *ARC1*.
transcripts (Fig. 4B). Taken together, the results of our analysis suggest that the requirement of ARC1 in SI is not universal. It is possible that, in B. rapa and A. thaliana, other ARC1-related genes may fulfil whatever function is played by ARC1 in B. napus. Further analysis is required to reconcile these discrepancies.

**Links between SI and defence against pathogens**

The parallels that exist between SI and ‘pathogen incompatibility’ have often been noted (for extensive reviews, see Hodgkin et al., 1988; Nasrallah, 2005; Sanabria et al., 2008). Although SI involves recognition and rejection of genetically similar (‘self’) pollen grains and pathogen resistance (PR) involves recognition and rejection of ‘non-self’ pathogens, both processes are based on highly specific molecular interactions. Just as recognition specificity in SI is achieved via the activity of highly polymorphic S-locus genes, recognition specificity in PR is achieved via highly polymorphic Resistance (R) genes. The similarities between PR involving initial basal resistance and SI in the Brassicaceae are particularly striking. Both processes occur at the epidermal cell surface where small peptide ligands, contributed by pathogen in PR and by ‘self’ pollen grains in SI, are perceived by plasma membrane-spanning receptor kinases. Both the SRK and the Flagellin Sensing 2 (FLS2) receptor, which functions in immunity, are single-pass trans-membrane serine/threonine kinases (Stein et al., 1991; Gómez-Gómez and Boller, 2000), the activation of which triggers signalling cascades that culminate in inhibited growth and penetration into subepidermal tissues of pollen tubes in SI and of fungal hyphae or bacteria in PR. Furthermore, SI and PR can have similar cytological manifestations, such as calcium fluxes and callose deposition at the site of contact between the interacting cells (Hodgkin et al., 1988; Elleman and Dickinson, 1999), and changes to the organization of the actin cytoskeleton and distribution of secretory vesicles (Iwano et al., 2007).

Recently, molecular linkages have been made between PR and SI, strengthening the hypothesis that the basal defence genetic network was co-opted for recognition of ‘self’ pollen in SI. Thus, programmed cell death, which is often triggered in PR, also occurs in the inhibition of ‘self’ pollen tubes in Papaver (Thomas and Franklin-Tong, 2004). In addition, similar molecules have been implicated in PR and the SI response of some plant families. A tobacco RNase a $^{32}$P-labelled ARC1 probe, and visualization of hybridization signals were performed as described previously by Nasrallah et al. (2004). The lower panel shows a blot of poly-A RNA isolated from the stigmas of a plant homozygous for the ARC1 allele and a plant homozygous for the arc1 hypomorphic allele. The blot was probed sequentially with an ARC1 probe and an actin control probe. Note that the arc1 homoyzgote has dramatically reduced levels of ARC1 transcripts, but it exhibits as robust an SI response as that exhibited by ARC1 homozygotes, as shown by the images of self-pollinated stigmas. Scale bars on micrographs = 50 μm.
belonging to the same family as the RNase that determines specificity in S-RNase-based SI systems was found to inhibit hyphal elongation of plant pathogens (Hugot et al., 2002). Another example is provided by the Cf-9-mediated gene-for-gene resistance of tomato, in which Cf-9, a receptor-like type I transmembrane R protein, and the Avr9 avirulence peptide from the fungal pathogen Cladosporium fulvum, elicit the hypersensitive response (including cell death) and confers resistance against the pathogen. In Cf-9–Avr9 resistance, a thioredoxin was found to be a negative regulator of Cf-9-mediated signalling (Rivas et al., 2004), similar to the situation in Brassica, where a thioredoxin negatively regulates SRK in the absence of an SCR ligand (Cabrillac et al., 2001). Interestingly, similarity in tertiary structure, but not primary sequence, exists between SCR proteins of the Brassicaceae SI system and plant (and animal) defensins, which function in immunity as broad-spectrum antimicrobial molecules (Segura et al., 1998; Schoopfer et al., 1999; Lay et al., 2003; Chookajorn et al., 2004). Furthermore, ACRE276/PUB17 and ACIK1, two proteins that share extensive sequence similarity with Brassica ARC1 and MLPK, respectively, are required for full disease resistance in the Cf-9–Avr9 interaction (Rowland et al., 2005; Yang et al., 2006). This finding provides additional evidence for the use of similar signalling pathways in SI and PR, if it is assumed that ARCI-like and MLPK-like genes, rather than the ARC1 and MLPK genes themselves, do function in SI.

Additional, albeit more indirect, links between PR and SI in the Brassicaceae are suggested by the observation that two members of the A. thaliana SRK-like (SRKL) gene family, ARK1 and ARK3, as well as several Brassica SRKL genes, are induced upon wounding and bacterial infection (Pastuglia et al., 2002). Based on microarray experiments, ARK1 and ARK3 were also identified as putative primary targets of a key regulator of systemic acquired resistance, NPR1, and other S-domain RLK genes were rapidly elicited by treatment with a peptide derived from bacterial flagellin (Navarro et al., 2004; Zipfel et al., 2004; Wang et al., 2005).

The possibility that the same signalling components are used in the SI and PR signalling pathways is readily testable in A. thaliana because a large number of PR mutants are available in this species. The strategy used for identifying signalling components that might be shared by the two pathways was similar to that described earlier for the PUB17 and APK1b genes. Transgenic self-incompatible A. thaliana SRKb–SCRb plants were crossed to plants carrying single-gene mutations at various loci known to function in plant defence. Progeny plants that carried both the SRK–SCR transgene and the mutant gene of interest were then assayed for SI. A gene would be inferred to function in SI if SRKb–SCRb plants homozygous for a mutation in this gene fail to exhibit the SI phenotype.

The following PR pathway mutations were tested: etr1-1 (Bleecker et al., 1988), ein2-1 (Guzmán and Ecker, 1990), npr1-1 (Cao et al., 1994), pad4-1 (Glazebrook et al., 1996), rar1-21 (Tornero et al., 2002), sgt1b (Tor et al., 2002), and eds1-1 (Parker et al., 1996). These mutations disrupt genes that function in all known types of pathogen-resistance pathways in A. thaliana, including gene-for-gene, salicylic-acid-dependent, ethylene- and jasmonic-acid-dependent, and induced systemic resistance pathways (Glazebrook, 2001). All of these mutations were generated in the Col accession, with the exception of eds1-1, which is in the Wassilewskija (Ws) accession. Initial crosses of these PR mutants were made to C24 plants carrying the A. lyrata SRKb–SCRb genes (Nasrallah et al., 2004), which exhibit stable SI. However, it was observed that the F1 generation plants resulting from the cross between self-incompatible C24 SRKb–SCRb plants and the Col PR mutant plants were very late-flowering when compared to either parent, even when grown under the long-day conditions known to hasten flowering in A. thaliana, a facultative long-day plant (Napp-Zinn, 1985). The very late-flowering phenotype of F1 plants of the C24×Col cross, as well as the highly divergent flowering time phenotypes of their F2 progenies, was previously reported to be caused by the combination of one copy of the late-flowering FRI allele from C24 and one copy of the late-flowering FLC allele from Col (Sanda and Amasino, 1995).

In order to avoid these and other potential secondary genetic effects that might affect interpretation of pollination phenotypes in inter-accession crosses, Col SRKb–SCRb plants were used in crosses to PR pathway mutants, since all but one of these mutants were generated in the Col background. The exception, eds1-1, is in theWs accession, which, like Col, expresses transient SI when transformed with both SRKb and SCRb genes (Nasrallah et al., 2004). Therefore, crosses between Ws and Col were expected to cause very little, if any, genetic variance, at least with regard to SI.

Each PR mutant was used as the female parent in crosses to Col SRKb–SCRb to ensure that any antibiotic (kanamy-cin, in this case)-resistant progeny would have resulted from successful crosses to the SRKb–SCRb parent rather than from spontaneous selfing of the SRKb–SCRb parent. Kanamycin-resistant F1 and F2 progeny plants were confirmed to carry the SRKb–SCRb transgenes by amplification of genomic DNA using SRKb- and SCRb-specific primers as well as kanamycin-specific primers (see Supplementary Table S1 at JXB online). The presence or absence of the PR mutant alleles was also assessed by amplification of the same genomic DNA using dCAPS marker primers (see Supplementary Table S1 at JXB online) designed using dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html; Neff et al., 2002), or in the case of sgt1b, gene-specific primers.

To evaluate the effect of PR mutations on SI, self-pollination assays were performed on stigmas of 1-stage floral buds, in which strong SI is expressed in SRKb–SCRb transformants of both Col and Ws. In the case of the dominant etr1-1 mutation, phenotypic analysis was performed on F1 plants. In the case of the remaining mutant alleles, all of which are recessive, self-pollination phenotypes were assessed in F2 plants homozygous for these mutant alleles and having at least one copy of the
SRKb–SCRb transgenes. For several plants, phenotypes were confirmed in the subsequent generation (F2 generation plants for etr1-1 and F3 generation plants for the other mutant alleles). A pollination was considered compatible if numerous pollen tubes (equivalent to the numbers obtained upon self-pollinations of wild-type and mutant non-transgenic plants) were observed in the transmitting tract of a pollinated pistil, or incompatible if 10 or fewer pollen tubes (equivalent to numbers obtained upon self-pollination of wild-type SRKb–SCRb transformants), were observed (Fig. 5). For each pollination assay, wild-type untransformed Col and wild-type transgenic Col SRKb–SCRb plants were used as controls.

Table 1 summarizes the pollination results from SRKb–SCRb plants homozygous for the recessive ein2-1, npr1-1, pad4-1, rar1-21, sgt1b, or eds1-1 alleles or SRKb–SCRb plants carrying at least one copy of the dominant etr1-1 allele; all of these plants were found to exhibit an SI response that was as robust and stable as that of the SRKb–SCRb parent plant (Table 1; Fig. 5). The observation that the SI response was not even weakened by the PR mutations strongly suggests that the ETR1, EIN2, NPR1, PAD4, RAR1, SGT1b, and EDS1 genes are not required for SI. It should be noted, however, that the results do not exclude the possibility that, for at least some of these genes, a related paralogue might function in SI. Simultaneous down-regulation of more than one member of the same gene family by gene-silencing methods such as RNA interference may help address this possibility. It is also possible that some of these PR genes might function in SI, but inactivation of individual genes may not be sufficient for abolishing or even weakening the SI response due to cross-talk among various pathways. Indeed, knocking out one PR pathway is not always sufficient to abolish resistance to pathogens (Zipfel et al., 2004). For example, NPR1, PAD4, and EDS1 are not universally required for all types of pathogen resistance: they are required for salicylic-acid-mediated resistance but, although they are up-regulated upon flagellin induction, they are not required for flagellin-induced basal resistance. Combining several mutations in SRK–SCR plants is required to test these possibilities.

Conclusions and perspectives

The transgenic self-incompatible A. thaliana SRK–SCR model is beginning to fulfill its promise as an excellent platform for investigating SI in the Brassicaceae. The transfer of several different SI specificities into A. thaliana, combined with the ability to generate large numbers of transformants and to perform efficient map-based cloning of induced and spontaneous mutations in this model species,

Table 1. Pollination phenotypes of SRKb–SCRb plants homozygous for PR pathway mutations

<table>
<thead>
<tr>
<th>PR mutant w/ SRKb–SCRb</th>
<th>Pollination phenotype</th>
<th>Total no. plants tested</th>
<th>No. families (No. plants/family)</th>
<th>No. sub-families (No. plants/sub-family)</th>
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<tr>
<td>etr1-1</td>
<td>SI</td>
<td>7</td>
<td>1 (7)</td>
<td>3 (3, 3, 1)</td>
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<tr>
<td>ein2-1</td>
<td>SI</td>
<td>8</td>
<td>1 (8)</td>
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<tr>
<td>npr1-1</td>
<td>SI</td>
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<td>2 (7, 6)</td>
<td>1 (7), 1 (6)</td>
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<td>SI</td>
<td>4</td>
<td>1 (4)</td>
<td>2 (3, 1)</td>
</tr>
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<td>12</td>
<td>2 (8, 4)</td>
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</tr>
<tr>
<td>eds1-1</td>
<td>SI d</td>
<td>9</td>
<td>1 (9)</td>
<td>1 (9)</td>
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a Pollination phenotype was determined by performing manual self-pollinations on stigmas of 1-stage floral buds, treating the buds with aniline blue to stain callose, and counting pollen tubes visualized with epifluorescence microscopy (see Fig. 5). For each plant, the functionality of stigma and pollen was confirmed via pollination assays using wild-type Col pollen and wild-type Col stigmas, respectively.

b For the vast majority of plants (47/63; ~75%), two stigmas were tested for pollination phenotype via self-pollination assays. For the remaining plants, one, three, or four stigmas were tested.

c For each PR mutation, one or two separate homozygous mutant plants were crossed to Col SRKb–SCRb to generate F1 plants, the latter of which were allowed to self-fertilize and produce F2 plants. In most cases, F2 plants were allowed to self-fertilize and produce F3 plants. A ‘family’ is defined as a group of plants that were generated from a single, initial crossing event, and a ‘sub-family’ is defined as the plants generated from one plant of a single family.

d One F2 SRKb–SCRb eds1-1 homozygous plant was scored as self-compatible in one pollination assay using two stigmas. However, this result seems to have been due to a failed pollination assay and not to the eds1-1 mutation, since the F3 progeny plants derived from this plant were all SI.
has allowed us to address longstanding issues in SI research. Analysis of SRK–SCR transfectants has elucidated aspects of the evolution of self-fertility in A. thaliana, and, more generally, the physiological and morphological changes that are associated with transitions between the out-crossing and inbreeding modes of mating in the Brassicaceae. In planta functional assays of site-directed SRK variants has pinpointed domains and amino acids responsible for SRK specificity. Chemical mutagenesis of SRK–SCR plants and analysis of the cryptic natural variation for expression of SI that differentiates various accessions have identified novel genes required for SI. In addition, the wealth of insertional and point mutations available in A. thaliana has allowed the use of a candidate-gene approach to assess the role in SI of the A. thaliana genes most closely related to genes previously reported to function in the SI response of Brassica and to explore potential molecular overlaps between SI and plant disease resistance pathways. None of the genes tested by this approach were found to be required for SI, a result that was unexpectedly corroborated in the case of ARC1 in B. rapa. Additional studies involving gene-silencing approaches, which are easily performed in the A. thaliana model, are required to exclude the possibility of genetic redundancy and provide definitive evidence for or against the involvement of specific genes in SI. There is every expectation that future molecular genetic studies of the A. thaliana SRK–SCR transgenic plants will continue to provide important insight into the mechanism and evolution of SI.

Supplementary data
Supplementary data are available at JXB online.

Supplementary Table S1. Primers used in analysis of SRK–SCR plants carrying PR pathway mutations.

Acknowledgements
We thank Jian Hua for the etr1-1, ein2-1, npr1-1, pad4-1, and edsl-1 mutant seed and the NPR1, PAD4, and EDS1 dCAPS marker primers, Jeff Dangl for the rarl-21 mutant seed, Jane Parker for the sgt1b mutant seed, Nathan Boggs for the SRKb- and SCRb-specific primers, Titima Tantikanjana for the kanamycin-specific primers, and Steve Tanksley for the use of his epifluorescence microscope and imaging system. T-DNA mutant seed was obtained from the Arabidopsis Biological Resource Centre at The Ohio State University (Columbus, OH). PL analysed the A. thaliana PUB17 and APK1b insertion mutants, JBN analysed ARC1 in B. rapa, and ACR analysed the PR pathway mutants. Research in the Nasrallah laboratory is supported by grants from the US National Science Foundation and the US Department of Agriculture.

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