Copyright © 2009 by the Genetics Society of America
DOI: 10.1534/genetics.109.102442

Expression of Distinct Self-Incompatibility Specificities in *Arabidopsis thaliana*

Nathan A. Boggs,* Kathleen G. Dwyer,† Paurush Shah,† Amanda A. McCulloch,† Jesper Bechsgaard,‡ Mikkel H. Schierup,‡ Mikhail E. Nasrallah* and June B. Nasrallah*,[1]

**Department of Plant Biology, Cornell University, Ithaca, New York 14853, †Department of Biology, University of Scranton, Scranton, Pennsylvania 18508 and ‡Bioinformatics Research Center, University of Aarhus, DK-8000 Aarhus, Denmark*

Manuscript received March 4, 2009
Accepted for publication May 28, 2009

ABSTRACT

The interplay of balancing selection within a species and rapid gene evolution between species can confound our ability to determine the functional equivalence of interspecific and intergeneric pairs of alleles underlying reproduction. In crucifer plants, mating specificity in the barrier to self-fertilization called self-incompatibility (SI) is controlled by allele-specific interactions between two highly polymorphic and co-evolving proteins, the *S*-locus receptor kinase (SRK) and its *S*-locus cysteine rich (SCR) ligand. These proteins have diversified both within and between species such that it is often difficult to determine from sequence information alone if they encode the same or different SI specificity. The self-fertile *Arabidopsis thaliana* was derived from an obligate outbreeding ancestor by loss of self-incompatibility, often in conjunction with inactivation of SRK or SCR. Nevertheless, some accessions of *A. thaliana* can express self-incompatibility upon transformation with an *SRK–SCR* gene pair isolated from its self-incompatible close relative *A. lyrata*. Here we show that several additional and highly diverged *SRK/SCR* genes from *A. lyrata* and another crucifer plant, *Capsella grandiflora*, confer self-incompatibility in *A. thaliana*, either as intact genes isolated from genomic libraries or after manipulation to generate chimeric fusions. We describe how the use of this newly developed chimeric protein strategy has allowed us to test the functional equivalence of *SRK/SCR* gene pairs from different taxa and to assay the functionality of endogenous *A. thaliana SRK* and *SCR* sequences.

MATING reactions in plants, fungi, and animals are strongly influenced by molecular recognition machineries that act as gauges of genetic relatedness (Brown and Casselton 2001; Nasrallah 2005; Yamazaki and Beauchamp 2007). Many plants with hermaphroditic flowers have evolved inbreeding avoidance mechanisms, known as self-incompatibility (SI) systems. These systems are based on the ability of the female reproductive apparatus (the pistil) to discriminate among genetically distinct pollen grains, resulting in the failure of self-pollination despite functional female and male reproductive structures. In the Brassicaceae (crucifers), specific recognition of pollen by the epidermal cells of the stigma (a structure located at the tip of the pistil) is controlled by haplotypes of the *S* locus, and activation of the SI response leading to inhibition of pollen tube growth occurs if pollen and stigma are derived from plants that express the same *S*-locus haplotype (*S* haplotype). Within self-incompatible crucifer species, the number of *S* haplotypes and corresponding SI specificities is usually high, with >50 reported in some species (Watanabe *et al.* 2000), and SI dictates that self-incompatible plants are typically heterozygous and carry two *S* haplotypes. Each *S* haplotype is composed of two highly polymorphic genes that are the determinants of SI specificity in stigma and pollen (Stein *et al.* 1991; Schopfer *et al.* 1999). The *S*-locus receptor kinase (SRK) gene encodes a single-pass transmembrane serine/threonine kinase localized on the surface of stigma epidermal cells, and the *S*-locus cysteine-rich protein (*SCR*) gene encodes a small peptide localized in the pollen coat. SCR is the ligand for SRK and will bind to the extracellular domain of SRK (hereafter eSRK) only if both proteins are encoded by the same *S*-locus haplotype (Kachroo *et al.* 2001; Takayama *et al.* 2001; Chookajorn *et al.* 2004). The binding of SCR to its cognate eSRK triggers an intracellular phosphorylation cascade that results in pollen rejection by a poorly understood mechanism.

A mechanistic understanding of the recognition phase of SI requires detailed structure–function analyses of SRK and SCR aimed at identifying the amino acid residues that determine their allele-specific interaction and explaining the puzzling dominance/recessive interactions exhibited by different *SRK* alleles in the

Supporting information is available online at: http://www.genetics.org/cgi/content/full/genetics.109.102442/DC1.

[1]*Corresponding author:* Department of Plant Biology, 412 Mann Library Bldg., Cornell University, Ithaca, NY 14853. E-mail: jbn2@cornell.edu

heterozygous stigmas of self-incompatible plants (Hatakeyama et al. 2001; Mable et al. 2003; Prigoda et al. 2005). Such structure–function studies require an experimental system that allows efficient *in vivo* functional analysis of large numbers of SRK and SCR sequence variants generated *in vitro* by site-directed mutagenesis or domain swapping between proteins that determine different SI specificities. The recent transfer of the SI trait into *Arabidopsis thaliana* has established this species as a model organism for mechanistic and evolutionary studies of mating systems in crucifers (Nasrallah et al. 2002, 2004). However, to date, only one SI specificity, that which is determined by the *Sb* haplotype of *A. lyrata*, has been successfully introduced into *A. thaliana* and shown to alter the plant's mating reaction from strict autogamy to full SI. To exploit fully the *A. thaliana* transgenic SI model, additional *S* haplotypes must be introduced into this species. In addition to facilitating mechanistic studies of the SRK–SCR interaction and dominance relationships, the expression of multiple SI specificities in *A. thaliana* promises to shed light on processes underlying the diversification of *SRK* and *SCR* genes. For example, expression in *A. thaliana* of SI specificities derived from different crucifer species will allow direct assays of the functional equivalence or nonequivalence of the corresponding *S* haplotypes, an issue that is difficult to resolve on the basis of sequence information alone.

Although conceptually simple, expressing different SI specificities by transformation with different *SRK/SCR* gene pairs is not a straightforward proposition. Difficulties stem largely from the availability of appropriate cloned *SRK/SCR* variants for use in transformation experiments. A large number of *SRK/SCR* gene pairs are available from Brassica species as a result of extensive and long-standing studies of SI. However, attempts to restore SI in transgenic *A. thaliana* using Brassica *S*-locus genes had met with failure (Bi et al. 2000; J. B. Nasrallah, unpublished data), possibly because of the inability of Brassica SRKs to interact productively with *A. thaliana* components of the SI signal transduction pathway. In the past few years, studies of SI were initiated in self-incompatible species more closely related to *A. thaliana*, such as *A. lyrata*, *A. halleri*, and *Capsella grandiflora*. However, with a few exceptions, these studies produced only partial *SRK* and *SCR* sequences amplified from genomic DNA (Schierup et al. 2001; Prigoda et al. 2005; Bechsgaard et al. 2006; Paetsch et al. 2006). The challenging task of cloning the very highly polymorphic *SCR* sequences and complete *SRK* and *SCR* genes, which requires genomic library construction and in many cases chromosome walking, has only been accomplished for two *S* haplotypes of *A. lyrata*, *Sb* (hereafter *AlSb*, which was used in previous transformation studies (Nasrallah et al. 2002, 2004), and *Sa* (*AlSa*; Kusaba et al. 2001), and for the *S7* haplotype of *C. grandiflora* (*CgS7*; Nasrallah et al. 2007).

In this article, we report the isolation of two new *SRK/SCR* gene pairs from genomic libraries of *A. lyrata* and expression of the corresponding SI specificities in *A. thaliana*. We also describe a novel strategy for rapid and efficient transfer of several distinct SI specificities into *A. thaliana*, which only requires knowledge of the *eSRK* sequence and *SCR* second-exon sequences that encode the mature SCR protein.

MATERIALS AND METHODS

Our strategy to express different SI specificities in *A. thaliana* was to transform *A. thaliana* plants with two types of constructs: (1) intact *SRK* and *SCR* gene pairs, either previously described or newly isolated from genomic libraries; and (2) chimeric *SRK* and *SCR* genes, which circumvent the laborious task of constructing genomic libraries for isolating *SRK* and *SCR* alleles from different *S* haplotypes.

***S* haplotypes used in this study:** Several functional *S* haplotypes were used as sources of *SRK* and *SCR* genes. These include the *C. grandiflora S7* haplotype and five functionally distinct *S* haplotypes from *A. lyrata*: the *Sa* and *Sb* haplotypes described in Kusaba et al. 2001 [also named *S20* and *S13*, respectively, by Charlesworth et al. (2003)]; and the *S6* (*AlS6*), *S16* (*AlS16*), *S25* (*AlS25*), and *S37* (*AlS37*) haplotypes (Charlesworth et al. 2003; Bechsgaard et al. 2006). Additionally, we used the nonfunctional *A. thaliana* Cape Verde Islands (Cvi-0) pseudo-*SB* (ΨSB) haplotype, which is the likely ortholog of the *AlS16* haplotype of *A. lyrata* (Bechsgaard et al. 2006).

Isolation of *SRK* and *SCR* genes from genomic libraries and construction of plant transformation vectors containing these genes: Genomic libraries were constructed in λDASH II (Stratagene; La Jolla, CA) from DNA isolated from *A. lyrata* plants containing the *S6* or *S25* haplotypes. The libraries were screened with a a single-copy probe derived from sequences 5′ of At4g21380 (*ARK3*) (Kusaba et al. 2001), a gene located at one flank of the *A. lyrata S* locus, often very close to *SRK*. Chromosome walking was then performed until an *SCR*-like sequence containing eight cysteines was identified. The *SRK* and *SCR* genes were sequenced at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY).

To construct plant transformation plasmids, the *AlSRK6* and *AlSCR6* were inserted together into the pCAMBIA1300 vector. In the case of *AlSRK25* and *AlSCR25*, fragments containing these genes were cloned separately into the pCAMBIA1300 vector, and introduced individually into *A. thaliana*, because repeated attempts to construct a plasmid containing both genes were unsuccessful. The *C. grandiflora CgSRK7* and *CgSCR7* genes had previously been isolated from a genomic library on one λ-clone containing an ∼13-kb insert (Nasrallah et al. 2007), and this insert was excised and cloned into the plant transformation vector pART27 (Gleave 1992).

Isolation of the complete *SCR16* sequence from the *A. lyrata S16* haplotype: Previous analysis of the *A. lyrata S16* and *S37* haplotypes had identified *SRK16*, *SRK37*, and *SCR37* sequences (Bechsgaard et al. 2006; Boggs et al. 2009), but not the *SCR16* exon-2 sequence. To isolate the complete *SCR16* sequence, we devised a polymerase chain reaction (PCR)-based strategy using genomic *S16* DNA as follows.

AlSRK16 shares 92% amino acid sequence similarity with its ortholog, the *A. thaliana* Cvi-0 Ψ*SRKB* allele. Furthermore, the Ψ*SRKB* and Ψ*SCRB* genes in Cvi-0 are oriented in a convergent manner and separated by very little intervening sequence

(Shimizu *et al.* 2008). On the basis of these results and on the assumption that the *AlS16* haplotype would have the same organization as the Cvi-0 Ψ*SB* haplotype, we designed PCR primers on the basis of the Ψ*SB* locus and used these primers to amplify a fragment starting from the second exon of *AlSCR16*, into the 3′ untranslated region of *AlSCR16* and to the 3′ end of *AlSRK16*. Amplified fragments containing *AlSCR16* sequences were cloned into pGemT-easy (Promega; Madison, WI), and inserts were sequenced.

Analysis of *eSRK* and *SCR* sequences: The sequences of the following genes (with accession numbers) were reported previously (Kusaba *et al.* 2001; Bechsgaard *et al.* 2006; Nasrallah *et al.* 2007): *AlSRKa* (AB052755) and *AlSCRa* (AB052753); *AlSRKb* (AB052756) and *AlSCRb* (AB052754); *CgSRK7* (EF530735) and *CgSCR7* (EF530736); *AlSRK16* (DQ520283); and *AlSRK37* (DQ520289) and *AlSCR37* (FJ752546). Sequences derived in this study have been deposited in GenBank under the following accession nos.: *AlSRK6*: GQ351354; *AlSCR6*: GQ351356; *AlSRK25*: GQ351355; *AlSCR25*: GQ351357; and *AlSCR16*: GQ351353. Amino acid alignments of the SRK extracellular domains and the mature SCR variants were performed using the sequence distance and tree function of MegAlign, a program in the Lasergene suite of applications (DNASTAR, Madison, WI; supporting information, Figure S1).

Construction of *SRK* and *SCR* chimeric genes: *SRK chimeric genes:* The *SRK* transcriptional unit consists of seven exons, the first of which encodes the signal peptide and the entire extracellular domain of SRK. Chimeric *SRK* genes were assembled in a pCAMBIA1300 derivative containing the *AtS1* promoter, which drives expression specifically in the stigma epidermis (Dwyer *et al.* 1992), fused to the *AlSRKb* transcriptional unit from its initiating methionine codon and including the six introns and 3′-UTR of the gene. To generate this derivative plasmid,the major part of *AlSRKb* exon 1 was amplified from the start codon (using a primer that incorporated a *Kpn*I restriction site) to the endogenous *Sac*I site located 1230 bp after the start codon and cloned as a *Kpn*I–*Sac*I fragment into pC*AtS1pr*. The rest of the *AlSRKb* gene including introns and 3′-UTR, isolated as a *Sac*I–*Xba*I fragment from a genomic clone containing *AlSRKb*, was then inserted downstream of *AleSRKb* exon-1 sequences. The resulting *AtS1::AlSRKb* cassette was cloned into pCAMBIA1300 as an *Eco*RI–*Xba*I fragment. The resulting plasmid was subsequently used as a backbone for replacement of the *eSRKb*-containing *Kpn*I–*Sac*I fragment with *eSRK* sequences amplified from functional *A. lyrata* and *C. grandiflora SRK* alleles and from the *A. thaliana* Ψ*SB* haplotype using specific forward and reverse primers that incorporated *Kpn*I and *Sac*I restriction sites, respectively.

Construction of the *AlSRKa::AlSRKb* and *CgSRK7::AlSRKb* chimeric genes was achieved by amplification of *eSRK* and *SCR* sequences from available genomic clones. In the case of *AlSRK16* and *AlSRK37*, for which genomic clones are not available, *SRK* chimeras were constructed using *eSRK* sequences amplified from genomic DNA of *A. lyrata* plants harboring each of these *S* haplotypes.

SCR chimeric genes: SCR genes consist of two exons, the first encoding the signal peptide and the second encoding the mature SCR protein. While the *A. lyrata SCRa* gene could be excised from a previously isolated *Sa* BAC clone (Kusaba *et al.* 2001) and cloned into the plant transformation vector pBIN-PLUS (van Engelen *et al.* 1995), constructs for expression of other *SCR* alleles corresponding to the *SRK*s described in the previous section, namely *A. lyrata SCR16*, *SCR37*, and *SCR6*, and *C. grandiflora SCR7*, were prepared as follows. An *SCR* expression cassette was constructed in pCAMBIA1300 using the promoter of the *Brassica rapa SCR8* gene (Schopfer *et al.* 1999) and the octopine synthase (OCS) terminator, between which *SCR* coding regions consisting of the *AlSCRb* signal peptide fused to *SCR* exon-2 sequences and generated by recombinant PCR were inserted.

Two additional *SCR* expression constructs based on the Ψ*SCRB* allele of the *A. thaliana* Cvi-0 accession were also prepared using the strategy described above: one construct was designed to express the SCRB protein predicted from the Ψ*SCRB* sequence, and another construct was designed to express a modified version of SCRB in which an extra cysteine residue located at position 10 of the mature peptide was replaced by the phenylalanine codon found in the corresponding site of the orthologous *SCR16* gene (see alignments in supporting information). Alignments of the predicted amino acid sequences of the SCR fusion proteins are shown in Figure S2.

Plant transformation and analysis of transgenic plants: *SRK* and *SCR* constructs were transformed into Agrobacterium strain GV3101 (Koncz and Schell 1986) and subsequently used to transform *A. thaliana* C24 plants, which express robust and developmentally stable SI upon transformation with *SRKb–SCRb* (Nasrallah *et al.* 2004), or Col-0 plants in the case of the *AlSCRa* construct, by the floral dip method (Zhang *et al.* 2006). The independent derivation of primary (T1) transformants and number of T-DNA integration were assessed by DNA gel blot analysis using probes derived either from the hygromycin- or kanamycin-resistance gene. Transgene expression was assessed in *CgS7* transformants by RT–PCR (reverse transcription–polymerase chain reaction) of total RNA isolated from stigmas as described previously (Nasrallah *et al.* 2002).

For each *SRK* and *SCR* construct, pollination responses were tested in an average of 12 plants per construct. Additionally, to confirm that the various *SRK/SCR* variants used in this study encode distinct SI specificities in transgenic *A. thaliana*, reciprocal cross-pollinations among the various *SRK* and *SCR* T2 plants transformants were performed in all combinations. In all cases, two sets of control pollinations were also performed: in one set, stigmas expressing each of the *SRK* chimeras were pollinated with pollen from untransformed *A. thaliana* plants, and in another set, pollen expressing each of the *SCR* chimeras was used to pollinate stigmas of untransformed *A. thaliana* plants. For all chimeric *SRK*- and *SCR*-expressing transformants, these control pollinations produced the expected large number of pollen tubes.

All of these pollination assays used pollen-free stigmas collected from buds just before anthesis, when the stigmas were receptive to pollen but before the pollen grains matured and were released from the anthers. Stigmas were manually pollinated, incubated for 2 hr, and subsequently stained with aniline blue and processed for observation by epifluorescence microscopy, as previously described (Kho and Baer 1968). Under these conditions, a strong incompatibility response is manifested by the absence or near-absence of pollen tubes ($<$5 tubes per pollinated stigma). A compatible response is evident by the growth of numerous pollen tubes ($>$50 pollen tubes per pollinated stigma).

Pollination assays were typically performed on at least five buds and repeated on at least two separate dates. For any construct that did not confer an incompatibility response, extensive pollination assays were performed on multiple stigmas with repetitions on separate dates. For example, for the *CgS7* construct, seven independent transformants were assessed by pollination assays at least four times on 2 separate days, and four of those lines were analyzed throughout stigma development by pollination assays in floral buds ranging from the immature stage to full maturity. Independent SRK- and SCR-expressing lines were tested in 12 independent T1 plants

on average and in the T2 generation (except for plants expressing AlSRKb, which were thoroughly tested in previous studies).

RESULTS

Isolation of novel *A. lyrata SRK/SCR* gene pairs that confer an incompatibility response in *A. thaliana*: Two *SRK/SCR* gene pairs (see sequence alignments in Figure S1) were isolated from genomic libraries constructed from plants harboring the *A. lyrata S6* and *S25* haplotypes. Figure 1 shows the organization of the *SRK* and *SCR* genes in the *AlS6* and *AlS25* haplotypes in comparison to the structure of the previously reported *AlSb*, *AlSa*, and *CgS7* haplotypes (Kusaba *et al.* 2001; Nasrallah *et al.* 2007). These comparisons highlight the extensive intraspecific structural heteromorphisms exhibited by *S* haplotypes, which are thought to contribute to reduced recombination in the region and maintenance of the tight genetic linkage of *SRK* and *SCR* (Boyes *et al.* 1997; Casselman *et al.* 2000; Nasrallah 2000). Indeed, the most structurally similar *S* haplotypes we analyzed are the *A. lyrata AlSa* and *C. grandiflora CgS7* haplotypes (Figure 1). In contrast, the *A. lyrata S* haplotypes differ from one another in gene content and organization. *AlSb*, but not the other *A. lyrata S* haplotypes, contains duplicated copies of *SCR* (Shimizu *et al.* 2004). Furthermore, the haplotypes differ drastically in the distances separating *SRK* from *SCR* and from markers that flank the *S* locus (in this case, the *ARK3* gene), as well as in the relative orientations of these genes. In particular, *SRK* can be separated from *ARK3* by as little as a few hundred base pairs as in *AlS6*, or by as much as 35 kb as in *AlSb*. Similarly, the interval between *SRK* and *SCR* varies from <1 kb in *AlS25* to ~22 kb in *AlSb*.

To determine if the newly isolated *AlSRK* and *AlSCR* genes, as well as the previously described *C. grandiflora CgSRK7* and *CgSCR7* genes, could confer an incompatibility response in *A. thaliana*, restriction fragments containing the *SRK* and *SCR* alleles were introduced into *A. thaliana* C24 plants. The *AlS6*- and *CgS7*-derived genes were introduced together on one plant transformation plasmid, but *AlSRK25* and *AlSCR25* were introduced individually on separate plasmids because plasmids containing both genes were unstable in bacteria (see materials and methods). Pollination assays of a minimum of 10 independent transformants per construct were performed by self-pollinating *AlSRK6-AlSCR6* and *CgSRK7-CgSCR7* transformants, or by pollinating the stigmas of *AlSRK25* transformants with pollen from *AlSCR25* transformants. Both the *AlS6*- and *AlS25*-derived genes conferred incompatibility in transgenic *A. thaliana*. Inhibition of self pollen was observed in 6 of 12 *AlSRK6-AlSCR6* independent transformants analyzed, and these plants produced a negligible amount of seed (typically 0–5 seeds per plant). Similarly, the stigmas of 10 of 11 independent *AlSRK25*

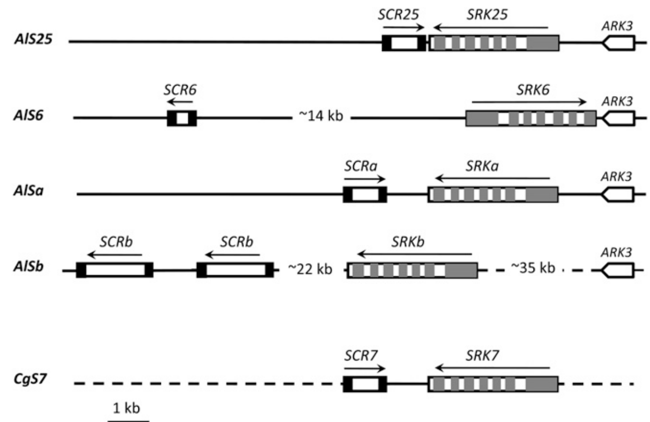


Figure 1.—Structure of the *S* haplotypes used in this study. The diagrams show the organization of the *SRK* and *SCR* genes in the *A. lyrata AlS25*, *AlS6*, *AlSa*, and *AlSb* haplotypes and the *C. grandiflora CgS7* haplotype. The structures of the *AlSa* and *AlSb* haplotypes were determined in Kusaba *et al.* (2001) and that of the *CgS7* in (Nasrallah *et al.* 2007). Note the drastic differences in the arrangements of the genes relative to each other and to the flanking gene *ARK3*. Intergenic distances that exceed 1 kb are indicated. The dashed lines in *AlSb* between *ARK3* and *SRKb* and between *SRKb* and *SCRb* indicate that the distances are not drawn to scale. The dashed lines flanking *SRK7* and *SCR7* in the *CgS7* haplotype indicate that these sequences have not been cloned. The *ARK3* gene is not drawn to scale.

transformants inhibited pollen from 7 of 10 independent *AlSCR25* transformants. In contrast, the *CgSRK7-CgSCR7* construct failed to confer SI: all 40 independent transformants analyzed produced wild-type levels of seed set, and self-pollination assays of seven of these plants failed to show inhibition of pollen tube development at any stage of stigma development.

Introduction of SI specificities into *A. thaliana* by expressing chimeric *SRK* and *SCR* genes: We used a chimeric *SRK* gene strategy designed to express, under control of the stigma-specific *AtS1* promoter (Dwyer *et al.* 1992), a fusion protein, designated eSRKx:AlSRKb, in which a particular eSRK (minus the last 23 amino acids) is fused to the last 23 amino acids of AleSRKb followed by the AlSRKb transmembrane and kinase domains (Figure 2A). In parallel, and in cases where *SCR* genomic clones were not available, we used chimeric intronless *SCR* genes, designed to express, under control of a *B. rapa SCR8* promoter (Schopfer *et al.* 1999), a mature SCR variant fused to the AlSCRb signal peptide (Figure 2B).

The effectiveness of the chimeric SRK expression system was first confirmed for two variants known to confer an incompatibility response in *A. thaliana*. Specifically, expression of reconstituted *AtS1pr::AleSRKb:AlSRKb* and *AtS1pr::AleSRK25:AlSRKb* chimeric genes conferred on transgenic stigmas the ability to inhibit pollen expressing *AlSCRb* and *AlSCR25*, respectively. The SRK and SCR expression system was subsequently used to test the functionality of *SRK* and *SCR* variants

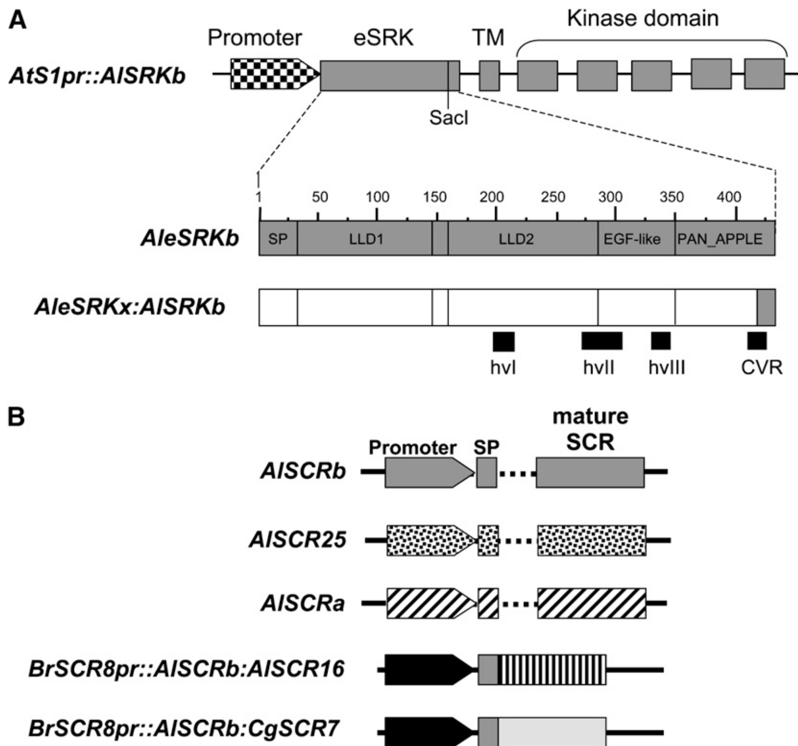


FIGURE 2.—Diagrams of the chimeric *SRK* genes and the *SCR* constructs used in this study. (A) *SRK* chimeric genes. At top is a schematic of the *AtS1pr::AlSRKb* backbone used for construction of *SRK* chimeric fusions, showing the *AtS1* promoter (checkered arrowhead) driving the *AlSRKb* transcriptional unit with its seven exons, which encode the *AlSRKb* extracellular domain (eSRK; exon 1), the transmembrane domain (TM; exon 2), and the kinase domain (exons 3–7), followed by its native 3′ untranslated sequences (not shown). The unique *Sac*I restriction site used for construction of chimeras is shown toward the 3′ end of the eSRK. Below are shown the structures of the AleSRKb (with numbers indicating amino acids) and of the eSRK of AleSRKbx:AlSRKb fusions. The vertical lines within the eSRKs delineate predicted structural subdomains in the eSRK (NAITHANI *et al.* 2007): SP, signal peptide; LLD1 and LLD2, lectin-like domains 1 and 2; EGF-like, epidermal growth factor-like domain; and PAN_APPLE domain. The location of hypervariable regions discussed in previous studies (KUSABA *et al.* 2001; NAITHANI *et al.* 2007) are indicated below the diagrams and correspond to the following regions in AlSRKb: 204–219 (hvI), 269–304 (hvII), 326–340 (hvIII), and 410–422 (C-terminal variable region/CVR). The unique SacI site corresponds to position 410 in the amino acid sequence, and the region C terminal to this position (shaded and spanning residues 411–434) was derived from AlSRKb in all AleSRKx:AlSRKb fusions. (B) *SCR* constructs. The *AlSCRb, AlSCR25*, and *AlSCRa* constructs were derived from λ-genomic fragments and contain the native *SCR* promoter, the two exons separated by an intron of variable length (dashed lines), and terminator. The intronless *BrSCR8::AlSCRb:AlSCR16* and *BrSCR8::AlSCRb:AlSCR7* constructs contain the *B. rapa SCR8* promoter (SCHOPFER *et al.* 1999) followed by the signal peptide sequence of the *AlSCRb* gene (shaded box) fused to exon-2 sequences of *AlSCR16* and *CgSCR7* and the OCS terminator (not shown). *SCR* constructs are not drawn to scale.

that had not been previously assayed in *A. thaliana*. In particular, we tested *A. lyrata* variants derived from the *AlSa* haplotype, for which *SRKa* and *SCRa* genomic clones are available (KUSABA *et al.* 2001) but attempts to construct *SRKa* transformation vectors had not been successful (J. B. NASRALLAH, unpublished data), as well as those derived from the *AlS16* and *AlS37* haplotypes (KUSABA *et al.* 2001; BECHSGAARD *et al.* 2006), for which genomic clones are not available. Furthermore, the expression system was used to determine if chimeric *SRK* and *SCR* genes could confer incompatibility in cases where native genes were ineffective, as in the case of the *C. grandiflora CgSRK7* and *CgSCR7* genes.

Each of the chimeric *SRK* and *SCR* genes was introduced individually into *A. thaliana* plants, and the stigmas of plants transformed with a particular *AtS1pr::eSRKx:AlSRKb* gene were tested by application of pollen from plants transformed with the cognate *SCR* construct. With the exception of pollinations between *AtS1pr::eSRK37:AlSRKb* and *BrSCR8::AlSCRb:SCR37* transformants, which were compatible, all other pollinations exhibited the expected incompatibility responses. As shown in Figure 3, stigmas expressing a particular eSRK:AlSRKb fusion inhibited pollen from plants expressing the eSRK's cognate SCR but not their own wild-type pollen. Similarly, pollen from plants expressing a particular SCR variant was inhibited on the stigmas of plants expressing the cognate eSRK: AlSRKb fusion, but produced many pollen tubes on wild-type stigmas (Figure 3). For each of the e*SRK* and *SCR* constructs tested, T1 plants were identified that exhibited a robust expression of the corresponding SI specificity and carried a single integration of the transgene. The T2 progenies of these plants recapitulated the pollination phenotype of T1 plants and were used for subsequent cross-pollination assays as described below.

Expression of six distinct SI specificities in *A. thaliana*: Although each of the *AlSb*-, *AlSa*-, *AlS6*-, *AlS16*-, *AlS25*-, and *CgS7*-derived *SRK* and *SCR* alleles conferred incompatibility in transgenic plants, it was important to determine if they bestowed distinct SI specificities in the *A. thaliana* genomic context. This is particularly critical in the case of the *AlSa* and *CgS7* haplotypes, which are more similar to each other than to other *A. lyrata* and *C. grandiflora S* haplotypes, both in overall organization and sequence of their *SRK* and *SCR* genes (NASRALLAH *et al.* 2007). Indeed, despite the divergence of *A. lyrata* and *C. grandiflora*, which are thought to have shared a common ancestor ∼10 million years ago, the exons of *AlSRKa* and *CgSRK7* on the one hand and of *AlSCRa* and *CgSCR7* on the other hand

♀ \ ♂	a	25	7	16	6	b
a	0 (6/8)	+++	+++	+++	+++	+++
25	+++	0 (9/13)	+++	+++	+++	+++
7	+++	+++	0 (8/16)	+++	+++	+++
16	+++	+++	+++	0 (11/16)	+++	+++
6	+++	+++	+++	+++	0 (6/12)	+++
b	+++	+++	+++	+++	+++	0 (1/2)

FIGURE 3.—Pollination phenotypes of *A. thaliana* plants transformed with the various e*SRK:AlSRKb* fusion constructs. The stigmas of first- (T1) and second- (T2) generation transgenic plants expressing each of the five SRK fusions and the *AlSRKb* gene were pollinated with pollen from plants expressing the cognate SCR and other SCRs. *SRK* variants are indicated in the column below the female symbol: *a*, *AleSRKa:AlSRKb*; *25*, *AleSRK25:AlSRKb*; *7*, *CgeSRK7:AlSRKb*; *16*, *AleSRK16:AlSRKb*; *6*, native *AlSRK6*; *b*, native *AlSRKb*. *SCR* variants are indicated in the row to the right of the male symbol: *a*, native *AlSCRa*; *25*, native *AlSCR25*; *7*, *AlSCRb:CgSCR7*; *16*, *AlSCRb:AlSCR16*; *6*, native *AlSCR6*; *b*, native *AlSCRb*. Ratios in parentheses indicate the number of T1 plants that expressed an incompatibility response toward pollen expressing cognate SCR over the total number of primary transformants analyzed. *AlS6* and *AlSb* transformants were tested by self-pollination because they expressed both *SRK* and *SCR* genes. Pollen of *AlSCR25* transformants was tested on the stigmas of plants expressing the AleSRK25:AlSRKb fusion and of plants expressing native AlSRK25. 0, an incompatible response (typically <5 pollen tubes per pollinated stigma); +++, a compatible response (typically >50 pollen tubes per pollinated stigma).

share an average of ~86 and 74% amino acid sequence identity, respectively. This degree of sequence identity is similar to the 88 and 71% amino acid identity shared by *SRK* and *SCR* alleles from functionally equivalent *S* loci identified in studies of the very closely related *B. rapa* and *B. oleracea* or Brassica and its sister genus Raphanus (KUSABA *et al.* 2001; KIMURA *et al.* 2002; SATO *et al.* 2003, 2004, 2006). Therefore, it is possible that the *AlSa* and *CgS7* haplotypes might have been derived from a common ancestral haplotype and could be functionally equivalent.

To determine if the various *SRK/SCR* variants used in this study encode distinct SI specificities in transgenic *A. thaliana*, reciprocal cross-pollinations among *SRK* and *SCR* T2 transformants were performed. As shown in Figure 3, in all cases, transformants expressing a particular eSRKx:AlSRKb fusion inhibited pollen expressing the cognate SCR but not pollen expressing any one of the other five SCR variants tested. Conversely, pollen of transformants expressing one SCR variant was inhibited on stigmas expressing the cognate eSRK:AlSRKb fusion, but not on stigmas of plants expressing an independently derived eSRK:AlSRKb fusion. This pattern was observed not only among transformants expressing *A. lyrata*-derived variants, but also for transformants expressing the *A. lyrata Sa*- and *C. grandiflora S7*-derived genes. This result demonstrates that the *AlSRKa/AlSCRa* and *CgSRK7/CgSCR7* gene pairs confer distinct SI recognition specificitites in *A. thaliana*. Interestingly, reciprocal pollinations of *A. thaliana* plants transformed with the *CgS7* genomic fragment containing the native *CgSRK7* and *CgSCR7* genes with plants expressing the *AtS1pr::CgeSRK7:AlSRKb* and *BrSCR8::AlSCRb:CgSCR7* chimeric genes demonstrated that the pollen of the *CgS7* transformants expressed the *S7* specificity (*i.e.*, it was inhibited on the stigmas of *AtS1pr::CgeSRK7:AlSRKb* transformants), while their stigmas did not (*i.e.*, they did not inhibit pollen from *BrSCR8::AlSCRb:CgSCR7*). Therefore, the lack of SI in *CgS7* transformants was due to the inability of the native *CgSRK7* gene to function in *A. thaliana*. The failure of this gene to confer SI was not due to lack of expression, however. Indeed, there was no significant difference between *CgSRK7* transcript levels in transgenic *A. thaliana CgS7* stigmas and *C. grandiflora S7* stigmas (Figure S3).

Attempts to reconstitute functional alleles from *A. thaliana SRK* and *SCR* pseudogenes: The chimeric gene expression system we developed allowed us to assess the extent of decay suffered by the nonfunctional Ψ*S* locus of the *A. thaliana* Cvi-0 accession. The Ψ*SB* haplotype was previously shown to contain a Ψ*SRK* allele that encodes a truncated open reading frame containing a full-length *eSRK* and terminating at the end of exon 2 (KUSABA *et al.* 2001; SHIMIZU *et al.* 2004) and a Ψ*SCRB* allele that encodes an apparently intact open reading frame, which had been suggested to have retained functionality. However, inspection of amino acid sequences of ΨSCRB with AlSCR16, AlSCRa, AlSCRb (SHIMIZU *et al.* 2008) and other SCRs used in this study (see Figure S1) shows that ΨSCRB is the only variant among these SCRs that contains an extra cysteine residue between the first and second of eight canonical cysteine residues (CHOOKAJORN *et al.* 2004). These eight conserved cysteines form four disulfide bridges critical for SCR structure and function, and it is possible that this additional cysteine might disrupt the configuration of the SCRB protein and thus affect its function.

The functionality in *A. thaliana* of the *AleSRK16:AlSRKb* and *AlSCRb:AlSCR16* chimeric genes, which are derived from the *A. lyrata* ortholog of Cvi-0 Ψ*SB*, provided us with the necessary tools to determine if the Ψ*SCRB or* Ψ*eSRKB* sequences of the Cvi-0 Ψ*SB* haplotype might have retained their mutual recognition or recognition of their *A. lyrata S16* orthologs. An attempt was made to "correct" the obvious mutations in the *A. thaliana* Ψ*SRKB* and Ψ*SCRB* sequences. We transformed *A. thaliana* with a construct containing a full-length chimeric *SRK* open reading frame in which

the Cvi-0 *eSRKB* sequence was fused to *AlSRKb* transmembrane and kinase domain sequences and with two Ψ*SCRB* chimeric constructs, one containing unmodified exon-2 sequences and another containing a modified exon 2 in which the extra cysteine residue was replaced with a phenylalanine residue as occurs at the equivalent position in *AlSCR16* (see Figure S2). None of the transformants exhibited an incompatibility response, either in reciprocal pollinations with plants transformed with the cognate "corrected" *BrSCR8::AlSCRb:AtSCRB* or *AtS1::AteSRKB:AlSRKb* genes, or with plants transformed with chimeric genes derived from their *A. lyrata SCR16* or *SRK16* orthologs.

DISCUSSION

This article describes the successful interspecific and intergeneric transfer of the self-incompatibility trait to *A. thaliana* by transformation with several newly isolated or previously described *SRK/SCR* allelic pairs from *A. lyrata* and *C. grandiflora*. This successful complementation was achieved using either the standard approach of transformation with intact *SRK* and *SCR* genes isolated from genomic libraries or the novel strategy of transformation with engineered genes designed to express chimeric proteins. Such a strategy is often used for analysis of receptor proteins in animals (*i.e.*, Bergwitz *et al.* 1996) but it has not been previously applied to the analysis of SRK/SCR function. Together, these approaches bring to six the number of SI specificities that have been transferred to *A. thaliana*. The five newly transferred specificities, *AlSa*, *AlS6*, *AlS16*, *AlS25*, and *CgS7*, differ in the relatedness of their SRKs and SCRs to each other and to AlSRKb/AlSCRb, the only SRK/SCR pair previously shown to confer SI in *A. thaliana*, ranging from 60 to 82% for SRKs and from 40 to 60% for SCRs (Figure S1). The observation that highly diverged SRK and SCR variants are functional in transgenic *A. thaliana* indicates that the *A. thaliana* pollen–stigma interface provides an adequate molecular environment for transfer of highly diverged SCRs from pollen to stigma, their transport across the stigma epidermal cell wall, and their binding to highly diverged cognate eSRKs.

The functionality of the majority of chimeric SRK molecules assayed here also demonstrates that highly diverged eSRKs can effect their specific recognition function when fused to the transmembrane and kinase domains of AlSRKb, which can differ by as much as 25% (AlSRKb *vs.* CgSRK7) from their native kinase domains. These results prove that specificity in the SRK–SCR interaction is solely determined by the eSRK domain (excluding the last 23 amino acids), with no contribution from the transmembrane domain, which has been reported to be required for high-affinity ligand binding *in vitro* (Shimosato *et al.* 2007) or the cytoplasmic juxtamembrane domain.

The fact that the *C. grandiflora S7* specificity could be expressed by transformation with the *CgeSRK7:AlSRKb* fusion, but not with an intact *CgSRK7* gene, was unexpected. The Capsella and Arabidopsis genera share a large number of trans-species polymorphisms (Paetsch *et al.* 2006). Because the *CgSRK7* gene is expressed and the eSRK7:AlSRKb fusion is functional, the nonfunctionality of *CgSRK7* reveals significant divergence between the two taxa either in processing of the SRK protein or in downstream targets of the receptor. Additional experiments are required to determine if the ineffectiveness of the *CgSRK7* gene in *A. thaliana* is a function of its transmembrane or kinase domains, and whether all *Capsella SRK* alleles fail to function in *A. thaliana*.

Despite successful expression of six distinct SI specificities, however, one *SRK/SCR* chimeric gene pair tested, *AleSRK37:AlSRKb* and *AlSCRb:AlSCR37*, failed to confer an incompatibility response in *A. thaliana*. This failure does not seem to be a function of overall sequence divergence, because AleSRK37 and AlSCR37 are not significantly more diverged from AleSRKb and AlSCRb than the variants that conferred an incompatibility response. At present, it is not known if failure to express incompatibility is due to the nonfunctionality of AleSRK37 or of AlSCR37. However, an earlier report had indicated that SRK variants bear polymorphisms that influence not only their ligand specificity, but also their ability to form homodimers and heterodimers (Naithani *et al.* 2007). The region that influences specificity in dimerization is located within the PAN_APPLE domain of eSRK (Naithani *et al.* 2007) at the junction of the SRK fusion constructs used in our study [C-terminal variable region (CVR) in Figure 3]. It is possible that receptor dimerization is negatively affected in the AleSRK37:AlSRKb fusion but not in the eSRK:AlSRKb fusions that conferred SI. Alternatively, the AleSRK37:AlSRKb protein might not assume a functional conformation and thus might fail to accumulate to appropriate levels or to be correctly targeted to the plasma membrane in *A. thaliana* stigmas.

Nevertheless, the chimeric protein approach is clearly a valuable strategy for analysis of SRK and SCR function. Not only has it allowed the transfer of several distinct SI specificities into *A. thaliana*, but it has also allowed us to determine that the eSRKs and SCRs of the *A. thaliana* Ψ*SB* haplotype have accumulated function-altering substitutions in addition to the previously noted obvious open reading frame disrupting mutations or rearrangements. At present, it is not possible to infer which mutations in these pseudogenes are primary mutations that might have caused loss of SI in the *A. thaliana* lineage and which mutations are secondary mutations that occurred after the switch to self-fertility due to relaxation of selective pressure on the *S* locus.

The chimeric protein approach also provides a facile means for testing the functional equivalence or non-

equivalence of *S* haplotypes from different species, as shown by our demonstration that *AlSa* and *CgS7* determine distinct SI specificities despite high similarity in the sequences and arrangement of their *SRK* and *SCR* genes. More generally, the chimeric gene expression system might provide a means for future transfer of SI specificities between various crucifer species. SI is a valuable trait in breeding schemes for hybrid seed production, and a reliable and efficient means of transferring SI specificities to agronomically important crucifers is desirable. Therefore, understanding the factors required for proper function of *SRK* and *SCR* in heterologous crucifer species, as may be achieved using the protein fusion expression system described here, is a critical goal of future research. In the short term, further *in planta* analysis of the various SI specificities already introduced into *A. thaliana* promises to provide a mechanistic understanding of several aspects of SRK and SCR function. In particular, this analysis may allow us to elucidate the molecular basis of dominance relationships between *SRK* alleles (Hatakeyama *et al.* 2001; Schierup *et al.* 2001; Mable 2003; Prigoda *et al.* 2005) and to delineate the residues in the eSRK and SCR that are required for specificity and functionality of these highly specific receptor-ligand pairs.

A. thaliana seed was obtained from the Arabidopsis Biological Resource Center in Columbus, Ohio. This article is based upon work supported by National Science Foundation grant IOS-0744579.

LITERATURE CITED

Bechsgaard, J. S., V. Castric, D. Charlesworth, X. Vekemans and M. H. Schierup, 2006 The transition to self-compatibility in *Arabidopsis thaliana* and evolution within S-haplotypes over 10 Myr. Mol. Biol. Evol. **23:** 1741–1750.

Bergwitz, C., T. J. Gardella, M. R. Flannery, J. T. Potts, Jr., H. M. Kronenberg *et al.*, 1996 Full activation of chimeric receptors by hybrids between parathyroid hormone and calcitonin. Evidence for a common pattern of ligand-receptor interaction. J. Biol. Chem. **271:** 26469–26472.

Bi, Y. M., N. Brugiere, Y. Cui, D. R. Goring and S. J. Rothstein, 2000 Transformation of Arabidopsis with a Brassica SLG/SRK region and ARC1 gene is not sufficient to transfer the self-incompatibility phenotype. Mol. Gen. Genet. **263:** 648–654.

Boggs, N. A., J. B. Nasrallah and M. E. Nasrallah, 2009 Independent *S*-locus mutations caused self-fertility in *Arabidopsis thaliana*. PLoS Genet. **5:** e1000426.

Boyes, D. C., M. E. Nasrallah, J. Vrebalov and J. B. Nasrallah, 1997 The self-incompatibility (S) haplotypes of Brassica contain highly divergent and rearranged sequences of ancient origin. Plant Cell **9:** 237–247.

Brown, A. J., and L. A. Casselton, 2001 Mating in mushrooms: increasing the chances but prolonging the affair. Trends Genet. **17:** 393–400.

Casselman, A. L., J. Vrebalov, J. A. Conner, A. Singhal, J. Giovannoni *et al.*, 2000 Determining the physical limits of the Brassica S locus by recombinational analysis. Plant Cell **12:** 23–33.

Charlesworth, D., C. Bartolome, M. H. Schierup and B. K. Mable, 2003 Haplotype structure of the stigmatic self-incompatibility gene in natural populations of *Arabidopsis lyrata*. Mol. Biol. Evol. **20:** 1741–1753.

Chookajorn, T., A. Kachroo, D. R. Ripoll, A. G. Clark and J. B. Nasrallah, 2004 Specificity determinants and diversification of the Brassica self-incompatibility pollen ligand. Proc. Natl. Acad. Sci. USA **101:** 911–917.

Dwyer, K. G., B. A. Lalonde, J. B. Nasrallah and M. E. Nasrallah, 1992 Structure and expression of AtS1, an Arabidopsis thaliana gene homologous to the S-locus related genes of Brassica. Mol. Gen. Genet. **231:** 442–448.

Gleave, A. P., 1992 A versatile binary vector system with a T-DNA organizational structure conducive to efficient integration of cloned DNA into plants genome. Plant Mol. Biol. **20:** 1203–1207.

Hatakeyama, K., T. Takasaki, G. Suzuki, T. Nishio, M. Watanabe *et al.*, 2001 The S receptor kinase gene determines dominance relationships in stigma expression of self-incompatibility in Brassica. Plant J. **26:** 69–76.

Kachroo, A., C. R. Schopfer, M. E. Nasrallah and J. B. Nasrallah, 2001 Allele-specific receptor-ligand interactions in Brassica self-incompatibility. Science **293:** 1824–1826.

Kho, Y. O., and J. Baer, 1968 Observing pollen tubes by means of fluorescence. Euphytica **17:** 298–302.

Kimura, R., K. Sato, R. Fujimoto and T. Nishio, 2002 Recognition specificity of self-incompatibility maintained after the divergence of *Brassica oleracea* and *Brassica rapa*. Plant J. **29:** 215–223.

Koncz, C., and J. Schell, 1986 The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of Agrobacterium binary vector. Mol. Gen. Genet. **204:** 383–396.

Kusaba, M., K. Dwyer, J. Hendershot, J. Vrebalov, J. B. Nasrallah *et al.*, 2001 Self-incompatibility in the genus Arabidopsis: characterization of the S locus in the outcrossing *A. lyrata* and its autogamous relative *A. thaliana*. Plant Cell **13:** 627–643.

Mable, B. K., 2003 Estimating the number, frequency, and dominance of S-alleles in a natural population of *Arabidopsis lyrata* (Brassicaceae) with sporophytic control of self-incompatibility. Heredity **90:** 422–431.

Mable, B. K., M. H. Schierup and D. Charlesworth, 2003 Estimating the number, frequency, and dominance of S-alleles in a natural population of Arabidopsis lyrata(Brassicaceae) with sporophytic control of self-incompatibility. Heredity **90:** 422–431.

Naithani, S., T. Chookajorn, D. R. Ripoll and J. B. Nasrallah, 2007 Structural modules for receptor dimerization in the S-locus receptor kinase extracellular domain. Proc. Natl. Acad. Sci. USA **104:** 12211–12216.

Nasrallah, J. B., 2000 Cell-cell signaling in the self-incompatibility response. Curr. Opin. Plant Biol. **3:** 368–373.

Nasrallah, J. B., 2005 Recognition and rejection of self in plant self-incompatibility: comparisons to animal histocompatibility. Trends Immunol. **26:** 412–418.

Nasrallah, J. B., P. Liu, S. Sherman-Broyles, R. Schmidt and M. E. Nasrallah, 2007 Epigenetic mechanisms for breakdown of self-incompatibility in interspecific hybrids. Genetics **175:** 1965–1973.

Nasrallah, M., P. Liu, S. Sherman-Broyles, N. Boggs and J. Nasrallah, 2004 Natural variation in expression of self-incompatibility in *Arabidopsis thaliana*: implications for the evolution of selfing. Proc. Natl. Acad. Sci. USA **101:** 16070–16074.

Nasrallah, M. E., P. Liu and J. B. Nasrallah, 2002 Generation of self-incompatible *Arabidopsis thaliana* by transfer of two S locus genes from *A. lyrata*. Science **297:** 247–249.

Paetsch, M., S. Mayland-Quellhorst and B. Neuffer, 2006 Evolution of the self-incompatibility system in the Brassicaceae: identification of *S-locus receptor kinase* (*SRK*) in self-incompatible *Capsella grandiflora*. Heredity **97:** 283–290.

Prigoda, N. L., A. Nassuth and B. K. Mable, 2005 Phenotypic and genotypic expression of self-incompatibility haplotypes in Arabidopsis lyrata suggests unique origin of alleles in different dominance classes. Mol. Biol. Evol. **22:** 1609–1620.

Sato, Y., R. Fujimoto, K. Toriyama and T. Nishio, 2003 Commonality of self-recognition specificity of S haplotypes between *Brassica oleracea* and *Brassica rapa*. Plant Mol. Biol. **52:** 617–626.

Sato, Y., S. Okamoto and T. Nishio, 2004 Diversification and alteration of recognition specificity of the pollen ligand *SP11/SCR* in self-incompatibility of Brassica and Raphanus. Plant Cell **16:** 3230–3241.

Sato, Y., K. Sato and T. Nishio, 2006 Interspecific pairs of class II S haplotypes having different recognition specificities between Brassica oleracea and Brassica rapa. Plant Cell Physiol. **47:** 340–345.

Schierup, M. H., B. K. Mable, P. Awadalla and D. Charlesworth, 2001 Identification and characterization of a polymorphic re-

ceptor kinase gene linked to the self-incompatibility locus of *Arabidopsis lyrata*. Genetics **158:** 387–399.

Schopfer, C. R., M. E. Nasrallah and J. B. Nasrallah, 1999 The male determinant of self-incompatibility in Brassica. Science **286:** 1697–1700.

Shimizu, K. K., J. M. Cork, A. L. Caicedo, C. A. Mays, R. C. Moore *et al.*, 2004 Darwinian selection on a selfing locus. Science **306:** 2081–2084.

Shimizu, K. K., R. Shimizu-Inatsugi, T. Tsuchimatsu and M. D. Purugganan, 2008 Independent origins of self-compatibility in *Arabidopsis thaliana*. Mol. Ecol. **17:** 704–714.

Shimosato, H., N. Yokota, H. Shiba, M. Iwano, T. Entani *et al.*, 2007 Characterization of the SP11/SCR high-affinity binding site involved in self/nonself recognition in brassica self-incompatibility. Plant Cell **19:** 107–117.

Stein, J. C., B. Howlett, D. C. Boyes, M. E. Nasrallah and J. B. Nasrallah, 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.

Takayama, S., H. Shimosato, H. Shiba, M. Funato, F. S. Che *et al.*, 2001 Direct ligand-receptor complex interaction controls Brassica self-incompatibility. Nature **413:** 534–538.

van Engelen, F. A., J. W. Molthoff, A. J. Conner, J. P. Nap, A. Pereira *et al.*, 1995 pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res. **4:** 288–290.

Watanabe, M., A. Ito, Y. Takada, C. Ninomiya, T. Kakizaki *et al.*, 2000 Highly divergent sequences of the pollen self-incompatibility (S) gene in class-I S haplotypes of Brassica campestris (syn. rapa) L. FEBS Lett. **473:** 139–144.

Yamazaki, K., and G. K. Beauchamp, 2007 Genetic basis for MHC-dependent mate choice. Adv. Genet. **59:** 129–145.

Zhang, X., R. Henriques, S. S. Lin, Q. W. Niu and N. H. Chua, 2006 Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat. Protoc. **1:** 641–646.

Communicating editor: J. Borevitz

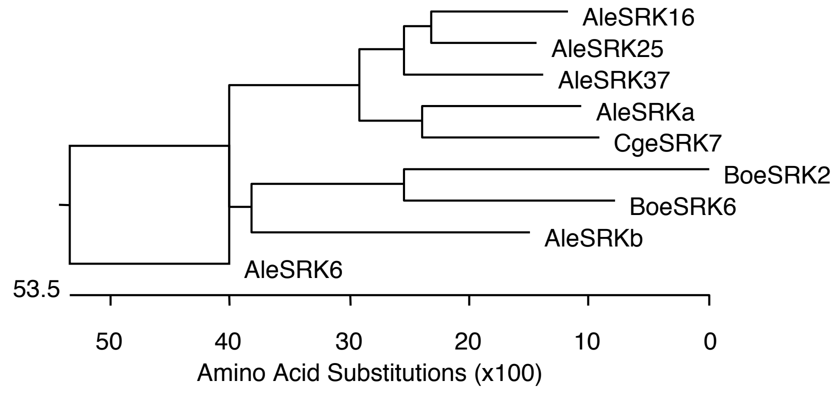
GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.109.102442/DC1

Expression of Distinct Self-Incompatibility Specificities in *Arabidopsis thaliana*

Nathan A. Boggs, Kathleen G. Dwyer, Paurush Shah, Amanda A. McCulloch,
Jesper Bechsgaard, Mikkel H. Schierup, Mikhail E. Nasrallah and June B. Nasrallah

Copyright © 2009 by the Genetics Society of America
DOI: 10.1534/genetics.109.102442


A
AleSRK16
AleSRK25
AleSRK37
AleSRKa
CgeSRK7
BoeSRK2
BoeSRK6
AleSRKb
AleSRK6
53.5
50
40
30
20
10
0
Amino Acid Substitutions (x100)

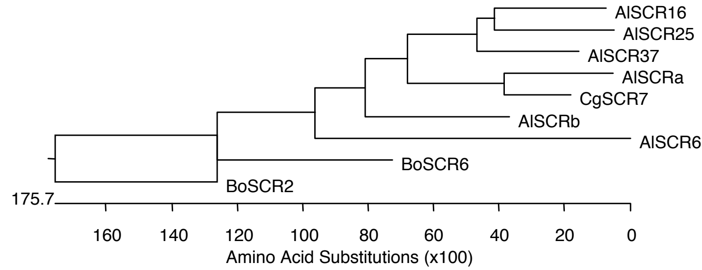
Percent Identity

	1	2	3	4	5	6	7	8	9		
1		82.3	78.6	71.3	71.6	56.3	58.0	61.0	59.8	1	AleSRK16
2			80.8	72.2	73.0	55.2	58.6	63.6	61.6	2	AleSRK25
3				71.6	71.4	55.0	59.9	61.9	63.1	3	AleSRK37
4					76.6	53.9	58.2	62.2	62.7	4	AleSRKa
5						51.4	55.1	60.3	57.3	5	CgeSRK7
6							67.1	58.1	56.4	6	BoeSRK2
7								60.9	60.2	7	BoeSRK6
8									61.0	8	AleSRKb
9										9	AleSRK6
	1	2	3	4	5	6	7	8	9		

B

```
AlSCRa  _MRCSVLFVVSYVIMSLLISHVQGMEDQKWKKVCNLEGNFP-GRC--VGNGDEQCKRDLTED-GNNPSK-CRC-RFRAGRRHCRCIYCEVFGM
AlSCRb   MRNATFFIVFYVFISLVLSNVQD-VTAQKN-KCMRSEMFPTGPC--GNNGEETCKKDFKNI-YRTPIQ-CKCLDKYDFARLCDCRFC
AlSCR25  MRCAALFMIFCVLIVFHINHGKE-VDAQKWKACRLRETFS-GTC--GHDGEIRCKNDITRNGGSPLPFECHC-EEFRRKRVCHCDKCLL
AlSCR6   MKSATLFMVAYVFMLIFLCRHVK-DLEAANFNCMWAGKFY-GPC-PLRNAGLSCAAEFSKRKSKEKPFNCYC-ANQGKWRVCRCLFC
AlSCR37  MRCVVLFMVSCLLIVLLINHFEE-VEAQKWKECNLRDIFP-GKCEHDANAKLRCKEDIAKNFRPSRPFECDC-QTFDQGRICYCKKCLV
CgSCR7   MRCGIFFVVSYVLMSFLISHVQGVETQKWKKECRGNFP---GRC--EGKGDEQCRHDLTEDGNKPSQ--CHC-TTHDLQRFCYCKYCKISV

AlSCR16   ------------------------QKWKACVIKQIFP-GSCRPDGYIRCKNDITKNGKQRPLECECKDVDGDRLCFCYKCLVLTTSDLTIS
AtΨSCRB MKQNK----FLGIISPCHFMKHILIEIEKACLIKQICP-GSCRTDGYIRCKNDITKNGKHRPFECKCKDVDGDRLCFCYKCLVLRASSDLTT
```



Percent Identity

	1	2	3	4	5	6	7	8	9		
1		53.4	53.4	38.3	37.5	39.6	22.2	36.0	25.5	1	AlSCR16
2			53.2	36.8	37.9	40.4	21.4	26.0	25.4	2	AlSCR25
3				38.6	46.6	36.8	25.0	26.0	30.0	3	AlSCR37
4					61.4	39.6	20.4	28.0	25.9	4	AlSCRa
5						40.7	23.6	33.3	27.3	5	CgSCR7
6							27.3	29.8	31.6	6	AlSCRb
7								31.1	22.2	7	BoSCR2
8									26.5	8	BoSCR6
9										9	AlSCR6
	1	2	3	4	5	6	7	8	9		

FIGURE S1.—Sequence analysis of the eSRK and SCR variants used in this study. Substitution trees and percent amino-acid identities shared by eSRK and SCR variants are shown. (A) eSRK comparisons. (B) SCR amino-acid alignments and comparisons. The majority (~420 amino acids) of the extracellular domain of SRK and the mature ligand region (~60 amino acids) of SCR were used in the analysis. *eSRK* and *SCR* sequences of the *Brassica oleracea S6* (BoeS6) and *S2* (BoeS2) are included for comparison.

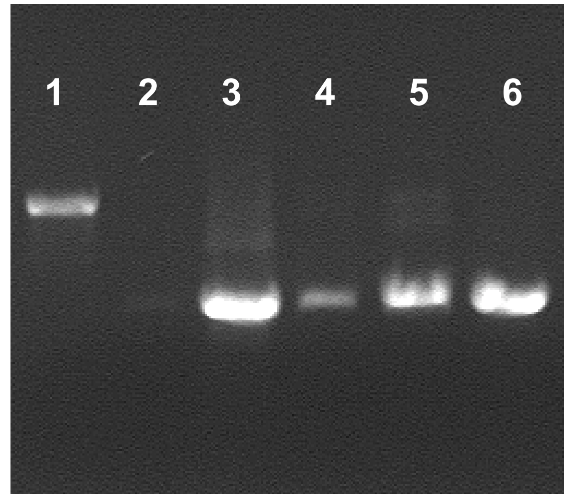


FIGURE S2.—Expression of the *CgSRK7* gene in transgenic *A. thaliana* stigmas. RNA was isolated from the stigmas of *A. thaliana CgS7* transformants and from *C. grandiflora S7* plants. After DNase treatment, equal amounts of RNA were used for amplification of *CgSRK7* transcripts by RT-PCR (reverse transcription-polymerase chain reaction) as described in the text using primers that flanked introns to distinguish between products amplified from RNA (lanes 3-6) and the larger products amplified from genomic DNA (lane 1).
The forward primer [5'TAGCGGCATGTCGGAGATTCAA3'] and the reverse primer [5'ACCAAGCCACTGGTTAGAAA3'] are complementary to sequences within exon 1 and exon 3, respectively.

Lane 1: PCR of *CgS7* DNA
Lane 2: PCR of *CgS7* RNA without reverse transcriptase
Lane 3-5: RT-PCR of stigma RNA from three independent *CgS7* transformants
Lane 6: RT-PCR of stigma RNA from a *C. grandiflora S7* plant.

```
AlSCRb     MRNATFFIVFYVFISLVLSNVQDVTAQKNKCMRSEMFPTGPCGNNGEETCKKDFKNIYRTPIQCKCLDKYDFARLCDCRFC.
           || :.||:| ||::|:::|:||:|.:|| |    .. || |.|..:|:| |:.|:.:  ..| ||.| ...|:.|:| |::|
CgSCR7     MRCGIFFVVSYVLMSFLISHVQGVETQKWKKECRGNFP-GRCEGKGDEQCRHDLTEDGNKPSQCHC-TTHDLQRFCYCKYCKISV.
                    ^10       ^20       ^30       ^40       ^50       ^60       ^70       ^80

AlSCRb     MRNATFFIVFYVFISLVLSNVQDVTAQKNKCMRSEMFPTGPCGNNGEETCKKDF-KNIYRTPIQCKCLDKYDFARLCDCRFC.
                                     .: : |: .::|| |:|  :|   ||:|: ||  : |::|.| |  | .||| |  |
AlSCR16    -------------------------QKWKACVIKQIFP-GSCRPDGYIRCKNDITKNGKQRPLECECKDV-DGDRLCFCYKCLVLTTSDLTIS.
                    ^10       ^20       ^30       ^40       ^50       ^60       ^70       ^80       ^90

AlSCRb     MRNATFFIVFYVFISLVLSNVQDVTAQK-NKCMRSEMFPTGPCGNNGEET--CKKDFKNIYR--TPIQCKCLDKYDFARLCDCRFC.
                                   |.||| :.|  .::|| | |.:::::..  ||.|: : :|   |::|.| :.:| :|:| |: |
AlSCR37    MRCVVLFMVSCLLIVLLINHFEEVEAQKWKECNLRDIFP-GKCEHDANAKLRCKEDIAKNFRPSRPFECDC-QTFDQGRICYCKKCLV.
                    ^10       ^20        ^30       ^40         ^50         ^60       ^70       ^80

AlSCRb     MRNATFFIVFYVFISLVLSN---VQDVTAQK-NKCMRSEMFPTGPCGNNGEET--CKKDFKNIYR--TPIQCKCLDKYDFARLCDCRFC.
           || ..:|:| .::| |::::   : :|.||| |||. .::|| | |.:::::..  ||.|: : :|   |::|:| :.:| : :| |: |
AtΨSCR1    MRCVVLFMVSCLLIVLLINHFEVITEVEAQKWNKCFLRDIFP-GKCEHDANAKLRCKEDIAKNFRPSRPFECNCQT-FDKGGICYCKKCLV.
                    ^10       ^20          ^30       ^40         ^50         ^60       ^70       ^80

AlSCRb     MRNATFFIVFYVFISLVLSNVQDVTAQKNKCMRSEMFPTGPCGNNGEETCKKDF-KNIYRTPIQCKCLDKYDFARLCDCRFC.
                    |  :||      : :.. .: |: .:: | |:| .:|   ||:|: ||  : |::||| |  | .||| |  |
AtΨSCRB     MKQNK----FLGIISPCHFMKHILIEIEKACLIKQICP-GSCRTDGYIRCKNDITKNGKHRPFECKCKD-VDGDRLCFCYKCLVLRASSDLTT.
    1               ^10       ^20       ^30       ^40       ^50       ^60       ^70       ^80       ^90

AlSCRb     MRNATFFIVFYVFISLVLSNVQDVTAQKNKCMRSEMFPTGPCGNNGEETCKKDF-KNIYRTPIQCKCLDKYDFARLCDCRFC.
                    |  :||      : :.. .: |: .::|| |:| .:|   ||:|: ||  : |::||| |  | .||| |  |
AtΨSCRB-C  MKQNK----FLGIISPCHFMKHILIEIEKACLIKQIFP-GSCRTDGYIRCKNDITKNGKHRPFECKCKD-VDGDRLCFCYKCLVLRASSDLTT.
                    ^10       ^20       ^30       ^40       ^50       ^60       ^70       ^80       ^90
```

FIGURE S3.—Amino-acid sequences of the AlSCRb:SCRx chimeras. Each of the SCR sequences is aligned with the AlSCRb sequence. The bold residues in AlSCRb correspond to the signal sequence that was fused to the sequence of each of the mature SCR variants (underlined). The yellow shading marks the cysteine at position 37 in AtYSCRB that was changed to a phenylalanine in AtYSCRB-C. Amino acids are numbered according to the AlSCRb sequence.