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Epigenetic Mechanisms for Breakdown of Self-Incompatibility in Interspecific Hybrids

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ABSTRACT

As a major agent of rapid speciation, interspecific hybridization has played an important role in plant evolution. When hybridization involves species that exhibit self-incompatibility (SI), this prezygotic barrier to self-fertilization must be overcome or lost to allow selfing. How SI, a normally dominant trait, is lost in nascent hybrids is not known, however. Here we demonstrate that hybrid self-fertility can result from epigenetic changes in expression of the *S*-locus genes that determine specificity in the SI response. We analyzed loss of SI in synthetic hybrids produced by crossing self-fertile and self-incompatible species in each of two crucifer genera. We show that SI is lost in the stigmas of *A. thaliana–lyrata* hybrids and their neo-allotetraploid derivatives and in the pollen of *C. rubella–grandiflora* hybrids and their homoploid progenies. Aberrant processing of *S*-locus receptor kinase gene transcripts as detected in Arabidopsis hybrids and suppression of the *S*-locus cysteine-rich protein gene as observed in Capsella hybrids are two reversible mechanisms by which SI might break down upon interspecific hybridization to generate self-fertile hybrids in nature.

THE origin of many plant species may be traced to sexual hybridization between more or less diverged species (Stebbins 1959; Rieseberg 1997, 2001). Stable self-fertile diploid (homoploid) hybrids are sometimes produced by hybridization between closely related species that have similar genomes and chromosome complements (Gross and Rieseberg 2005). More frequently, however, several barriers to gene flow between species must be overcome before fertile interspecific hybrids are generated. Most commonly discussed are postzygotic barriers that lead to sterility in F_1 hybrids (Bushell *et al.* 2003), often resulting from aberrant meiotic pairing between highly divergent parental genomes. In these cases, chromosome doubling restores normal meiosis and generates fertile allopolyploids, a process that is thought to underlie at least 4% of speciation events in flowering plants (Rieseberg 2001). Neither homoploid nor allopolyploid hybrids can form, however, unless prezygotic barriers to hybridization are overcome, including pollination barriers that prevent pollen tubes from forming at the stigma surface, growing within the pistil, and reaching the ovules. A major prezygotic pollination barrier is genetic self-incompatibility (SI), which, although primarily known as an intraspecific barrier to self-fertilization in many obligate outcrossing plants, is also important in the context of interspecific hybridization. Indeed, when interspecific hybridization involves self-incompatible species, the generation of self-fertile hybrids, whether homoploid or allopolyploid, is dependent on the breakdown of SI.

The crucifer (Brassicaceae) family, which includes predominantly self-fertilizing species and self-incompatible species, provides several examples of self-fertile interspecific hybrids that occurred spontaneously or were produced artificially in breeding programs. Self-fertile allopolyploids are particularly common. Examples of allotetraploids include *Brassica napus,* derived by hybridization between *B. oleracea* and *B. campestris* (syn. *B. rapa*), and *Arabidopsis suecica*, derived by hybridization between self-fertile *A. thaliana* and self-incompatible *A. arenosa* (Mummenhoff and Hurka 1995; O'Kane *et al.* 1996). In addition, self-fertile homoploid F_1 hybrids occur in this family. For example, crosses between self-incompatible *Capsella grandiflora* and self-fertile *C. rubella* produce self-fertile diploid F_1 hybrids that can be selfed to establish F_2 populations (Riley 1934; Acarkan *et al.* 2000; Koch and Kiefer 2005).

The crucifer family is particularly well suited for investigating the breakdown of SI in interspecific hybrid progenies of self-incompatible species, not only because of the prevalence of interspecific hybridization events in this family, but also because the crucifer SI system is

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well characterized. SI specificity has been shown to be determined by the highly polymorphic protein products of two genes that are tightly linked within the *S*-locus complex (Nasrallah 2005; Takayama and Isogai 2005). Allele-specific interaction between two proteins, the stigma-expressed *S*-locus receptor kinase (SRK) and its pollen ligand, the *S*-locus cysteine-rich protein (SCR, also known as SP11; Takayama and Isogai 2005), triggers a signaling cascade within the stigma epidermis that leads to pollen inhibition. Importantly, these two proteins have also been shown to be the primary determinants of the outcrossing mating system in the family as demonstrated by the successful transfer of the SI trait into self-fertile *A. thaliana* by transformation with an *SRK–SCR* gene pair from self-incompatible *A. lyrata* (Nasrallah *et al.* 2002, 2004).

To understand the molecular basis of breakdown of SI in interspecific hybrids, we focused on interspecific hybrids of Arabidopsis and Capsella. Previous studies had shown that crosses between *A. thaliana* and *A. lyrata*, on the one hand, and between *C. rubella* and *C. grandiflora*, on the other hand, produced interspecific hybrids in controlled pollinations (Acarkan *et al.* 2000; Nasrallah *et al.* 2000). Here, we document the behavior of pollen tubes in the hybrids upon self-pollinations and reciprocal crosses to the parental species. We also report on the isolation of *S*-locus genes from *C. grandiflora* and the use of these genes and of previously isolated *A. lyrata SRK* and *SCR* genes for a molecular analysis of interspecific hybrids. We demonstrate that both *A. thaliana–lyrata* and *C. rubella–grandiflora* hybrids exhibit a loss of SI, on the stigma side in the first case and on the pollen side in the second case. The expression patterns of *S*-locus genes in these hybrids suggest at least two different mechanisms by which SI might break down to generate fertile interspecific hybrids in nature.

MATERIALS AND METHODS

Plant materials and generation of interspecific hybrids: For interspecific hybridization of Arabidopsis species, we used *A. thaliana* accession Col-0 and self-incompatible *A. lyrata SaSb* plants (described by Kusaba *et al.* 2001), which were descended from accessions collected in Michigan (kindly provided by Charles Langley, University of California at Davis). The generation of *A. thaliana–lyrata* hybrids by pollinating *A. thaliana* stigmas with pollen from *A. lyrata*, followed by ovule rescue, was described previously (Nasrallah *et al.* 2000). The *C. rubella* and *C. grandiflora* strains used in this analysis were described previously (Acarkan *et al.* 2000) and *C. rubella–grandiflora* hybrids were generated by manual cross-pollinations, which produced seed that could be germinated directly in soil.

Pollination analyses and determination of SI phenotype: Stigmas were examined for absence of contaminating pollen under a stereoscope, and appropriate pollen was manually applied to their surface. Two hours after pollination, flowers were fixed for 30 min in a 3:1 mixture of ethanol and acetic acid, softened for 30 min in 1 M NaOH at 65°, washed for 30 min in water, stained in decolorized aniline blue, and mounted on slides for examination by epifluorescence microscopy (Kho and Baer 1968). Under these conditions, an incompatible response is typically manifested by <10 pollen tubes per stigma while compatible pollinations exhibit numerous pollen tubes per stigma.

Construction and screening of genomic libraries: For genomic library construction, *C. grandiflora S7S8* DNA was partially digested with *Sau*3A1, and a fraction containing fragments of 9–20 kb was cloned into the *Bam*HI site of the λDASHII vector (Stratagene, LaJolla, CA). The library was screened with a ^{32}P-labeled probe containing a mixture of fragments derived from the first exons of the *A. lyrata SRKa* and *SRKb* genes (Kusaba *et al.* 2001).

DNA gel-blot analysis: DNA was isolated from leaves according to Murray and Thompson (1980). Digested DNA (~3 μg) was run on 0.8% (w/v) agarose gels, transferred to GeneScreen Plus membrane (DuPont-New England Nuclear, Boston) using an alkaline transfer method. The blots were prehybridized and hybridized at 65° in 10% (w/v) dextran sulfate, 330 mM sodium phosphate, pH 7.0, 10 mM EDTA, and 5% (w/v) SDS. Probes were labeled with ^{32}P using the Random Priming kit (Roche, Indianapolis). After washing in a solution containing 0.2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% (w/v) SDS at 65°, the blots were exposed to phosphor screens and developed using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager.

RNA analysis: *SRK* transcripts were detected in poly(A)+ RNA isolated from stigmas as previously described (Kusaba *et al.* 2001), while *SCR* transcripts were detected in total RNA isolated from anthers using the TRIZOL reagent (Invitrogen, San Diego). RNA gel blot analysis was performed as previously described (Kusaba *et al.* 2001) by subjecting the RNA [~1 μg of poly(A) RNA for *SRK* detection and ~15 μg of total RNA for *SCR* detection] to denaturing electrophoresis on 1% (w/v) agarose, transfer to GeneScreen Plus membrane (DuPont-New England Nuclear, MA), and hybridization with ^{32}P-labeled *SRK* or *SCR* probes as described above. Quantitation of *SRK* and *SCR* signal intensity was performed with a Molecular Dynamics PhosphorImager using the ImageQuant software package and normalization of hybridization signals was performed using an actin probe.

For reverse transcription–polymerase chain reaction (RT–PCR) analysis of *SRKa* transcripts, stigma RNA was treated with DNase I to eliminate contaminating genomic DNA, reverse transcribed, and amplified using the SuperScript one-step RT–PCR kit (Invitrogen) and *SRKa*-specific intron-flanking primers. The effectiveness of DNase digestion was verified by RT–PCR using actin intron-flanking primers, and only samples lacking contaminating DNA were used for RT–PCR of *SRKa*.

Sequence analysis and database searches: DNA sequencing was performed at the Cornell University BioResource Center using an Applied Biosystems (Foster City, CA) automated sequencer. Sequences were manipulated and aligned using DNASTAR Lasergene software (DNASTAR, Madison, WI). BLAST searches were performed on the National Center for Biotechnology Information website (http://www.ncbi.nih.gov).

RESULTS

***A.thaliana–lyrata* interspecific hybrids:** *Analysis of SI in A. thaliana–lyrata hybrid stigmas:* We used two hybrids produced from a cross between *A. thaliana* Col-0, which is homozygous for a defective *S* haplotype (Kusaba *et al.*

TABLE 1

Pollination analysis of Arabidopsis species hybrids and parental species

	♂ *A. lyrata SaSa*	♂ *A. lyrata SbSb*	♂ *A. thaliana–lyrata*	
			F_1[b]	Allotetraploid *SaSaS0S0*
♀ *A. lyrata SaSa*	<10[a]	+++	MS	<10
♀ *A. lyrata SbSb*	+++	<10	MS	+++
♀ *A. thaliana–lyrata*				
F_1[b]	+++	+++	MS	ND
BC[c]	<10	+++	MS	ND
Allotetraploid SaSaS0S0	+++	+++	MS	+++

[a] The number of pollen tubes per pollinated stigma: <10, incompatible pollination; +++, compatible pollination; MS, male sterile; ND, not determined because previous generations were no longer available.
[b] F_1, interspecific hybrids from the *A. thaliana* × *A. lyrata* cross.
[c] BC, plants from a backcross of *A. thaliana–lyrata* hybrids to *A. lyrata*.

2001) designated *S0*, and an *A. lyrata SaSb* plant (Nasrallah *et al.* 2000). On the basis of gel-blot analysis of genomic DNA, the two hybrids were determined to have inherited the *A. lyrata Sa* haplotype (data not shown) and were therefore designated *SaS0*. These hybrids failed to produce pollen and were male sterile, consistent with the divergent chromosome number and genome organization of the two parental species (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005). While male sterility precluded analysis of pollen from these *SaS0* hybrids, hybrid stigmas were functional, allowing assays of cross-incompatibility responses by manual pollination with *A. lyrata SaSa* and *SbSb* pollen. Both pollinations resulted in equally prolific pollen tube growth (Table 1), demonstrating that the stigmas of *A. thaliana–lyrata SaS0* hybrids failed to recognize and reject *Sa* pollen. Interestingly, backcrossing of these hybrids to *A. lyrata* restored the stigma SI response within one generation (Table 1).

Loss of SI was also exhibited by a neo-allotetraploid that arose spontaneously on one *A. thaliana–lyrata SaS0* hybrid (Nasrallah *et al.* 2000). This allotetraploid, which was shown by cytological analysis to have 26 chromosomes (or double the chromosome number of the *A. thaliana–lyrata* F_1 hybrids) (Nasrallah *et al.* 2000), produced functional pollen due to restoration of normal meiosis upon chromosome doubling. Selfing of this plant generated self-fertile allotetraploid progeny (Nasrallah *et al.* 2000). Reciprocal pollinations to *A. lyrata SaSa* plants demonstrated that the pollen of the allotetraploid and its progeny retained *Sa* specificity while its stigmas allowed confluent *Sa* pollen tube growth (Table 1). This pollination phenotype was stable and persisted over four allotetraploid generations analyzed. Thus, loss of SI in allotetraploids—and, by inference, in the original *A. thaliana–lyrata* hybrid—was stigma specific.

Molecular basis of SI breakdown in A. thaliana–lyrata hybrids: To investigate the molecular basis of breakdown in hybrid stigmas, we compared expression of the *SRKa* gene in each of the two *A. thaliana–lyrata SaS0* hybrids and in *A. lyrata* by gel-blot analysis of stigma poly(A)+ RNA using an *SRKa*-specific probe derived from the *SRKa* first exon. Self-incompatible *A. lyrata Sa* stigmas exhibit an *SRK* transcript profile (Kusaba *et al.* 2001) consisting of a fully spliced 3-kb *SRKa* transcript that encodes the full-length SRKa receptor and a 10-fold less abundant 1.6-kb alternative transcript derived from the first exon of the gene, which results from the use of an alternative poly(A) addition site within the first intron of *SRK* and encodes a soluble form of the SRK ectodomain. The stigmas of *A. thaliana–lyrata SaS0* hybrids and allotetraploids differed from those of *A. lyrata Sa* stigmas in two respects (Figure 1). They exhibited an additional 4-kb transcript species (Figure 1), at least some of which correspond to unspliced *SRKa* transcripts (expected size: 3.920 kb) on the basis of RT–PCR using

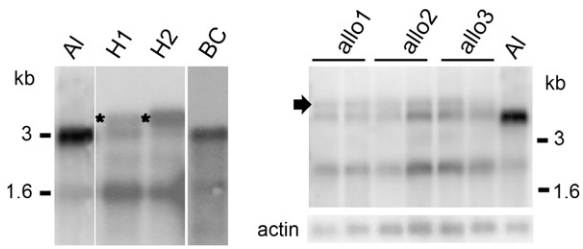

Figure 1.—Association of aberrant *SRK* transcript processing with self-fertility in *A. thaliana–lyrata* hybrids and allotetraploid derivatives. Gel blots of stigma poly(A)+ RNA were hybridized with a probe derived from exon 1 of *A. lyrata SRKa* as previously described (Kusaba *et al.* 2001). Al, *A. lyrata*; H1 and H2, first-generation hybrids; BC, a first-generation plant from a backcross of H2 to *A. lyrata*; allo1, allo2, and allo3, three consecutive allotetraploid generations. The 3- and 1.6-kb transcripts typically produced by *SRK* genes are indicated. Note the 4-kb aberrant *SRKa* transcripts produced by stigmas of F_1 hybrids (asterisks) and allotetraploid derivatives (arrow).

TABLE 2

Pollination analysis of Capsella species hybrids and parental species

			♂ F_2		
	♂ *C. rubella S0S0*	♂ *C. grandiflora S7S7*[b]	*S0S0*	*S7S0*	*S7S7*
♀ *C. rubella S0S0*	+++[a]	+++	+++	+++	+++
♀ *C. grandiflora S7S7*[b]	+++	<10	+++	+++	<10
♀ F_2					
S0S0	+++	+++	+++	+++	+++
S7S0	+++	<10	+++	+++	<10
S7S7	+++	<10	+++	+++	<10

[a] The number of pollen tubes per pollinated stigma: <10, incompatible pollination; +++, compatible pollination; MS, male sterile; ND, not determined because previous generations were no longer available.

[b] *C. grandiflora S7S7* homozygotes were used because the *C. rubella–grandiflora* F_2 population segregated for the *S7* allele and only pollination assays with *S7S7* plants are relevant.

intron-flanking primers (data not shown). They also exhibited dramatically reduced levels of the fully spliced 3-kb transcripts, which at best equaled those of the alternative 1.6-kb transcripts (Figure 1). Importantly, these 3-kb *SRKa* transcripts accumulated to levels that were <10% (6.4% ± 0.5) of those detected in *A. lyrata Sa* stigmas.

To examine further the association of aberrant *SRK* transcript profiles with loss of SI, we analyzed *SRKa* expression in the stigmas of self-incompatible plants produced by backcrossing each of the *A. thaliana–lyrata SaS0* hybrids to the *A. lyrata* parent. Interestingly, the correct *SRK* transcript profile (Figure 1) was restored along with SI (Table 1) in first-generation backcross plants. These observations suggest that aberrant splicing of *SRKa* transcripts, and in particular the dramatic reduction in correctly spliced full-length transcripts, is the cause of the breakdown of SI in *A. thaliana–lyrata SaS0* hybrids.

***C. rubella–grandiflora* interspecific hybrids:** A cross between a *C. rubella* plant and a *C. grandiflora* plant produced fertile F_1 hybrids that set seed spontaneously, as previously described (Riley 1934; Acarkan *et al.* 2000; Koch and Kiefer 2005). The fertility of *C. rubella–grandiflora* hybrids is not surprising, given that these plants have the same basic chromosome number ($n = 8$) and are very similar at the molecular level (Hurka and Neuffer 1997). Despite these similarities, however, *C. rubella* and *C. grandiflora* are recognized as separate species (Hurka and Neuffer 1997). In this context, it should be noted that the concept of a strict biological species, which is defined by the ability of its members to produce viable and fertile progeny upon cross-hybridization, has not been recognized by plant biologists as a useful species concept (Grant 1981), and hybridization between plants belonging to well-recognized species is common (Arnold 1997).

Genetic analysis of SI in C. grandiflora, C. rubella, and interspecific hybrid populations: Prior to analysis of *C. rubella–grandiflora* interspecific hybrids, it was necessary to perform a genetic analysis of the parental strains used in our interspecific cross. First, we generated a *C. grandiflora* population of plants that segregated for SI specificity by forced self-pollination in young floral buds prior to the developmental onset of SI in stigmas. These plants were analyzed by microscopic examination of pollen tube growth in self-pollinations and reciprocal cross-pollinations among all siblings and with the parental plant. On the basis of these pollinations, we confirmed early reports (Bateman 1955) that the SI system of Capsella, like that of other crucifers, is under single-locus sporophytic control, whereby the pollen SI phenotype is determined by the diploid genotype of its parent plant. We also determined that the *C. grandiflora* plant used in our interspecific cross carried two SI specificities, arbitrarily designated *S7* and *S8*, with *S7* and *S8* exhibiting codominance in the stigma and *S7* being dominant to *S8* in pollen (Table 2). Finally, we analyzed several progeny derived from the spontaneous selfing of the *C. rubella* parent and confirmed that all of these plants were self-compatible, as expected (Table 2).

Genetic analysis was then carried out on the *C. rubella* × *C. grandiflora* cross. Starting with a *C. rubella–grandiflora* F_1 hybrid, we produced an F_2 population of 74 plants that segregated for SI and self-compatibility (SC). Analysis of the F_1 and F_2 plants by self-pollinations, autonomous seed set, and reciprocal pollinations with *C. grandiflora S7S7* and *S8S8* tester plants showed that this population inherited the *S7* allele and that SC segregated as a simple dominant trait (53 SC:21 SI, approximating a 3:1 ratio; $\chi^2 = 0.45$; $P = 0.5$) linked to the *S* locus (Table 2). Reciprocal pollinations of the self-incompatible F_2 plants with tester *C. grandiflora S7S7* and *S8S8* homozygotes showed that pollinations with *S8S8* plants were compatible while those with *S7S7* plants were incompatible, indicating that the plants expressed *S7* specificity in stigma and pollen (Table 2). The stigmas of 36 (or 2/3) of the self-compatible F_2 plants inhibited pollen from the *S7S7* tester, but the

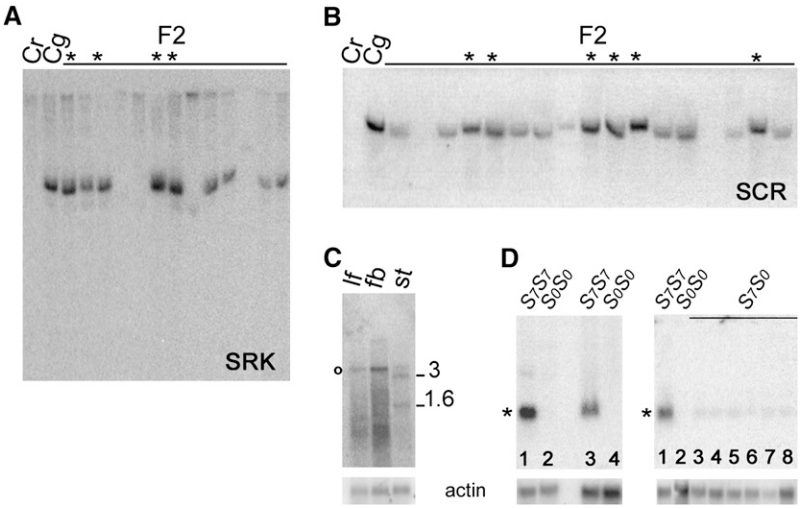

FIGURE 2.—Molecular analysis of self-incompatibility and self-fertility in Capsella species hybrids. (A and B) DNA gel blots of *C. rubella* (*Cr*), *C. grandiflora* (*Cg*), and two populations of *C. rubella–grandiflora* F_2 plants probed with the *CgSRK7* exon 1 probe (A) and *CgSCR7* (B). Only F_2 plants indicated with asterisks were self-incompatible and genotypically *S7S7* on the basis of pollination assays. All other F_2 plants were self-compatible and could be grouped into two classes on the basis of pollination analysis: self-fertile F_2 plants that hybridized with the probes were *S7S0* while those that did not hybridize were *S0S0*, as with *C. rubella*. The small differences in electrophoretic mobility of the hybridizing fragments observed between some of the lanes are due to irregular migration of DNA fragments, which is often associated with the use of relatively impure DNA obtained by mini-preparation methods. (C) Stigma-specific expression of the 3.0- and 1.6-kb *CgSRK7* transcripts detected by gel-blot analysis of poly(A)+ RNA from leaf tissue (lf), floral buds with pistils removed (fb), and stigmas (st) of *C. grandiflora S7S7*. The cross-hybridizing band (circle) common to the three samples represents transcripts from an *SRK*-related gene. (D) Gel-blot analysis of *CgSCR7* in total RNA from *C. grandiflora* (*S7S7*) and *C. rubella* (*S0S0*) whole floral buds (left, lanes 1 and 2) and anthers (left, lanes 3 and 4) and in the anthers of a representative sample of *C. rubella–grandiflora* F_2 plants (right). Note the lack of hybridization signal in *S0S0* plants and the drastic reduction in the steady-state levels of *CgSCR7* in the anthers of *S7S0* F_2 plants (right, lanes 3–8). Hybridization with actin was used as a loading control.

pollen of these plants germinated and produced pollen tubes on *S7S7* stigmas (Table 2). The remaining 17 (or 1/3) of the self-compatible F_2 plants were reciprocally cross-compatible with both *S8S8* and *S7S7* plants (Table 2).

The data, confirmed by seed counts and self-pollinations in 106 additional F_2 plants, are consistent with the following interpretation. The self-fertile *C. rubella* parent is homozygous for a nonfunctional *S* haplotype, designated *S0*, and crossing it to the *C. grandiflora S7S8* plant produces an *S7S0* F_1 hybrid. In the F_2 generation, *S7S7* plants are self-incompatible, *S0S0* plants are self-compatible, and *S7S0* plants express *S7* specificity in the stigma (their stigmas inhibit pollen from *S7S7* plants) but are self-compatible due to the breakdown of SI in pollen (their pollen germinates and produces tubes on *S7S7* stigmas).

Molecular cloning of S-locus genes from C. grandiflora: The availability of *SRK* and *SCR* genes is essential for a molecular analysis of SI and its breakdown. To isolate *SRK* and *SCR* genes from the *C. grandiflora S7* haplotype, a *C. grandiflora* genomic library constructed from an *S7S8* plant was screened with a probe derived from the first exons of the *A. lyrata SRKa* and *SRKb* genes (KUSABA *et al.* 2001). Sequence analysis of a subset of positive clones revealed them to contain an *SRK*-like gene in close proximity to an *SCR*-like gene. DNA gel-blot analysis with probes from these genes confirmed that they were derived from the *S7* haplotype. A probe corresponding to exon 1 of the *C. grandiflora SRK*-like gene hybridized most strongly with plants previously classified by pollination analysis as carrying the *S7* haplotype, and it detected a restriction fragment length polymorphism that cosegregated with the *S7* haplotype in *C. grandiflora* F_2 plants segregating for the *S8* and *S7* haplotypes (data not shown). In the *C. rubella* × *C. grandiflora* F_2 population, the *SRK* and *SCR* probes hybridized only to plants carrying the *S7* haplotype (Figure 2B) but not to plants predicted to be homozygous for the *C. rubella*-derived *S0* haplotype. This lack of hybridization, even with the *SRK* probe, suggests that the nonfunctional *S0* haplotype carries either deleted or highly diverged SI genes.

Our molecular cloning of *S*-locus genes from *C. grandiflora* confirmed the expectation that SI in this genus has the same molecular basis as other crucifers. The *C. grandiflora SRK7* and *SCR7* genes (hereafter designated *CgSRK7* and *CgSCR7*), which represent the first *SRK–SCR* gene pair identified in the Capsella species, contain all sequence motifs characteristic of *SRK* and *SCR* genes (Figure 3). The *CgSRK7* sequence represents a new allele distinct from the partial *C. grandiflora SRK* sequences that have been reported recently (PAETSCH *et al.* 2006). BLAST searches show *CgSRK7* and *CgSCR7* to be most similar to the *SRKa* and *SCRa* genes of *A. lyrata* (KUSABA *et al.* 2001). The exons of *CgSRK7*, on average, share ~84% nucleotide and ~86% amino-acid sequence identity with the corresponding exons of *AlSRKa*, while *CgSCR7* exons 1 and 2 are ~85% and ~74% identical to *AlSCRa* exons 1 and 2 in nucleotide and amino-acid sequence, respectively. Interestingly, the *C. grandiflora S7* haplotype is also similar to the *A. lyrata Sa* haplotype in the position and spacing of the *SRK* and *SCR* genes relative to each other (KUSABA *et al.* 2001). In both *S* haplotypes, the two genes are arranged tail to tail and are separated by a relatively small intergenic region (1006 bp in *C. grandiflora S7* and 1500 bp in *A. lyrata Sa*). However, neither the region between *CgSRK7* and *CgSCR7* nor the introns

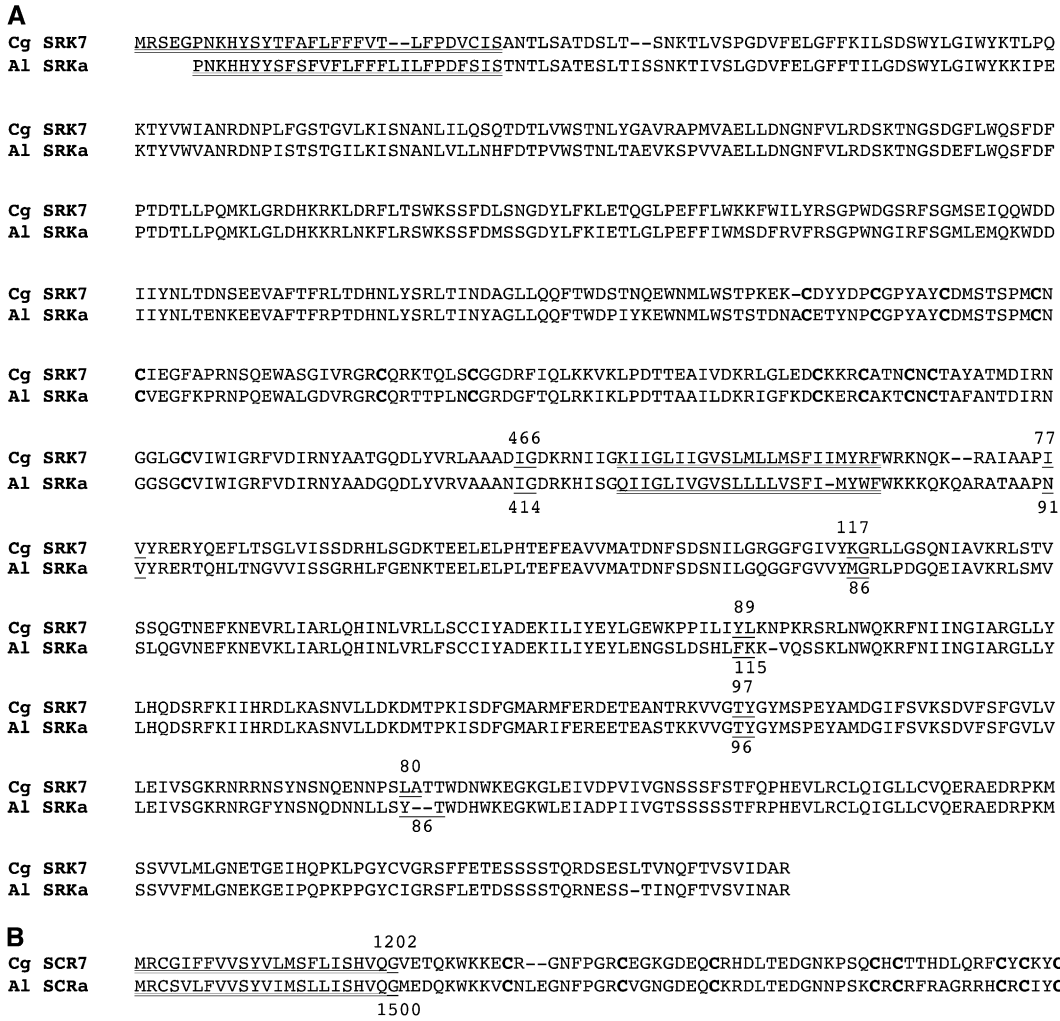

FIGURE 3.—Alignment of the predicted SRK (A) and SCR (B) amino-acid sequences from the *C. grandiflora S7* haplotype (CgSRK7 and CgSCR7; accession nos. 892031 and 894476) and the *A. lyrata Sa* haplotype (KUSABA *et al.* 2001) [AlSRKa (accession no. AB052755) and AlSCRa (accession no. AB052753)]. Cysteine residues that are conserved in SRKs and SCRs are shown in boldface type. Signal sequences and transmembrane domains are double underlined. The location of introns in the genomic sequences is shown by underlined residues that flank each intron with numbers indicating intron size in base pairs.

of the two genes, which are similar in size and placement (Figure 3), share appreciable sequence identity according to pairwise BLAST alignments (data not shown).

Molecular basis of SI breakdown in C. rubella–grandiflora hybrids: The molecular cloning of *CgSRK7* and *CgSCR7* allowed us to compare their expression in self-incompatible and self-compatible plants. In self-incompatible plants, these genes exhibited hybridization patterns characteristic of other *SRK* and *SCR* genes. *CgSRK7* transcripts were detected in the pistils, but not in the leaves or anthers, of plants carrying the *S7* haplotype (Figure 2C), irrespective of whether the plants were self-incompatible (*i.e.*, *S7S7*) or self-compatible (*i.e.*, *S7S0*). *CgSCR7* transcripts were detected in anthers or young floral buds of *C. grandiflora S7S7* plants and self-incompatible *S7S7 C. rubella–grandiflora* F_2 plants (Figure 2D). In contrast, all 4 *S0S0* F_2 plants analyzed failed to hybridize with the *CgSCR7* probe and all 10 *S7S0* F_2 plants analyzed produced only a weak hybridization signal that was 10- to 12-fold lower in self-compatible *S7S0* plants relative to their self-incompatible *S7S7* sibs (Figure 2D).

DISCUSSION

We investigated two cases of breakdown of SI upon interspecific hybridization in Arabidopsis and Capsella. In both cases, loss of SI was due to reversible changes in expression of the *S*-locus recognition genes, *SRK* or *SCR*. In *A. thaliana–lyrata SaS0* hybrids and their allotetraploid derivatives, stigmas exhibited aberrant *SRKa* transcript profiles. In particular, the levels of the fully spliced 3.0-kb *SRKa* transcripts were reduced to ~6% of the levels detected in *A. lyrata Sa* stigmas, while the levels of the 1.6-kb transcripts were slightly enhanced. The role of the soluble forms of the extracellular domain of SRK (designated eSRK) that are encoded by the 1.6-kb

transcripts is not understood. However, there is no evidence that they have a negative effect on SRK function; rather, they might contribute to the stabilization of the full-length SRK receptor, as previously suggested for the Brassica S-locus glycoprotein SLG, which shares a high degree of sequence similarity with the extracellular domain of SRK (Dixit *et al.* 2000). Thus, any increased accumulation of eSRKa that might result from the slight increase in 1.6-kb transcript levels observed in *A. thaliana–lyrata SaS0* hybrid stigmas is not expected to cause loss of SI through dominant-negative effects. In contrast, the ~94% reduction in the levels of fully spliced 3.0-kb *SRKa* transcripts, which is much greater than the 75% reduction that was previously shown to cause loss of SI in Brassica (Conner *et al.* 1997), is expected to result in absence or suboptimal accumulation of SRKa protein and, consequently, in the breakdown of SI in the stigmas of *A. thaliana–lyrata* hybrids and their neo-allotetraploid derivatives. Strong support for this conclusion is provided by the observation that reestablishment of SI in the first-generation backcross of *A. thaliana–lyrata SaS0* hybrids to *A. lyrata* was accompanied by restoration of normal *SRKa* transcript profiles.

Species-specific differences in pre-mRNA splicing have been reported (Laverdiere *et al.* 2000; Pan *et al.* 2005), but their consequences for gene expression in interspecific hybrids had not been previously explored. The production of some 3-kb *SRKa* transcripts in *A. thaliana–lyrata* hybrids indicates that the correct *SRKa* initiation, termination, and splice sites are utilized, albeit inefficiently, by the transcription and processing machinery of hybrid stigmas. Similarly, the production of 1.6-kb *SRKa* transcripts indicates that this machinery recognizes and utilizes the alternative poly(A) addition site within the *SRKa* first intron that generates this transcript species. At present, the cause of aberrant *SRKa* transcript processing is unclear. It cannot be due to global defects in transcript synthesis and processing in all tissues of *A. thaliana–lyrata* hybrids, because these hybrids do not exhibit major developmental or physiological abnormalities. Nor can it be ascribed to dilution of the *A. lyrata*-derived machinery by *A. thaliana* factors that cannot process *A. lyrata SRK* transcripts, because the *SRKb* transgene is processed correctly in *A. thaliana* stigmas (Nasrallah *et al.* 2002). Although analysis of additional *A. lyrata SRK* alleles and their processing in *A. thaliana–lyrata* hybrids is required, it is possible that this phenomenon reflects incompatibilities between the *A. thaliana* and *A. lyrata* RNA processing machineries resulting from independent changes that accumulated in the two species since their divergence from a common ancestor ~5 million years ago (Koch *et al.* 2000).

While breakdown of SI occurred in the stigmas of *A. thaliana–lyrata* hybrids, it occurred in pollen of *C. rubella–grandiflora* hybrids in correlation with a 10- to 15-fold reduction in *CgSCR7* gene expression in *S7S0* anthers. This suppression of *CgSCR7* is similar to, albeit not as complete as, the silencing of *SCR* alleles from pollen-recessive *S* haplotypes observed in intraspecific heterozygotes (Kusaba *et al.* 2002; Shiba *et al.* 2002, 2006; Fujimoto *et al.* 2006). Notably, a functional *SCR* allele has been reported in Brassica, which is silenced in the presence of a nonfunctional *SCR* allele (Fujimoto *et al.* 2006), similar to the situation described here. Why particular combinations of *SCR* alleles cause silencing while others do not is not understood. In any case, it is evident that pollen recessiveness based on *SCR* suppression, which allows an *S* haplotype to evade *SRK* surveillance in intraspecific pollinations, can also cause loss of SI upon interspecific hybridization.

Interestingly, the sequences of *CgSRK7* and *CgSCR7* are most similar to the *SRK* and *SCR* genes of the *A. lyrata Sa* haplotype, which also exhibits pollen recessiveness resulting from silencing of its *SCRa* gene, at least in *SaSb* heterozygotes (Kusaba *et al.* 2001). In previous studies, comparison of *S* haplotypes from Brassica and Raphanus identified several cases of trans-genus polymorphisms, whereby pairs of *S* haplotypes were found to determine the same recognition specificity (Sato *et al.* 2004). In one of these intergeneric pairs, the *SRKs* and *SCRs* exhibited ~88% and ~70% amino-acid sequence identity, respectively (Sato *et al.* 2004). *CgSRK7* and *CgSCR7* exhibit similarly high 86 and 74% amino-acid sequence identity to the *A. lyrata SRKa* and *SCRa* genes, respectively. On the basis of this extensive sequence identity and on the basis of a similar genomic organization of *SRK* and *SCR* genes, it is possible that the *C. grandiflora S7* and *A. lyrata Sa* haplotypes may be descended from one ancestral haplotype that existed before the divergence of Capsella and Arabidopsis species ~10 million years ago (Koch *et al.* 2000).

The 3:1 ratio of self-compatible to self-incompatible progeny observed in the *C. rubella–grandiflora* F_2 population is noteworthy for two reasons. First, the dominance of SC over SI inferred from this ratio is unusual for a loss-of-function trait and it also violates the general, albeit not absolute, rule of dominance of SI over SC inferred from intraspecific crosses (Nasrallah 1974; Nasrallah *et al.* 1992, 2004). Second, the 3:1 ratio, although clearly consistent with the segregation of self-fertility as a single *S*-locus-linked trait, is not a simple case of dominance of one allele over another. Rather, this ratio has a more complex basis, with two different phenomena contributing to the majority self-fertile class: homozygosity for the nonfunctional *C. rubella S0* locus, on the one hand, and suppression of the *SCR7* gene in *S7S0* heterozygotes, on the other hand. In any case, the segregation of self-fertility as a single *S*-locus-linked trait in the *C. rubella–grandiflora* F_2 population indicates that the *C. rubella* strain used in our study has not suffered major mutations at other loci required for SI. The strain is therefore similar to the *A. thaliana* C24 accession, which becomes self-incompatible when transformed with functional *SRK* and *SCR* genes from *A.*

lyrata (Nasrallah *et al.* 2004). Whether other strains of *C. rubella*, like other accessions of *A. thaliana* (Nasrallah *et al.* 2002, 2004), have accumulated additional mutations at SI modifier loci remains to be determined.

In summary, our analysis has revealed two mechanisms for loss of SI upon interspecific hybridization in crucifers. While the well-documented changes in genome structure and gene expression (Song *et al.*1995; Soltis and Soltis 1999; Wendel 2000; Ranz *et al.* 2004; Lai *et al.* 2006; Wang *et al.* 2006) that occur upon merging of divergent genomes have been reasonably proposed to underlie phenotypic variability in interspecific hybrids and their allopolyploid derivatives, our results provide a concrete case in which *de novo* changes in expression of specific genes are correlated directly with a specific and adaptive change in phenotype. Although analysis of additional interspecific hybrids generated by crossing plants carrying different *S* haplotypes is required, it is unlikely that the aberrant *SRK* RNA processing in hybrid stigmas and downregulation of *SCR* in hybrid anthers that we observed are restricted to the *AlSRKa* and *CgSCR7* alleles analyzed in this study. Indeed, *C. rubella–grandiflora S8S0* heterozygotes also exhibit breakdown of SI in pollen, suggesting that the *CgSCR8* allele might also be silenced in heterozygous anthers. However, confirming this hypothesis will require the isolation of the *CgSCR8* allele, which has not been accomplished as yet. Because of the extensive polymorphisms of *SRK* and *SCR*, the behavior of individual *S* haplotypes in interspecific hybrids will have to be determined empirically on a case-by-case basis.

The two mechanisms underlying breakdown of SI described here do not result from changes in DNA sequence, and they are reversible and therefore epigenetic in nature. These mechanisms can allow establishment of stable self-fertile hybrid genotypes, promote their reproductive isolation from parental species, and thus facilitate hybrid speciation in nature. Unlike mutation or deletion of *S*-locus recognition genes, which cause irreversible evolutionary switches from SI to self-fertility, these epigenetic processes can cause reversible loss of SI. In addition to allowing self-fertile interspecific hybrids to act as bridges for transfer of functional *S* haplotypes between species, this reversibility would provide nascent interspecific hybrids with flexibility in their selection of mating system, possibly contributing to reproductive success in the face of uncertain ecological conditions.

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