Recent Loss of Self-Incompatibility by Degradation of the Male Component in Allotetraploid *Arabidopsis kamchatica*

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Abstract

The evolutionary transition from outcrossing to self-fertilization (sselfing) through the loss of self-incompatibility (SI) is one of the most prevalent events in flowering plants, and its genetic basis has been a major focus in evolutionary biology. In the Brassicaceae, the SI system consists of male and female specificity genes at the S-locus and of genes involved in the female downstream signaling pathway. During recent decades, much attention has been paid in particular to clarifying the genes responsible for the loss of SI. Here, we investigated the pattern of polymorphism and functionality of the female specificity gene, the S-locus receptor kinase (SRK), in allotetraploid *Arabidopsis kamchatica*. While its parental species, *A. lyrata* and *A. halleri*, are reported to be diploid and mainly self-incompatible, *A. kamchatica* is self-compatible. We identified five highly diverged SRK haplogroups, found their disomic inheritance and, for the first time in a wild allotetraploid species, surveyed the geographic distribution of SRK at the two homeologous S-loci across the species range. We found intact full-length SRK sequences in many accessions. Through interspecific crosses with the self-incompatible and diploid congener *A. halleri*, we found that the female components of the SI system, including SRK and the female downstream signaling pathway, are still functional in these accessions. Given the tight linkage and very rare recombination of the male and female components on the S-locus, this result suggests that the degradation of male components was responsible for the loss of SI in *A. kamchatica*. Recent extensive studies in multiple Brassicaceae species demonstrate that the loss of SI is often derived from mutations in the male component in wild populations, in contrast to cultivated populations. This is consistent with theoretical predictions that mutations disabling male specificity are expected to be more strongly selected than mutations disabling female specificity, or the female downstream signaling pathway.

Introduction

The evolutionary transition from outcrossing to predominant self-fertilization (selfing) is one of the most prevalent events in flowering plants [1–4]. Although increased homozygosity caused by selfing often leads to reduced fitness in the offspring (inbreeding depression), the inherent transmission advantage would favor selfing [5,6]. This is because selfers can transmit gametes in three ways—as both the ovule and pollen donor for their own selfed progeny and as the pollen donor for outcrossed progeny—whereas outcrossers cannot serve as pollen donors for their selfed progeny. Thus, an allele promoting selfing has a 3:2 transmission advantage relative to an allele promoting outcrossing. Selfing would also be advantageous, because selfers can reproduce when pollinators or mates are scarce (“reproductive assurance” [6–9]). Theory suggests that selfing should evolve when these advantages outweigh the costs of inbreeding depression [6,10,11].

In many flowering plants, predominant selfing evolved through the loss of self-incompatibility (SI) [2,3]. SI systems have evolved multiple times in diverse lineages of flowering plants. They generally consist of female and male specificity genes at the S-locus and other genes involved in signaling pathways [12–14]. In SI species, the S-locus region is subject to negative frequency-dependent selection, which is a classic example of multiallelic balancing selection [15]. The molecular basis of the SI system has been studied extensively and is well characterized in the Brassicaceae. Here, female specificity is known to be determined by the S-locus receptor kinase (SRK) and male specificity is determined by the S-locus cysteine-rich protein (SCR; also known as S-locus protein 11, SP11). SRK is a transmembrane serine/threonine receptor kinase that functions on the stigma, and SCR is a small cysteine-rich protein present in the pollen coat that acts as a ligand for the SRK receptor protein [12–14,16–19]. SRK and
Author Summary

Since Charles Darwin recognized the extraordinary diversity of plant mating systems, deciphering their origins has been a central theme for evolutionary biologists. Among various sexual systems, the evolution of self-fertilization (selfing) from cross-fertilization is one of the most frequent transitions in flowering plants, but the genetic basis responsible for these changes is still poorly understood. Here, we have focused on the evolution of selfing in the Brassicaceae, where cross-fertilization is usually enforced by a self-incompatibility (SI) system, determining specific recognition between the pistil (female component) and the pollen (male component). Through sequencing analysis and crossing experiments, we studied the genetic changes leading to the loss of SI in selfing species Arabidopsis kamchatka. We found that the female components of the SI system remain functional in many accessions, suggesting that degradation of the male component was responsible for the loss of SI. Recent studies in the Brassicaceae suggest that such a male-driven loss of SI is more common in wild than in cultivated populations. Furthermore, this pattern is consistent with theories predicting that mutations disabling male specificity have a higher probability of leading to successful mating and are thus more likely to spread than those disabling female specificity.

SCR are tightly linked at the S-locus, where dozens of highly divergent sequence groups, called S-haplogroups (or S-haplotypes or S-alleles), are segregating. S-haplogroups confer specificity in self-recognition: direct interaction between SRK and SCR of the same S-haplogroup leads to the inhibition of pollen germination at the stigma [13,20]. In the Brassicaceae, several genes involved in the female downstream signaling pathway of SRK have also been reported, such as M-locus protein kinase (MLPK) and ARM-repeat containing 1 (ARC1) [13,21–23].

The genetic basis for the recurrent loss of SI has been a major focus from both theoretical and empirical viewpoints [4,24–29]. In particular, much attention has been paid to clarifying which mutations are responsible for the loss of SI, and whether these mutations are in the female specificity gene, the male specificity gene or in downstream signaling pathways [4,24–29]. With the advantage of a suite of molecular tools, the most extensively studied species in the Brassicaceae is the diploid self-compatible Arabidopsis thaliana [4,29–42]. Whereas a number of mutations disabling the male and female components have been identified in wild accessions of A. thaliana [30,33,36,38,39], many accessions still retain full-length and expressed SCR sequences [38,41]. Importantly, interspecific crossings using the self-incompatible Arabidopsis halleri revealed that some A. thaliana accessions, including Wei-1, retain a functioning female SI reaction, demonstrating that all female components including SRK and the female downstream signaling pathway are still functional [41]. In addition, Tsuchimatsu et al. [41] reported that a 213-base-pair (bp) inversion (or its derivative haplotypes) in the SCR gene is found in 95% of European accessions. When the 213-bp inversion in SCR was inverted and expressed in transgenic Wei-1 plants, the functional SCR restored the SI reaction. These results suggested that degradation of SCR (the male specificity gene) was primarily responsible for the evolutionary loss of SI of the S-haplogroup in European A. thaliana, while other mutations at genes involved in the downstream signaling pathway might have contributed to some extent [35,39].

To understand whether such mutations in the male components of the S-locus are common in the recurrent evolution of self-compatibility in the Brassicaceae, empirical examples need to be investigated in other self-compatible species. In addition to A. thaliana, there are a few reports on the pattern of polymorphism at the S-locus in self-compatible species in Brassicaceae, such as Capsella rubella [43] and Lepidium th充实loides [44]. However, the genetic and molecular bases responsible for the loss of SI are still unknown in these species. A major obstacle to charting the history of the S-locus in self-compatible species has been in distinguishing the primary inactivating mutation from subsequent decay of the nonfunctional S-haplogroups by further mutations. This is because all genes involved in this signaling pathway are expected to be released from selection pressure and to evolve neutrally once the SI system ceases to function [4,28,39,41], although pleiotropy of these genes could play a role in maintaining the functionality of the signaling pathway [42,45]. To study the primary mutations, a powerful approach would be to combine experiments confirming gene function with population genetic analyses finding gene-disruptive mutations.

Arabidopsis kamchatka would be a good model system to address this issue. It is a self-compatible species, and originated through allopolyploidization of two species, A. halleri and A. lyrata, which are reported to be diploid [46–51]. Shimizu-Inatsugi et al. [48] reported that multiple haplotypes of nuclear and chloroplast sequences of A. kamchatka are identical to those of their parental species, indicating that multiple diploid individuals of A. halleri and A. lyrata contributed to the origin of A. kamchatka. In particular, A. kamchatka and A. halleri share four chloroplast haplotypes, strongly suggesting that at least four diploid individuals of A. halleri contributed independently to the multiple origins of A. kamchatka [48]. The two parental species are predominantly self-incompatible, although some of North American populations of A. lyrata are known to be self-compatible [26,29]. Their SI systems have been extensively studied [26,52–61]. Most of these studies have focused on SRK to characterize S-haplogroups, because novel SRK haplogroups can be isolated relatively easily by using PCR primers that were designed in the conserved regions of SRK [52,55], while much fewer SCR sequences have been isolated because of its extreme polymorphism [30,40,62,63]. To date, 40 and 30 SRK haplogroups have been reported in A. lyrata and A. halleri, respectively, and studies of nucleotide polymorphisms and divergence using large sets of SRK sequences revealed various characteristics of the S-locus, such as the spatial distribution of S-haplogroups, complex dominance interactions and transspecific sharing of S-haplogroups among species [55–57,59,60,64]. The wealth of knowledge available on these parental species enabled us to investigate nucleotide polymorphisms of the S-locus in self-compatible A. kamchatka. In addition, A. kamchatka would be a novel model to investigate the mechanism underlying the loss of SI in polyploid species. The relationship between self-compatibility and polyploidy has been debated for more than 60 years, as it is argued that polyploids have higher selection rates than their diploid relatives [1,65] (but see also [66] for controversy). Hypotheses have been proposed to explain this association, such as: (1) self-compatible individuals in polyploids would not suffer from limitation of mates of the same ploidy level [1,66–70]; and (2) inbreeding depression would be reduced by having multiple gene copies [10,66,71,72]. Despite numerous ecological and evolutionary studies, the molecular mechanisms underlying the evolution of self-compatibility of polyploid species are still poorly understood.

To understand the mechanisms underlying the loss of SI in A. kamchatka, we first isolated SRK haplogroups from A. kamchatka by examining 48 populations across its distribution range (Table S1).
Based on the analyses of nucleotide divergence from parental species and the distribution of SRK haplogroups with respect to population structure, we studied how S-haplogroups in *A. kamchatctica* have originated from the parental species. To understand the approximate timescale of this evolutionary event, we also estimated the divergence time of *A. kamchatctica* from its parental species based on the nucleotide divergence of multiple nuclear genes other than SRK. This is because speciation time would be used as the upper boundary of the time estimate of the evolution of self-compatibility in a species, when the progenitor species was self-incompatible [43,73]. We tested the function of SRK haplogroups through interspecific crossing with self-incompatible *A. halleri*, and confirmed the disomic inheritance and allelic relationships of SRK haplogroups by segregation analyses in experimental and natural populations of *A. kamchatctica*. Most importantly, our interspecific crossing with *A. halleri* also indicated the retained function of the female component of SI in *A. kamchatctica*, suggesting that the degradation of male components was responsible for the loss of SI. We suggest that the degradation of male components among the Brassicaceae might represent a general trend in the evolution of self-compatibility in wild populations in contrast to cultivated populations.

**Results**

**Five highly diverged SRK-like sequences in *A. kamchatctica* revealed by PCR–based screening**

Through PCR-based screening, we obtained five partial SRK-like sequences from *A. kamchatctica*, named *AhSRK-A*, *AhSRK-B*, *AhSRK-C*, *AhSRK-D* and *AhSRK-E*. Our five SRK sequences were aligned with S-domain sequences available for SRK from *A. halleri* and *A. lyrata* [52,54,55,58,59,61], and a phylogenetic tree including a total of 76 SRK sequences was generated using the neighbor-joining method (Figure 1). While previous studies reported that the SRK haplogroups are transspecifically shared among *A. halleri*, *A. lyrata* and *A. thaliana* [59,62], the tree clearly shows that the SRK haplogroups are also transspecifically shared between *A. kamchatctica*, *A. halleri* and *A. lyrata* (Figure 1).

In *A. lyrata* and *A. halleri*, SRK sequences that presumably share the same specificities are highly homologous, while sequences with different specificities show at most 91–92% nucleotide sequence identity [59,74]. Here, four out of the five *A. kamchatctica* SRK sequences (*AhSRK-A*, *AhSRK-C*, *AhSRK-D* and *AhSRK-E*) showed more than 98% sequence identity to SRK sequences previously reported in both *A. halleri* and *A. lyrata* (Figure 1), suggesting that they also share specificities with the corresponding *A. halleri* and *A. lyrata* SRK sequences. In contrast, no previously reported sequence showed any particularly high similarity with *AhSRK-B*. The most similar ones were *AhSRK12* (81% identity over 576 bp) and *AhSRK23* (87% identity over 558 bp), suggesting that they are unlikely to share specificity with *AhSRK-B*. Using specific primers for *AhSRK-B*, we successfully amplified a sequence from an *A. halleri* plant (lowland habitat in Western Honshu, Japan; No. 61 in Table S1) that showed 100% sequence identity to *AhSRK-B* of *A. kamchatctica* and, as shown later by the interspecific crosses, they shared the functional specificity of SI. This newly discovered *A. halleri* ortholog was named *AhSRK-B* (Figure 1). Using specific primers, we also isolated orthologous sequences of *AhSRK-A* and *AhSRK-C* from *A. halleri*, which were nearly identical to *AhSRK26* and *AhSRK01*, respectively (Figure S1). These sequences were named *AhSRK26-Ibuki* and *AhSRK01-Ibuki*, respectively (see the section “Retained full-length SRK sequences as well as multiple gene-disruptive mutations” for details).

**Nucleotide divergence of *AkSRK* from orthologous genes of the parental species *A. halleri* and *A. lyrata***

Despite their transspecificity, several species-specific nucleotide substitutions have been reported within the same S-haplogroups in *A. lyrata* and *A. halleri*, respectively [61]. Thus, to obtain insight into which parental species the SRK-like sequences found in *A. kamchatctica* were derived from, we compared the nucleotide divergence of SRK-like sequences of *A. kamchatctica* with corresponding orthologous genes from *A. halleri* and *A. lyrata*. *AkSRK-A* and *AkSRK-C* were closer to their orthologs of *A. halleri* (*AhSRK26* and *AhSRK01*, respectively) than to those of *A. lyrata* (*AlSRK22* and *AlSRK01*, respectively) (Figure 2; Figure S1; Table S2). Conversely, *AkSRK-D* and *AkSRK-E* showed higher sequence identity to orthologs from *A. lyrata* (*AlSRK42* and *AlSRK17*, respectively) (Figure 2; Figure S1; Table S2). These results suggest that *AkSRK-A* and *AkSRK-C* are derived from *A. halleri* and that *AkSRK-D* and *AkSRK-E* are derived from *A. lyrata*. In addition, because we found that *AhSRK-B* in *A. halleri* showed 100% identity to *AkSRK-B* in *A. kamchatctica*, *AkSRK-B* is most likely derived from *A. halleri*. Based on this pattern of nucleotide divergence from the parental species, mutually exclusive distribution of SRK haplogroups and a segregation analysis in the *F2* population (see below), hereafter we denote *AkSRK-A*, *AkSRK-B* and *AkSRK-C* as the “*A. halleri*-derived SRK” and *AkSRK-D* and *AkSRK-E* as the “*A. lyrata*-derived SRK” (see below and Discussion).

**Distribution and frequency of five S-haplogroups revealed by PCR–based genotyping**

Using PCR-based genotyping of SRK haplogroups, we investigated the frequencies and geographic distribution of the five S-haplogroups identified in this study. Altogether, 49 accessions from 46 populations were genotyped by primer pairs that could specifically amplify *AkSRK-A*, *AkSRK-B*, *AkSRK-C*, *AkSRK-D* or *AkSRK-E* (Figure 3; Figure 4; Table S1; see Table S3 for the primer pairs used). Two copies of SRK were amplified from all accessions except those from Hokkaido in Japan that had only one copy (Nos. 25, 27 and 28), and one from Kamchatka in Russia that had three copies (No. 33). This finding is consistent with reports that *A. kamchatctica* is a self-compatible allotetraploid, which usually harbors two homeologs from the parental species, supposing rare heterozygosity because of selfing and rare duplication [46,48].

The distributions of *AkSRK-A*, *AkSRK-B* and *AkSRK-C* were mutually exclusive except for one accession (No. 33, see below) and showed a strong geographic structure. *AkSRK-A* and *AkSRK-B* were found in about half of our samples (46.9% and 41.8%, respectively; Figure 3; Figure 4); all *AkSRK-A* were found in the southwestern part of the distribution range of the species (Taiwan and Japan), whereas *AkSRK-B* was mainly located in the northern and eastern part of the species range (North America and the Kamchatka Peninsula in Russia; Figure 3; Figure 4; Table S1). In contrast, the frequency of *AkSRK-C* was lower than those of *AkSRK-A* and *AkSRK-B* (3.1%; Figure 3; Figure 4), and was found only in three accessions from the Kamchatka Peninsula (Figure 3; Figure 4). One of them (No. 33) harbored both *AkSRK-B* and *AkSRK-C*, indicating heterozygosity or duplication of the *A. halleri*-derived SRK. *AkSRK-B* and *AkSRK-C* sequences. We further genotyped nine additional individuals in that population (Table S4) and found that *AkSRK-B* and *AkSRK-C* segregated at intermediate frequencies (*AkSRK-B*: 0.55; *AkSRK-C*: 0.45). This is consistent with the hypothesis that No. 33 is a rare heterozygote of *AkSRK-B* and *AkSRK-C*, because it would occasionally arise even by rare outcrossing events under predominant selfing, in particular when the allele frequencies were intermediate. Moreover, the distributions of *AkSRK-D* and *AkSRK-E* were
completely mutually exclusive in our 49 samples and AkSRK-D was nearly fixed (91.8% frequency; Figure 3; Figure 4).

The geographic distribution of the A. halleri-derived S-haplogroups is concordant with the population structure inferred from polymorphisms of four other nuclear loci (two homeologous genes each of WER and CHS) (Figure 3). The high values of the mean posterior probability of data lnP(X|K), AK and the symmetric similarity coefficient supported the clustering of K = 2, which reflect the spatial structure of the distribution well [Figure 3; Figure S2; Table S1]. The distributions of A. halleri-derived S-haplogroups—AkSRK-A, AkSRK-B and AkSRK-C—are significantly correlated with the population structure (Cramer’s coefficient: 1; p = 7.62 × 10^{-15}); that is, most of accessions bearing AkSRK-A belong to cluster 1 (orange in Figure 3) and most of others belong to cluster 2 (blue in Figure 3). This significant correlation indicates that the pattern of distribution of these A. halleri-derived S-haplogroups is consistent with the genome-wide pattern of polymorphism. The correlations were also significant with the clustering of K = 3 (Cramer’s coefficient: 0.675; p = 6.37 × 10^{-11}) and of K = 4 (Cramer’s coefficient: 0.577; p = 5.39 × 10^{-15}). In contrast, the distributions of A. lyrata-derived S-haplogroups—AkSRK-D and AkSRK-E—are not correlated significantly with the
population structure (Cramer’s coefficient: 0.302; \( p = 0.109 \)). The correlations were also not significant with the clustering of \( K = 3 \) (Cramer’s coefficient 0.194; \( p = 0.393 \)) and of \( K = 4 \) (Cramer’s coefficient 0.163; \( p = 0.517 \)). In fact, the frequency of \( \text{AkSRK-D} \) is markedly higher than that of \( \text{AkSRK-E} \) and it is widespread throughout the geographically wide range, resulting in no significant correlation between these \( A. \text{lyrata} \)-derived \( S \)-haplogroups and population structure.

In \( A. \text{kamchatka} \) subsp. \( kawasakiana \) (formerly, \( A. \text{lyrata} \) subsp. \( kawasakiana \)), a previous survey of \( \text{SRK} \) identified two sequences, \( \text{Aly13-1} \) and \( \text{Aly13-22} \) [57]. The result of our PCR-based genotyping is consistent with those findings, as we also found \( \text{AkSRK-A} \) (orthologous to \( \text{Aly13-22} \)) in all accessions of \( A. \text{kamchatka} \) subsp. \( kawasakiana \) (Table S1; Figure 3). However, \( \text{AkSRK-C} \) (orthologous to \( \text{Aly13-1} \)) was not found in our samples of subsp. \( kawasakiana \) but only in subsp. \( kamchatka \).

Estimation of the divergence time from parental species

Using the nucleotide divergence estimate \( K \) and the synonymous substitution rate \( K_s \) of four nuclear loci (two homeologous genes each of \( \text{CHS} \) and \( \text{WER} \); Table S5), we estimated the divergence time of \( A. \text{kamchatka} \) from the parental species (Table S6). When we employed the mutation rate given by Koch et al. [75], which is based on the synonymous substitution rate calibrated by fossil records, the mean divergence time was 20,417 years (with a 95% confidence interval of 0–75,460 years). When we employed the mutation rate given by Ossowski et al. [76], estimated using mutation accumulation lines, the mean divergence time was 245,070 years (with a 95% confidence interval of 37,385–532,953 years). Overall, estimates based on the mutation rate given by Koch et al. [75] were smaller than those based on Ossowski et al. [76], although the 95% confidence intervals overlapped.

Two clusters were suggested in the analysis of population structure (Figure 3). Whereas here we estimated the divergence averaged over population clusters, it is possible that it varies between clusters, given that population structure is profoundly affected by the multiple origins of \( A. \text{kamchatka} \) [48]. We also calculated the clustertoever nucleotide divergence from \( A. \text{halleri} \) and \( A. \text{lyrata} \) but no significant difference from each other was found in the current dataset and clustering (Table S5).

\( \text{AkSRK-A} \) and \( \text{AkSRK-B} \) are allelic with each other and disomically inherited

Based on the pattern of nucleotide divergence from the parental species and the mutually exclusive distribution of \( \text{SRK} \), we predicted the following allelic relationships: the \( A. \text{halleri} \)-derived
SRK (AkSRK-A, AkSRK-B and AkSRK-C) should be allelic and the A. lyrata-derived SRK (AkSRK-D and AkSRK-E) should be allelic with each other, respectively. This prediction also assumes the disomic inheritance of the A. halleri- and A. lyrata-derived SRK, respectively.

To confirm our predictions, we investigated the pattern of segregation of the A. halleri-derived SRK (AkSRK-A and AkSRK-B) in an F2 population that was generated by crossing individuals bearing AkSRK-A and AkSRK-D, and individuals bearing AkSRK-B and AkSRK-D (Table 1). We genotyped 95 F2 individuals with specific primers for AkSRK-A and AkSRK-B, and compared the goodness-of-fit of four inheritance models: (1) disomic and allelic, (2) disomic and nonallelic, (3) tetrasomic and allelic and (4) tetrasomic and nonallelic (Table 1). We found that the observed pattern of segregation better fitted model (1), i.e., the disomic and allelic model, rather than the other three models (p = 0.27; Table 1). We also confirmed the amplification of AkSRK-D in all 95 F2 plants and in the F1 generation, which indicates that AkSRK-D segregates neither with AkSRK-A nor with AkSRK-B in the F2 population.

Retained full-length SRK sequences as well as multiple gene-disruptive mutations

We isolated entire coding sequences of SRK from several A. kamchatica individuals for each haplogroup (three AkSRK-A, three AkSRK-B, one AkSRK-C, five AkSRK-D and one AkSRK-E; Figure 5 and Table S1). In addition, we also isolated entire coding sequences of orthologs of AkSRK-A, AkSRK-B and AkSRK-C from A. halleri. Alignment of the coding regions of SRK from A. kamchatica and A. halleri indicates that at least one accession of four haplogroups—A, B, D and E—retains the full-length SRK, without any apparent disruptive mutations such as frameshifts or inverted repeats. Furthermore, four single nucleotide polymorphisms were identified among three sequences of AkSRK-A and 13 among five sequences of AkSRK-D (Figure 5).

No obvious gene-disruptive mutations were found in the sequences of A. halleri, but we found three in multiple haplogroups of A. kamchatica (Figure 5; Figure S3). First, we found that AkSRK-A from an accession from Bivako, a lowland region of Japan, contained an approximately 1,700-bp insertion in exon 6. PCR-based genotyping revealed that this insertion is shared by all the accessions of A. kamchatica subsp. kawasakiana living in lowland regions in western Japan (Table S1; see Table S3 for the PCR primers used). We also found a 1-bp deletion in AkSRK-C from a Kamchatka accession that caused a frameshift. In addition, we found that AkSRK-D of an accession from mountains in central Honshu contains a 45-bp deletion in exon 1. Although this deletion does not change the reading frame, it is likely to abolish the recognition function, because it lies within the S-domain and...
encompasses one of the 12 conserved cysteine residues suggested to be important for protein structure [77,78] (Figure 5; Figure S3; see also the section on interspecific crosses for the degraded female SI in the Murodo accession).

Interspecific crosses between *A. halleri* and *A. kamchatica* suggest that degradation of the male components was responsible for the loss of SI

To test whether these full-length SRK sequences and other components involved in the female signaling pathway are functional in *A. kamchatica*, we first conducted interspecific crosses between *A. halleri* (male) and *A. kamchatica* (female). As *S*-haplogroups are shared transspecifically among *A. lyrata*, *A. halleri* and *A. kamchatica* (Figure 1), an incompatible reaction should occur even in interspecific crosses in which pollen and stigma share the same haplogroup [41]. We used the three most frequent haplogroups in *A. kamchatica*—A, B and D—as all of the accessions surveyed contain at least one of them.

In eight accessions of *A. kamchatica*, incompatible reactions were observed when pollen of *A. halleri* was used to pollinate pistils of *A. kamchatica* sharing the same haplogroups (Figure 6). These interspecific crosses verified the shared specificities of the SI system between *A. halleri* and *A. kamchatica*. More importantly, these results indicate that the female components of the SI system are functional in these accessions of *A. kamchatica*. Specifically, we found incompatible reactions in the following combinations of crosses: pollen of *A. halleri* bearing haplogroup A with pistils of *A.
kamchatka accessions bearing AkSRK-A from Murodo and Tsurugigozen; pollen of A. halleri bearing haplogroup D with pistils of A. kamchatka bearing AkSRK-D from Biwako and Potter; and pollen of A. halleri bearing haplogroup B with pistils of A. kamchatka bearing AkSRK-B from Darling Creek and Potter. In the Potter accession, incompatible reactions were observed when haplogroups B and D of A. halleri were pollinated, respectively, suggesting that these haplogroups are codominant on pistils.

As control experiments, we also carried out crosses of the following combinations: (1) where the $S$-haplogroups of pollen and stigmas differ, and (2) where the SRK of A. kamchatka possessed any gene-disruptive mutations (Figure 6). In these combinations, we consistently observed compatible reactions, indicating that the reduced pollen growth observed in this experiment was caused not by interspecific reproductive isolation between A. halleri and A. kamchatka, but by the SI system.

It is worth noting that, in the Murodo accession, incompatible reactions were observed when plants were pollinated by A. halleri containing haplogroup A, but not by those containing haplogroup D. Because all components of the signaling pathway except for SRK are thought to be shared among different specificities, the data strongly suggest that a mutation in AkSRK-D—most likely the 43-bp deletion—was responsible for this decay of the female SI reaction. Likewise, compatibility of haplogroup A of the Biwako population is also attributable to a mutation in SRK, the approximately 1,700-bp insertion, because the functionality of the downstream signaling pathway was shown by crosses with A. halleri containing haplogroup D.

Because all these accessions of A. kamchatka are self-compatible and retain functional female components of SI including downstream signaling pathways, the data indicate that the male components of $S$-haplogroups A, B and D are not functional in these accessions. To confirm the degradation of the male components, we conducted interspecific crosses between A. halleri (female) and A. kamchatka (male), using A. halleri bearing haplogroups A, B or D as pistil donors and A. kamchatka as a pollen donor. In these combinations, we consistently observed compatible reactions, indicating that the male components of S-haplogroups A, B and D are not functional in these accessions of A. kamchatka (Figure S4). In addition, we conducted intraspecific crosses among A. kamchatka, with the Biwako accession as a pollen donor and the Murodo accession, which retains the functional female specificity of haplogroup A, as a pistil donor (Table S7). Incompatible reactions were not observed, thus excluding the possibility that the male components of haplogroup A of the Biwako accession remain functional. Similarly, we found that the male components of haplogroup D of Murodo are also not functional. All these data confirm that the male components of haplogroups A, B and D are not functional in these accessions of A. kamchatka. These nonfunctional male components and functional female components including downstream signaling pathways suggest that degradation of the male components was primarily responsible for their loss of SI. We do not exclude the possibility that recombination events between the male and female components on the S-locus may have also been involved, although their occurrence is reported to be very rare (reviewed in [56]).

Discussion

A. halleri– and A. lyrata–derived homologs of the S-locus in allotetraploid A. kamchatka

In this study, we identified the full-length SRK sequences of five S-haplogroups in A. kamchatka (Figure 1). Through interspecific crosses with A. halleri, we confirmed that the intact SRK sequences of the three most frequent S-haplogroups in our dataset—AkSRK-A, AkSRK-B and AkSRK-D—are indeed associated with the female specificities of SI. Although associations with SI specificities for the other less frequent haplogroups—AkSRK-C and AkSRK-E—were not confirmed experimentally, they also exhibited very high similarities with known SRK sequences from A. lyrata and A. halleri (>98%), suggesting that specificities of SI are shared between species [59,74].

Our investigation on nucleotide polymorphism and divergence, as well as the pattern of segregation in natural and experimental populations, allows us to address how these five S-haplogroups in allotetraploid A. kamchatka originated from their parental diploid species, A. halleri and A. lyrata. First, AkSRK-A and AkSRK-C of A. kamchatka are closer to the orthologs of A. halleri than to those of A. lyrata, and AkSRK-D and AkSRK-E of A. kamchatka are closer to the orthologs of A. lyrata than to those of A. halleri (Figure 2; Figure S1; Table S2). We also found that AkSRK-B in A. kamchatka showed 100% identity to AkSRK-B in A. halleri, although its ortholog of A. lyrata was not isolated in the present study. Second, the distributions of AkSRK-A, AkSRK-B and AkSRK-C were mutually exclusive, as were those of AkSRK-D and AkSRK-E, except for a minor presumable heterozygote of AkSRK-B and AkSRK-C (Figure 3; Figure 4). Third, the pattern of segregation in the F2 population significantly supports the model in which AkSRK-A and AkSRK-B are allelic while AkSRK-D is not allelic to them (Table 1). In addition, the pattern of polymorphism within a Kamchatka population is consistent with the model in which AkSRK-B and

Table 1. The pattern of segregation of AkSRK-A and AkSRK-B in 95 F2 individuals.

|          | A/B | A/- | -/B | -/- | $\chi^2$ | P-value |
|----------|-----|-----|-----|-----|----------|---------|
| Observed | 53  | 25  | 17  | 0   |          |         |
| Expected under the disomic and allelic model | 47.5 (2/4) | 23.5 (1/4) | 23.5 (1/4) | 0 (0/4) | 2.62 | 0.27 |
| Expected under the disomic and nonallelic model | 53.4 (9/16) | 17.8 (3/16) | 17.8 (3/16) | 5.9 (1/16) | 8.88 | 0.031 |
| Expected under the tetrasomic and allelic model | 89.7 (34/36) | 2.64 (1/36) | 2.64 (1/36) | 0 (0/36) | 92.73 | <2.2×10^{-16} |
| Expected under the tetrasomic and nonallelic model | 89.8 (1225/1296) | 2.57 (35/1296) | 2.57 (35/1296) | 0.073 (1/1296) | 102.84 | <2.2×10^{-16} |

A/B, Amplified by both the AkSRK-A- and AkSRK-B-specific primers. A/-, Amplified only by the AkSRK-A specific primer. -/B, Amplified only by the AkSRK-B-specific primer. -/-, Amplified by neither the AkSRK-A- nor the AkSRK-B-specific primer. Expected frequencies in each category are shown in parentheses. We confirmed the amplification of AkSRK-D in all 95 F2 plants and in the F1 plants. doi:10.1371/journal.pgen.1002838.t001
AkSRK-A are also allelic and segregating in a local population (Table S4). These independent lines of evidence suggest that AkSRK-A, AkSRK-B and AkSRK-C are S-alleles of the A. halleri-derived S-locus and that AkSRK-D and AkSRK-E are S-alleles of the A. lyrata-derived S-locus. Although we confirmed the association between AkSRK-D and the SI specificity of A. halleri in this study, the specificity is also likely to be shared with A. lyrata (discussed in [59,61,62]). While there are a few reports on the evolutionary history of the S-locus in cultivated allotetraploid species, particularly Brassica napus [79,80], to our knowledge, this study is the first to demonstrate clearly how multiple S-haplogroups in a wild allotetraploid species originated from the parental species and spread throughout a geographically wide area.

**Pattern of nucleotide polymorphism and divergence of the S-locus**

Shimizu-Inatsugi et al. [48] reported that multiple haplotypes of nuclear and chloroplast sequences were shared between allotetraploid A. kamchatica and its parental diploid species, suggesting independent origins of A. kamchatica. In particular, A. kamchatica and A. halleri share four identical chloroplast haplotypes, suggesting that at least four diploid individuals of A. halleri contributed independently to the multiple origins of A. kamchatica [48]. As three of the four suggested independent origins are manifested as distinct clusters of population structure, independent origins combined with range expansion out of Asia appear to affect the population structure of A. kamchatica profoundly [48]. A comparison between the geographic distribution of S-haplogroups and the population structure inferred from other loci thus illustrates how independent origins of A. kamchatica contributed to form the current pattern of polymorphism of two S-loci: the A. halleri-derived S-locus and the A. lyrata-derived S-locus. We found that the distribution of three A. halleri-derived S-haplogroups was significantly correlated with population structure. Given that the population structure of A. kamchatica is profoundly affected by its multiple independent origins, the concordance between population structure and the

![Figure 5. Entire coding sequences of SRK of five S-haplogroups from A. kamchatica and three S-haplogroups from A. halleri.](doi:10.1371/journal.pgen.1002838.g005)
### A. kamchatica (4n)

| Location                  | Haplogroup A | Haplogroup D | Biwako (1 accession) |
|---------------------------|--------------|--------------|----------------------|
| Murodo (2 accessions)     | 0/5          | 0/4          | 5/5                  |
| Tsurugi-Gozen (3 accessions) | NP           | 0/6          |                      |
|                           | 3/3          | 6/6          | 6/6                  |

### A. halleri (2n)

| Location                  | Haplogroup A | Haplogroup D | Biwako (1 accession) |
|---------------------------|--------------|--------------|----------------------|
|                           | 5/5          | 0/6          |                      |
|                           | 3/3          | 6/6          |                      |

### A. kamchatica (4n)

| Location                  | Haplogroup B | Haplogroup D |
|---------------------------|--------------|--------------|
| Darling Creek (1 accession) | 0/5          | NP           |
| Potter (1 accession)      | 0/5          | NP           |

### A. halleri (2n)

| Location                  | Haplogroup B | Haplogroup D |
|---------------------------|--------------|--------------|
|                           | NP           | NP           |
|                           | 5/5          | NP           |
distribution of A. hallieri-derived S-haplogroups suggests that the composition of the gene pool of the A. hallieri-derived S-locus would be explained at least partially by the independent origins of this species. In contrast, there was no significant correlation found for A. kamchatica sequence of Biwako has a 45-bp deletion including one of the 12 conserved cysteine residues (see text). The A. kamchatica-A sequence of Biwako has a ~1,700-bp insertion in exon 6 (see text and Table S1). Note that full-length sequences of AksRK-D from Tsurugi-Gozen were not confirmed and that crosses with A. hallieri bearing haplogroup D were not conducted (indicated by asterisks). (D) Crosses using A. kamchatica from Darling Creek and Potter, bearing AksRK-B and AksRK-D. Two A. hallieri plants that do not bear AksRK-A, AksRK-B or AksRK-D were also used as pollen donors (indicated as “Other haplogroups”; see Methods). (C, D) Numerators denote crosses where more than 20 pollen tubes penetrated the stigma, indicating compatible reactions. Denominators show the total number of crosses conducted in each combination. NP: not performed.

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Figure 6. Interspecific crosses between A. hallieri and A. kamchatica. (A, B) Representative incompatible (A) and compatible (B) reactions on A. kamchatica stigmas. Crosses were carried out between A. hallieri bearing haplogroup B and A. kamchatica bearing haplogroup B (B), and between A. hallieri not bearing haplogroup B and A. kamchatica bearing haplogroup B (B). A bundle of 20 pollen tubes indicates a compatible reaction (yellow arrow). Scale bars = 0.1 mm. (C) Crosses using A. kamchatica from Murodo, Tsurugi-Gozen and Biwako, bearing AksRK-A and AksRK-D. The AksRK-D sequence of Murodo has a 45-bp deletion including one of the 12 conserved cysteine residues (see text). The AksRK-A sequence of Biwako has a ~1,700-bp insertion in exon 6 (see text and Table S1). Note that full-length sequences of AksRK-D from Tsurugi-Gozen were not confirmed and that crosses with A. hallieri bearing haplogroup D were not conducted (indicated by asterisks). (D) Crosses using A. kamchatica from Darling Creek and Potter, bearing AksRK-B and AksRK-D. Two A. hallieri plants that do not bear AksRK-A, AksRK-B or AksRK-D were also used as pollen donors (indicated as “Other haplogroups”; see Methods). (C, D) Numerators denote crosses where more than 20 pollen tubes penetrated the stigma, indicating compatible reactions. Denominators show the total number of crosses conducted in each combination. NP: not performed.

Dominance interactions among S-haplogroups and the loss of SI

The molecular mechanism underlying the loss of SI in polyploid species is still not well understood, although the relationship between polyploidy and self-compatibility has been debated for more than 60 years [1,63,66]. Whereas polyploidization is known to disrupt SI almost invariably in gametophytic SI systems where specificities are determined by haploid genomes of gametes [97,98], in sporophytic SI systems where specificities are determined sporophytically by the diploid parental genotypes, polyploidization in itself does not necessarily induce the loss of SI (e.g., [57]). We speculate that, in sporophytic SI systems such as those of the Brassicaceae, dominance interactions among S-haplogroups might have played an important role in the loss of SI in polyploid species, as is also implied in a study of synthetic interspecific hybrids by Nasrallah et al. [99]. In the Brassicaceae, complex dominance interactions among S-haplogroups have been reported [52,55,56,60,100–103]. Given that the female components are functionally intact in A. kamchatica, as shown by our interspecific crosses, both homeologous copies of the SCR gene ought to have lost their function. However, gene-disruptive mutations at both of the homeologous SCR genes might not necessarily be required if the dominant SCR harboring a gene-disruptive mutation suppresses the expression of the recessive SCR [30,104]. Thus, dominance interactions could facilitate the evolution of self-compatibility by a single mutation in polyploid species. This is indeed suggested for Brassica napus, which is also an allotetraploid species that originated from hybridization of B. rapa and B. oleracea [80]. In a cultivar of B. napus, neither of the homeologous SP11/SCR genes is expressed. In artificially synthesized B. napus lines, SP11/SCR alleles from B. rapa showed dominance over SP11/SCR alleles from B. oleracea, suggesting that the nonfunctional dominant SP11/SCR allele suppressed the expression of the recessive SP11/SCR allele [80].

In A. lyrata and A. hallieri, such dominance relationships between S-haplogroups have been characterized. Thus, the S-haplogroup of S12 in A. hallieri, which is orthologous to haplogroup D of A. kamchatica, has been suggested to belong to a relatively dominant class [60]. Given that the dominance relationship is consistent between A. hallieri and A. kamchatica, haplogroup D would also be dominant in A. kamchatica and might have suppressed the expression of its homeologous counterparts, such as haplogroup C, which is orthologous to the most recessive S-haplogroup of S1 in A. lyrata and A. hallieri [52,60,61,103]. While we showed that haplogroups B and D are codominant in pistils in A. kamchatica
(Figure 6), their dominance rank might be different in pollen, as dominance is known to occur more frequently in pollen than in pistils [60].

It is worth noting that haplogroup C, which is orthologous to the most recessive S-haplogroup of S1 in A. lyrata and A. halleri, was found at a relatively low frequency in A. kamechatica (5.1%). In contrast, S1 shows the highest frequencies in self-incompatible populations of A. lyrata (53%; [53]) and A. halleri (26.3%; [60]), because recessive haplogroups are hidden from negative frequency-dependent selection in heterozygotes, whereas the most dominant S-haplogroups are always exposed to selection [60,105,106]. While random genetic drift due to population bottlenecks could explain this contrasting change in frequency, we hypothesize that dominant haplogroups are more likely to be found in self-compatible populations. This is because a dominant haplogroup with gene-disruptive mutations should repress the expression of another specificity, and thus spread more rapidly than a recessive self-compatible mutation [42]. Consistent with this hypothesis, a nearly fixed haplogroup in A. thaliana has been shown experimentally to be a dominant allele in A. halleri [42,60].

The molecular basis of the dominance relationship has recently been unveiled in Brassica, where the expression of the recessive haplogroups is specifically silenced by methylation of promoter Brassica been unveiled in A. kamchatica [61]. A. thaliana hypothesis, a nearly fixed haplogroup in than a recessive self-compatible mutation [42]. Consistent with this haplogroup with gene-disruptive mutations should repress the function of the male component, possibly SCR. Because extensive studies in wild Brassicaceae species demonstrate that the evolution of self-compatibility tends to be driven by mutations in the male rather than in the female components (Table 2).

In contrast, in cultivated Brassica, extensive functional analyses have identified gene-disruptive mutations both in male and in female components, and the pattern is significantly different from that observed in wild populations (Table 2; Table S8; two-tailed Fisher’s exact test \( p = 0.0476; \) one-tailed \( p = 0.0230; \) [79,80,111,112]). This contrasting pattern suggests that the male-skewed frequency of mutations found in recent studies of wild populations could be attributed to the process of natural selection and spread in wild populations [25,29], rather than to the mechanistic natures of mutations, such as differences in mutation rate between male and female components. If such mechanistic reasons were important, similar patterns would have been observed in both wild and cultivated populations. Indeed, the context of selective pressure would be very different between wild and cultivated populations. In a wild population, as mentioned above, mutations disabling SI would be selected in terms of the transmission advantage, i.e., number of compatible mates in a population [25,29]. In cultivated populations, these gene-disruptive mutations would be positively selected by humans based on the self-compatible phenotype per se. Moreover, because the female SRR gene is about 10 times longer than the male SCR, it may decay even faster than the male component during domestication, as indeed shown in cultivated Brassica (Table 2; Table S8).

Conclusion and future perspectives

Our combination of population genetic analyses and crossing experiments suggests that degradation of the male components was primarily responsible for the loss of SI in A. kamechatica. As we have demonstrated in the present study, functional confirmations, such as crossings between individuals sharing the same SI specificity, will further corroborate the genetic basis for the loss of SI in other selfing species. First, sequence analysis alone cannot provide definitive evidence of gene function. For example, in previous studies of the evolution of self-compatibility in A. thaliana, a splicing mutation in the female gene SRR-B was found in the Cvi-0 accession while the male gene SCR-B did not have any obvious gene-disruptive mutations [30]. However, transgenic experiments suggested that SCR-B was also nonfunctional, possibly due to some amino acid changes, so it is not clear whether the primary mutation occurred in male or female components [40]. Second, functional analyses may reveal multiple origins of self-compatibility within species and contribute to an increase in the number of empirical examples, although in the present study, which took a conservative approach, each wild species was

Table 2. Numbers of examples in which the primary mutations involved in the loss of SI are attributable to male or female components of self-incompatibility.

| Wild populations | Cultivated populations |
|------------------|-----------------------|
| Male component   | Female component      |
| Wild populations†| 4                      | 0                      |
| Cultivated       | 1                      | 5                      |

The pattern is significantly different between wild and cultivated populations (two-tailed Fisher’s exact test \( p = 0.0476; \) one-tailed \( p = 0.0238; \)).

†Wild populations: Arabidopsis thaliana [41], A. kamechatica (this study), Capsella rubella [43] and Leavenworthia alabamica [44]. Cultivated populations: Brassica napus [79,80], B. oleracea [111] and B. rapa [112]. See Table S8 for details of mutations involved in the loss of SI in cultivated Brassica populations.

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counted as one example (Table 2). While more empirical examples in various species should be gathered to better understand the general pattern of mutations in the evolution of self-compatibility, functional confirmations and analyses of the pattern of polymorphisms are both essential to disentangle mutational histories in the loss of SI.

Materials and Methods

Plant materials

*Arabidopsis kamchatatica* consists of two subspecies, *A. kamchatatica* subsp. *kamchatica* and *A. kamchatatica* subsp. *kawasakiana* [46]. *A. kamchatatica* subsp. *kamchatica* is a perennial, described originally from Kamchatka, Russia. It is also reported from East Asia (Far East Russia, China, Korea, Japan and Taiwan) and North America (Alaska, Canada and the Pacific Northwest of the USA). The second subspecies, *A. kamchatatica* subsp. *kawasakiana*, is an annual found in sandy open habitats along seashores or lakeshores in lowlands in western Japan. Tetraploid chromosome number counts (2n = 32 and n = 16q) were reported from samples in Japan, Far East Russia, Alaska and Canada, representing both subspecies [46,48].

Altogether, 48 populations of *A. kamchatatica* were sampled (43 from *A. kamchatatica* subsp. *kamchatica* and five from *A. kamchatatica* subsp. *kawasakiana*), including one to three individuals per population, giving a total of 60 accessions (Table S1). The sample locations included Far East Asia (Taiwan and Japan), Far East Russia (Kamchatka Peninsula and Okhotsk), Alaska, Canada and the northwest of the USA (Washington), covering the majority of the distribution range of both subspecies. In addition, five accessions of *A. halleri* were used for interspecific crosses with *A. kamchatica* and for obtaining full-length sequences of *SRK* (Table S1; see also the section “Interspecific crosses between *A. halleri* and *A. kamchatica*” for details).

General methods for isolation of genomic/complementary DNA, genotyping, sequencing, and construction of phylogenetic trees

Genomic DNA was isolated from young leaves using Plant DNeasy Mini kits (Qiagen, Hilden, Germany). Total RNA was extracted from floral buds and flower tissues with RNAeasy kits (Qiagen). The 5’ and 3’ ends of complementary DNA (cDNA) sequences were isolated by 5’ and 3’ Rapid Amplification of cDNA Ends (RACE) with the 5’/3’ 2nd Generation RACE Kit (Roche Applied Science, Indianapolis, IN, USA). PCR was performed with ExTaq (TaKaRa Bio Inc., Shiga, Japan) or iProof High Fidelity DNA Polymerase (Bio-Rad, Hercules, CA, USA). Primers used for amplification and genotyping are shown in Table S3, with the respective annealing temperatures and elongation times. Genotyping was based on the presence or absence of PCR products. Direct DNA sequencing was conducted at the Institute of Plant Biology, University of Zurich, using a PRISM 3730 48-capillary automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequence assemblies and alignments were performed in CodonCode Aligner 3.7.1 (CodonCode, Dedham, MA, USA) and BioEdit v 7.0.5 [113]. Minor adjustments to optimize the alignments were made by eye. Sequence data have been deposited in GenBank under accession numbers JX114752-JX114778. MEGA 5 was used for the construction of a phylogenetic tree [114]. Phylogenetic trees were obtained using the neighbor-joining method on pairwise proportions of nucleotide divergence. The evolutionary distances were computed using the Kimura two-parameter method.

PCR–based screening and sequencing of the entire coding region of *SRK*

To survey the *S*-haplogroups in *A. kamchatatica* across its distribution range, we performed a PCR–based screening of the *SRK* gene using two kinds of primer sets: (1) “general primers”, designed in conserved regions of the *S*-domain of *SRK* and known to amplify *SRK* sequences that belong to a number of haplogroups (“Aly13F1” and “SLGR” [52,55]); and (2) “haplogroup-specific primers”, designed in polymorphic regions of the *S*-domain of *SRK* [55]. Based on the obtained sequences, new haplogroup-specific primers were designed for each haplogroup (Table S3). Although we found sequences in several accessions that were highly similar to the *Aly13-7* sequence, Mable et al. [55] reported that *Aly13-7* is not associated with SI. Thus, these sequences were excluded from further analyses.

To investigate the functionality of *SRK*, the entire coding sequences of *SRK* were obtained by RACE PCR using the haplogroup-specific primers designed in the *S*-domain of *SRK*. The primers used for RACE PCR are listed in Table S3.

Bayesian clustering for the inference of population structures

To examine the associations between the geographic distribution of the *S*-haplogroups and the population structure (see the next section for details), we reexamined the population structure of *A. kamchatatica*, which was originally inferred by Shimizu-Inatsugi et al. [48], by adding the Okhotsk population that was not included in the former study. From two accessions of this population, we obtained nucleotide sequences of the genes *WER* (WEREWOLF) and *CHS* (CHALCONE SYNTHASE). By including these data in the dataset of Shimizu-Inatsugi et al. [48], population structure was inferred by the Bayesian clustering algorithm implemented in the InStruct program [115]. The programs CLUMPP [116], DISTRUCT [117] and *AK* statistic [118] were used to summarize and interpret the outputs. The parameters used were the same as in Shimizu-Inatsugi et al. [48], except for the number of independent runs: 10 instead of 20. In addition to the software InStruct, we also used the software STRUCTURE 2.2.3, which is not able to consider inbreeding explicitly [119]. (See Text S1 and Figure S5 for details.)

Association analysis of *SRK* genotypes and population structures

To examine associations between the geographic distribution of the *S*-haplogroups and the population structure inferred from polymorphisms of other nuclear loci (*WER* and *CHS*), Cramer’s coefficient *V* (also called *φ*) was calculated for two sets of *SRK* (*A. halleri*-derived *AksRK-A, AksRK-B* and *AksRK-C* and *A. lyrata*-derived *AlySRK-D* and *AlySRK-E*). Cramer’s coefficient *V* measures the strength of association or interdependence between two categorical variables [120,121]. Its statistical significance was assessed using Fisher’s exact test. (See Table S9 for cluster assignments based on *K* = 2, 3, or 4.) Heterozygotes or accessions that did not bear corresponding *SRK* were excluded from the analysis. All statistical analyses were conducted with R 2.10.0 [122].

Calculation of nucleotide divergence of *SRK*

To obtain insight into which parental species *SRK* of *A. kamchatatica* were derived from, we calculated mean values of *K*, *Ks* (the proportion of synonymous substitutions per synonymous site) and *Ka* (the proportion of nonsynonymous substitutions per nonsynonymous site) from the outgroups *A. halleri* and *A. lyrata* for all *SRK* sequences obtained in *A. kamchatatica*, using MEGA 5 [114]. We used all the sequences of *A. kamchatatica* and *A. halleri* obtained in
this study (see Table S1 for which accessions were used). In addition, we used the following publicly available sequences for the calculation: AlSRK22 [32], AlSRK26 [59], AlSRK01 [61], AlSRK01 [61], AlSRK43 [59], AlSRK12 [50], AlSRK17 [54] and AlSRK02 [50]. Specifically, we calculated the nucleotide divergence of the following sequence pairs: (1) AlSRK-A from AlSRK22 and from AlSRK26; (2) AlSRK-B from AlSRK17; (3) AlSRK-C from AlSRK01 and from AlSRK01; (4) AlSRK-D from AlSRK43 and from AlSRK12; and (5) AlSRK-E from AlSRK17 and from AlSRK02.

Estimation of divergence time from two parental species

To understand the approximate timescale of the evolutionary loss of SI in *A. kamchatica*, the divergence time of *A. kamchatica* from two parental species was calculated based on four nuclear loci (*CHS*-lyr, *CHS*-hal, *WER*-lyr and *WER*-hal). We first calculated the nucleotide divergence on these loci using publicly available data [48] as well as newly obtained sequence data (see the section “Bayesian clustering for the inference of population structures”). For *A. lyrata*, two accessions from Far East Russia were reported to show the highest nucleotide similarities with *A. lyrata*-derived homeologs of *A. kamchatica* among those surveyed by Shimizu-Inatsugi et al. [48], suggesting that they are closest to one of the founding parents of *A. kamchatica*. In contrast, other individuals of *A. lyrata* from Europe and North America were not as close to the *lyrata*-derived homeolog of *A. kamchatica*. Therefore, we calculated the divergence of *A. kamchatica* from these two *A. lyrata* accessions from Far East Russia, as this would better represent the split from the parental species. Standard errors and 95% confidence intervals were calculated for all estimates. All estimates were corrected using the Jukes–Cantor method [123]. As two clusters were suggested in the InStruct analysis (Figure 3), we also calculated the clustervise nucleotide divergence from *A. halleri* and *A. lyrata* (Table S5). (See the above section, “Association analysis of SRK genotypes and population structures”, for the cluster assignment.)

Based on the calculated nucleotide divergence, we estimated the divergence time between *A. kamchatica* and the two parental species *A. lyrata* and *A. halleri*. The estimation was based on the expression

\[ T = \frac{K}{2\gamma}, \]

where \( T \) is the time to the most recent common ancestor, \( K \) is the nucleotide divergence and \( \gamma \) is the substitution rate [124–126]. Note that our estimation of divergence time assumes a constant rate of evolution throughout the tree [127]. Here we employed two estimates of mutation rates: the synonymous mutation rate of \( 1.5 \times 10^{-8} \text{ per site per year} \) [75], which is commonly used in studies of the evolution of self-compatibility [44,62], and a mutation rate of \( 7.1 \times 10^{-9} \text{ per site per generation} \), which was estimated using mutation accumulation lines [76]. While we assumed a generation time of two years [128], we note that this could cause an overestimation or an underestimation, because some accessions of *A. kamchatica* are reported to be annual plants [47,48] and because an effect of seed dormancy was not considered, respectively. Note that estimates based on the mutation rate given by Koch et al. (2000) are independent from the generation time, because its unit is base pair per year, not per generation. For calculating the 95% upper and lower bounds of divergence time, the 95% upper and lower bounds of nucleotide divergence and the 95% lower and upper bounds of mutation rates were used, respectively. For estimating the divergence time, we used the nucleotide divergence values of *CHS*-hal and *WER*-hal from corresponding orthologs in *A. halleri*, and those of *CHS*-lyr and *WER*-lyr from corresponding orthologs in *A. lyrata* from Far East Russia.

F₂ generation segregation analysis

To confirm the disomic inheritance and the allelic relationship of AlSRK-A and AlSRK-B, F₂ segregation analysis was conducted using *A. kamchatica*. F₁ plants were generated using the Biwako accession from Japan as the pistil donor and the Potter accession from Alaska as the pollen donor. (See Table S1 for the detailed geographic locations of these accessions.) Ninety-five F₂ plants were generated by selfing of two F₁ plants and genotyped using the haplogroup-specific primers for AlSRK-A, AlSRK-B and AlSRK-D (Table S3). While AlSRK-A and AlSRK-B were amplified in the Biwako accession and AlSRK-B and AlSRK-D were amplified in the Potter accessions (see Table S1 for details), all of AlSRK-A, AlSRK-B and AlSRK-D were amplified in F₁ plants. Using \( \chi^2 \) tests with \( R = 2.10 \times 10^2 [122] \), the goodness-of-fit for each of the following inheritance models was calculated: (1) disomic and allelic, (2) disomic and nonallelic, (3) tetrasomic and allelic and (4) tetrasomic and nonallelic. The expected frequencies of segregants are described in Table 1.

Supporting Information

Figure S1 Phylogenetic trees of SRK sequences from haplogroups A (A), B (B), and C (C) from *A. halleri*, *A. lyrata* and *A. kamchatica* to test whether the full-length SRK sequences and other components involved in the female signaling pathway are functional, and also to test whether the male components are not functional in *A. kamchatica*. To screen *A. halleri* plants bearing haplogroups A, B or D, genotypes of AlSRK were surveyed in *A. halleri* from Mt Ibuki (35.42°N, 136.40°E), Ohara (35.16°N, 135.04°E) and Tada-Ginzan (34.90°N, 135.35°E) in Japan. We used them as both pollen and pistil donors for the crossing experiments, because individuals that bear SRK-A, SRK-B or SRK-D should bear the haplogroup A, B or D of the S-locus (encompassing SCR-A, SCR-B or SCR-D), respectively, giving the tight linkage between SCR and SRK. Three *A. halleri* plants were used for each haplogroup. The haplogroup-specific primers listed in Table S3 were used for this screening. Two *A. halleri* plants from Boden and Beride in Switzerland, which bear neither SRK-A, SRK-B nor SRK-D, were also used as pollen donors (Table S1). To confirm the SRK genotypes, eight *A. kamchatica* accessions were used for the crossing experiments (Table S1). Three of these eight accessions are reported to be capable of selling [48], and the other five accessions were confirmed in this study (data not shown).

Plants used in the pollination assay were grown at 22°C under a 16 h light/8 h dark cycle. We removed anthers from flower buds and carefully confirmed that stigmas were not contaminated by self-pollen using a stereomicroscope. Flowers were harvested 2–3 h after pollination when *A. kamchatica* was the pistil donor, or 24 h after pollination when *A. halleri* was the pistil donor. Harvested flowers were fixed in a 9:1 mixture of ethanol and acetic acid, softened for 10 min in 1 M NaOH at 60°C and stained with aniline blue in a 2% K₃PO₄ solution. Pistils were mounted on slides to examine the pollen tubes using epifluorescence microscopy [41,129]. In compatible crosses, more than 100 pollen tubes typically penetrate the stigma and penetration of <20 pollen tubes was considered as a criterion of incompatible crosses, following the common criteria of previous work in *Arabidopsis* [31,41]. The results did not change even if a more stringent criterion of <10 pollen tubes was used. Although pollen tube growth was observed in most of the crosses to evaluate incompatible and compatible reactions, in a few combinations of crosses where *A. halleri* was the pistil donor, we alternatively used the lengths of siliques as a criterion of incompatible crosses (see Figure S4). A siliques length of <5 mm was considered to be the criterion of an incompatible cross.
This phylogeny was obtained by the neighbor-joining method on pairwise proportions of nucleotide divergence. In total, 552 (A), 567 (B) and 449 (C) nucleotide positions were used. The evolutionary distances were computed using the Kimura two-parameter method. See Table S10 for accession numbers of these sequences deposited in GenBank. SRK sequences obtained in this study are shown in red.

Figure S2 Results of population clustering based on the data for the nuclear WER and CHS genes using InStruct software. (A) Mean posterior probability of the data ln P(X|K) over 10 runs for each K-value. (B) Plot of AK for each K.

Figure S3 Alignment of the predicted amino acid sequences of AkSRK-A, AkSRK-B, AkSRK-C, AkSRK-D and AkSRK-E, deduced from their DNA sequences. A 1-bp deletion causing a frameshift mutation in AkSRK-C is shown as a red “X” (position 67) and subsequent amino acids are shown as if the frameshift did not happen. AkSRK-D* denotes AkSRK-D from Murodo bearing a deletion of 15 amino acids caused by a 45-bp deletion in AkSRK-D genomic DNA. The site of an approximate 1,700-bp insertion in Orange [77,78]. Asterisks denote stop codons. Subsequent amino acids are shown as if the insertion did not happen. Twelve conserved cysteine residues are indicated in orange [77,78]. Asterisks denote stop codons.

Figure S4 Interspecific crosses between A. halleri (pistil donor) and A. kamchatica (pollen donor), and control crosses within A. halleri. Unless indicated by asterisks, numerators denote crosses where more than 20 pollen tubes penetrated the stigma (compatible crosses). If indicated, denominators denote crosses where the length of siliques was >5 mm (see Methods). Denominators denote the total number of crosses conducted in each combination.

Figure S5 Results of population clustering based on the data of cpDNA and nuclear WER and CHS genes using STRUCTURE software. See the caption for Figure S1 for details. (A) Inference of population structure for the clustering of K=2, 3, and 4. (B) Mean posterior probability of the data ln P(X|K) over 10 runs for each K-value. (C) Plot of AK for each K.

Table S1 Geographic locations of materials used in this study, list of accessions used for each analysis, and the results of PCR-based genotyping.

Table S2 Nucleotide divergence of SRK from two parental species, A. halleri and A. lyrata.

Table S3 Primer list.

Table S4 Pattern of intrapopulation segregation in a Kamchatka population from Petropavlovsk Kamchatskii, Mishenaya gora.

Table S5 Nucleotide divergence of the CHS and WER genes from the two parental species A. halleri and A. lyrata.

Table S6 Estimated divergence time in years from parental species under two estimates of the mutation rate; Koch et al. [75] and Ossowski et al. [76].

Table S7 Intraspecific crosses between two accessions to exclude the possibility that the male components of haplogroup D of the Murodo accession and of haplogroup A of the Biwako accession remain functional.

Table S8 Summary of mutations suggested to be responsible for the loss of SI in cultivated Brassica populations.

Table S9 Cluster assignments of each accession based on the analyses by InStruct and STRUCTURE, given the number of clusters K=2, 3 or 4. The geographic location of each accession is also shown.

Table S10 GenBank accession numbers of sequences used to generate phylogenetic trees (Figure 1; Figure S1).

Text S1 Population Structure Based on the Software STRUCTURE.

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Author Contributions

Conceived and designed the experiments: TT PK KKS. Performed the experiments: TT PK C-LY JBB KKS. Analyzed the data: TT PK KKS. Wrote the paper: TT PK JBB KKS.

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