Genomic Conflicts that Cause Pollen Mortality and Raise Reproductive Barriers in *Arabidopsis thaliana*

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ABSTRACT Species differentiation and the underlying genetics of reproductive isolation are central topics in evolutionary biology. Hybrid sterility is one kind of reproductive barrier that can lead to differentiation between species. Here, we analyze the complex genetic basis of the intraspecific hybrid male sterility that occurs in the offspring of two distant natural strains of *Arabidopsis thaliana*, Shahdara and Mr-0, with Shahdara as the female parent. Using both classical and quantitative genetic approaches as well as cytological observation of pollen viability, we demonstrate that this particular hybrid sterility results from two causes of pollen mortality. First, the Shahdara cytoplasm induces gametophytic cytoplasmic male sterility (CMS) controlled by several nuclear loci. Second, several segregation distorters leading to allele-specific pollen abortion (pollen killers) operate in hybrids with either cytoplasm. The complete sterility of the hybrid with the Shahdara cytoplasm results from the genetic linkage of the two causes of pollen mortality, *i.e.*, CMS nuclear determinants and pollen killers. Furthermore, natural variation at these loci in *A. thaliana* is associated with different male-sterility phenotypes in intraspecific hybrids. Our results suggest that the genomic conflicts that underlie segregation distorters and CMS can concurrently lead to reproductive barriers between distant strains within a species. This study provides a new framework for identifying molecular mechanisms and the evolutionary history of loci that contribute to reproductive isolation, and possibly to speciation. It also suggests that two types of genomic conflicts, CMS and segregation distorters, may coevolve in natural populations.

KEYWORDS cytoplasmic male sterility; hybrid sterility; pollen killer; reproductive barrier; segregation distorsion

REPRODUCTIVE isolation is an important component of species differentiation, and mechanisms that create reproductive barriers between once-conspecific organisms have attracted interest since evolutionary biology emerged as a field of study (Coyne 1992; Orr 1996). Hybrid incompatibility, due to genetic divergence between the hybridizing parents, as theorized by Bateson, Dobzhansky, and Muller (Orr 1996), is commonly observed between subspecies or distinct populations of the same species (Cutter 2012). Hybrid incompatibility is therefore thought to contribute to the differentiation between incipient species. Reproductive barriers translate into interspecific hybrid unviability, weakness, or sterility, and recent studies on several types of hybrid incompatibility have shed light on their mechanisms in various taxa (Maheshwari and Barbash 2011). The genetic basis of hybrid sterility has been studied in yeast (Chou *et al.* 2010), fruit flies (Dobzhansky 1936; Larracuente and Presgraves 2012), mice (Mihola *et al.* 2009), and several plants, including *Solanum* (Moyle and Nakazato 2008), rice (Oryza) (Ouyang *et al.* 2010), *Mimulus* (Barr and Fishman 2010; Lowry and Willis 2010), *Arabidopsis lyrata* (Leppala and Savolainen 2011), and *A. thaliana* (Torjek *et al.* 2006; Durand *et al.* 2012). In this last species, male sterility observed in the progeny of crosses between C24 or Shahdara (Sha) and Col-0 accessions results from an incompatible interaction between two duplicated genes located on different chromosomes (Durand *et al.* 2012), as in a classical two-locus Bateson–Dobzhansky–Muller (BDM) interaction. In rice, many reproductive barriers have been observed between

doi: 10.1534/genetics.115.183707
Manuscript received October 27, 2015; accepted for publication April 28, 2016; published Early Online May 2, 2016.
Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.183707/-/DC1
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Table 1 Fertility phenotype of genotypes from crosses between Sha and Mr-0

	Plant fertility score	Pollen viability score	Figure
[Mr-0]Sha	4	**4**	Figure 1, A and C
[Sha]Mr-0	0	0.1	Figure 1, A and C
Sha × Mr-0 F_1	0	0.1	Figure 3
Mr-0 × Sha F_1	4	1.6	Figure 3
[Sha]$L1^HL3^H$	0 to >2[a]	0 to >1.5[a]	Figure 6
[Mr-0]$L1^HL3^H$	4	2.1	Figure 6
[Sha]$L1^ML3^M$	4	3.6	—

[a] During their development, these plants are first sterile and then later produce some siliques with seeds.

species and subspecies, including several types of hybrid sterility (Ouyang and Zhang 2013). Classical two-locus BDM interactions between differentially inactivated duplicated genes appear to cause pollen sterility in hybrids between *Oryza sativa* and *O. glumaepatula* (Yamagata *et al.* 2010) and between the two *O. sativa* subspecies *japonica* and *indica* (Mizuta *et al.* 2010). In both of these BDM interactions, a particular combination of alleles present at the two loci is deleterious at the haploid stage, leading to the production of deficient pollen grains in the hybrid plant. In addition, at least two examples of one-locus hybrid sterility have been reported in rice (Long *et al.* 2008; Yang *et al.* 2012). In these cases, hybrid sterility results from the abortion of the gametophytes (pollen grains in one case, embryo sacs in the other) that carry one of the parental alleles, when in the presence of the other allele in the hybrid. Such loci, leading to segregation distortions, were named gamete killers (Cameron and Moav 1957). Segregation distorters are considered selfish genetic elements because they enhance their own transmission to progeny at the expense of the fitness of the organism, creating an intragenomic conflict (Frank 2000).

Another type of hybrid sterility involves cytoplasmic male sterility (CMS), which generates an intragenomic conflict between the cytoplasmic and nuclear genomes. In this case, pollen abortion is induced by the presence of maternally inherited mitochondrial genes, which enhance their fitness by impairing resource allocation to the male function (Cosmides and Tooby 1981; Saur Jacobs and Wade 2003). Nuclear restorers of fertility (Rf) that inhibit the action of sterilizing mitochondrial genes are selected under the pressure to restore male function. In some cases, Rf genes have a deleterious effect on fitness compared with their nonrestorer alleles, resulting in a cost of restoration (McCauley and Bailey 2009). When Rfs are not fixed in populations, gynodioecy (the cooccurrence of females and hermaphrodites) is observed. However, fixation of Rfs leads to undetectable, cryptic CMS, revealed only by crosses between individuals from allopatric hermaphrodite populations. Cryptic CMS has been reported in *Mimulus* (Fishman and Willis 2006) and *A. lyrata* (Leppala and Savolainen 2011; Aalto *et al.* 2013). In *A. thaliana*, we previously discovered a cryptic CMS by crossing two distant *A. thaliana* accessions, Sha and Mr-0, originating respectively from Tajikistan and Sicily. The reciprocal F_1 hybrids give different reproductive phenotypes: F_1 plants with the Sha cytoplasm are unable to produce pollen and, consequently, seeds (because *A. thaliana* is a selfing species). In contrast, the reciprocal F_1 plants with Mr-0 cytoplasm have full seed set. A gene present in the mitochondrial genome of Sha, called *orf117Sha*, has been identified as the cytoplasmic factor that induces male sterility; in the nuclear genome, two large regions on chromosomes 1 and 3 are associated with the sterility phenotype (Gobron *et al.* 2013).

Here, we dissect the complex genetic bases of the Sha × Mr-0 hybrid male sterility. We confirm that the Sha cytoplasm induces male sterility in the presence of Mr-0 nuclear alleles and narrow down the two main nuclear regions involved in the hybrid sterility. We also uncover nuclear segregation distorters that lead to the death of pollen grains carrying Sha alleles (pollen killers; PKs) at the same loci previously identified as involved in the CMS. By conducting a QTL analysis, we identify several additional genomic regions involved in Sha × Mr-0 hybrid sterility. Finally, by studying other crosses in *A. thaliana*, we explore the links between the sterility phenotypes of the hybrids and natural variation at the loci involved in the sterility. Our results indicate that the hybrid sterility observed in the Sha × Mr-0 F_1 results from the combination of gametophytic CMS and PK effects whose nuclear determinants are genetically linked.

Materials and Methods

Nomenclature

Hereafter, crosses are always written in the following, conventional order: female parent × male parent. When needed, the origin of the cytoplasmic (mitochondrial and chloroplastic) genomes of the crossed plants is indicated in brackets before the name that designates the nuclear genotype: a plant carrying the cytoplasm from parent A and the nuclear genome from parent B is designated [A]B. The genotype at a specified locus Lx is designated Lx^M, Lx^S, or Lx^H for homozygous Mr-0, homozygous Sha, or heterozygous, respectively. BCp denotes paternal backcross, and BCm denotes maternal backcross.

Plant materials

The [Sha]Mr-0 and [Mr-0]Sha cytolines were obtained after recurrent paternal backcrosses of Sha × Mr-0 and Mr-0 × Sha F_1s followed by genotyping with 384 SNP markers distributed throughout the genome (see Supplemental Material, File S1). The SNPs used are available at https://www.versailles.inra.fr/ijpb/crb/anatool/ (Simon *et al.* 2012).

Due to the poor fertility of plants heterozygous on chromosome 1 and chromosome 3 in the Sha cytoplasm, segregating populations needed in this work were obtained through complex cross plans described below.

Two populations, PopL1 and PopL3, were constructed with the aim of narrowing down the intervals containing the two main loci involved in hybrid sterility, hereafter named L1 and L3 (Figure S1). They had the Sha cytoplasm and a nuclear background mainly homozygous for the Sha alleles, except

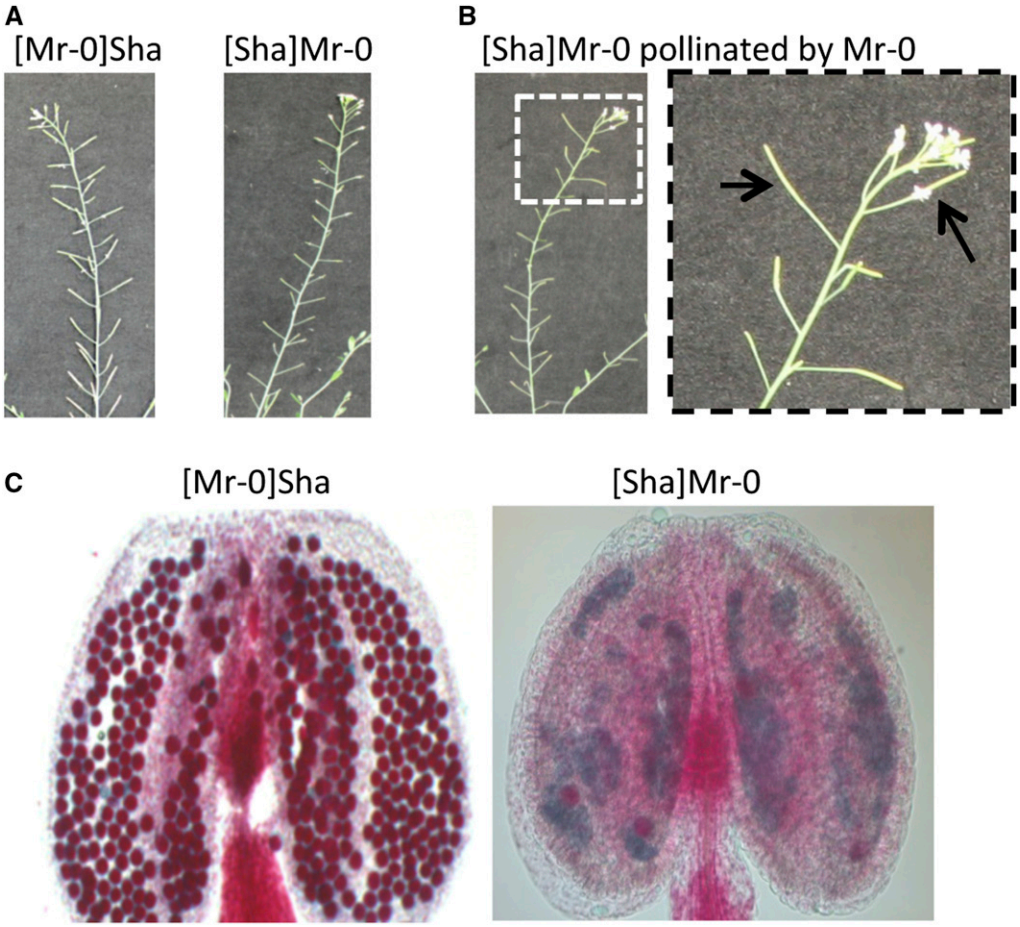

Figure 1 Reproductive phenotypes of reciprocal cytolines. (A) The [Mr-0]Sha cytoline presents normal fruit (silique) development whereas the [Sha]Mr-0 cytoline is totally sterile. (B) Manual pollination of the sterile [Sha]Mr-0 cytoline with Mr-0 pollen (dashed box) leads to a fertile, restored phenotype (arrows). (C) Alexander staining (observation under a light microscope, ×10) of anthers. The cytoline with the Mr-0 cytoplasm has only viable (red) pollen grains, whereas its reciprocal shows no viable pollen grains.

on chromosome 1 and chromosome 3. In PopL1, chromosome 1 segregated heterozygous and homozygous Sha genotypes between 10.7 Mb and the south telomere, whereas chromosome 3 was homozygous Mr-0 from 15.2 Mb to the south telomere. Conversely, in PopL3, chromosome 1 was homozygous Mr-0 from 10.7 Mb to the south telomere, and chromosome 3 segregated heterozygous and homozygous Sha genotypes between 15.2 Mb and the south telomere.

Near-isogenic lines with a Sha nuclear background but heterozygous at L1, L3, or both L1 and L3 were created in the two cytoplasmic backgrounds, in order to investigate segregation distortion at these two loci (see File S1, Figure S1, and Figure S2).

The Sha × Mr-0 F_2* mapping population was composed of plants carrying the Sha cytoplasm, homozygous Mr-0 at L1 and L3, and segregating elsewhere. To obtain this population, a plant [Sha]$L1^{M}L3^{M}$, selected from the progeny of the [Sha]$L1^{H}L3^{H}$ × [Mr-0]$L1^{H}L3^{H}$ cross, was first crossed with Mr-0 in both ways. The Sha × Mr-0 F_2* population was then obtained by crossing the two resulting F_1 plants, using the F_1 carrying the Sha cytoplasm as the mother, because it produces very few seeds via selfing.

Growth conditions

Before sowing, seeds were stratified in the dark at 4°C for 3 days in a water solution containing 0.1% agar and 7 mM KNO_3 to overcome the dormancy that is particularly strong in seeds with Mr-0 alleles. Plants were grown in soil in a greenhouse under long-day conditions (16-h day, 8-h night) with additional artificial light (105 μE/m^2/sec) when necessary.

Genotyping

SNP genotyping was performed at the genomics facility at INRA Toulouse, France (http://www.genotoul.fr); genomic DNA preparation, genotyping, and data analysis were carried out as described by Simon *et al.* (2012). For microsatellite genotyping, DNA extractions were conducted on leaves from 15-day-old seedlings as described by Loudet *et al.* (2002); the microsatellite markers used are described in Table S1. The presence of *orf117Sha* was detected by PCR as described by Gobron *et al.* (2013). Allele-specific PCRs at L1 and L3 were performed with the primer pairs described in Table S1 (L1MrF/L1MrR and L1ShaF/L1ShaR, specific to Mr-0 and Sha alleles, respectively, at L1, and L3MrF/L3MrR and L3ShaF/L3ShaR, specific to Mr-0 and Sha alleles, respectively, at L3) on equal amounts of DNA; pollen was isolated as described by Honys and Twell (2004).

Phenotyping

Plant overall fertility was scored using a visual estimate of the number and size of the fruits (siliques) that developed. Scores were based on the following scale (with fractional scores if necessary): 0, plant completely sterile (no developed

siliques); 1, a few developed siliques; 2, roughly half of the siliques are developed; 3, a few aborted siliques; 4, plant fully fertile (Figure S3). Sha and Mr-0 plants were included in every experiment as fully fertile controls. Scoring was carried out independently by two experimenters. For each plant, scoring was carried out after three stems had flowered. To take into account the possible variation in the fertility phenotype during development, plants were scored one or two times per week for at least 2 or 3 weeks to obtain a minimum of five scores per plant, averaged to obtain the final score.

Pollen viability was estimated from flower buds harvested just before anthesis. Sha and Mr-0 plants were used as fertile controls. For each plant, anthers of two buds (8–12 anthers/plant) were dissected and observed under a light microscope after Alexander staining (Alexander 1969). The cytoplasm of viable pollen grains was colored in red, and the pollen wall was colored in green, thus dead pollen grains appeared greenish. For each anther, pollen viability was scored using the following scale: 0, all pollen grains aborted; 1, a few viable pollen grains; 2, about half of the pollen grains are viable; 3, a few aborted pollen grains; 4, all pollen grains are viable. The final score of the plant was the average of all anther scores.

According to their cytoplasms and nuclear genotypes, mainly at L1 and L3, the plants from crosses between Sha and Mr-0 showed different degrees of overall and/or pollen fertility. The fertility of the genotypes, scored at the overall plant level and at the pollen viability level as described above, is summarized in Table 1. Some plants with a maximum overall fertility score (*i.e.*, 4) showed low pollen viability.

L1 and L3 mapping

At both L1 and L3, the Sha homozygous state was associated with an overall fertility score of 3 or higher, whereas heterozygotes were mainly sterile (overall fertility score of 1 or lower). Genotyping of the 276 plants from PopL1 led to the identification of 85 recombinants between markers CIW1 (18.4 Mb) and F5I14 (24.4 Mb). Similarly, genotyping of the 294 plants from PopL3 identified 43 recombinants between markers MSAT3.58 (18.7 Mb) and MSAT3.70 (23.4 Mb). Because we suspected that the sterility phenotype of homozygotes could necessitate several determinants, we used only sterile recombinants to reduce the intervals. Therefore, for L1 and L3 mapping, markers homozygous for Sha alleles in mainly sterile recombinants (an overall fertility score of 1 or lower) were excluded from the candidate interval.

QTL analysis

Phenotyping and genotyping data of the Sha × Mr-0 F_2* population were analyzed with the R/qtl package (Broman *et al.* 2003), using the Haley–Knott regression method. The QTL model was implemented and refined using the addqtl, addint, and refineqtl functions, by adding significant new QTL and/or interactions to the QTL first detected with the scanone function.

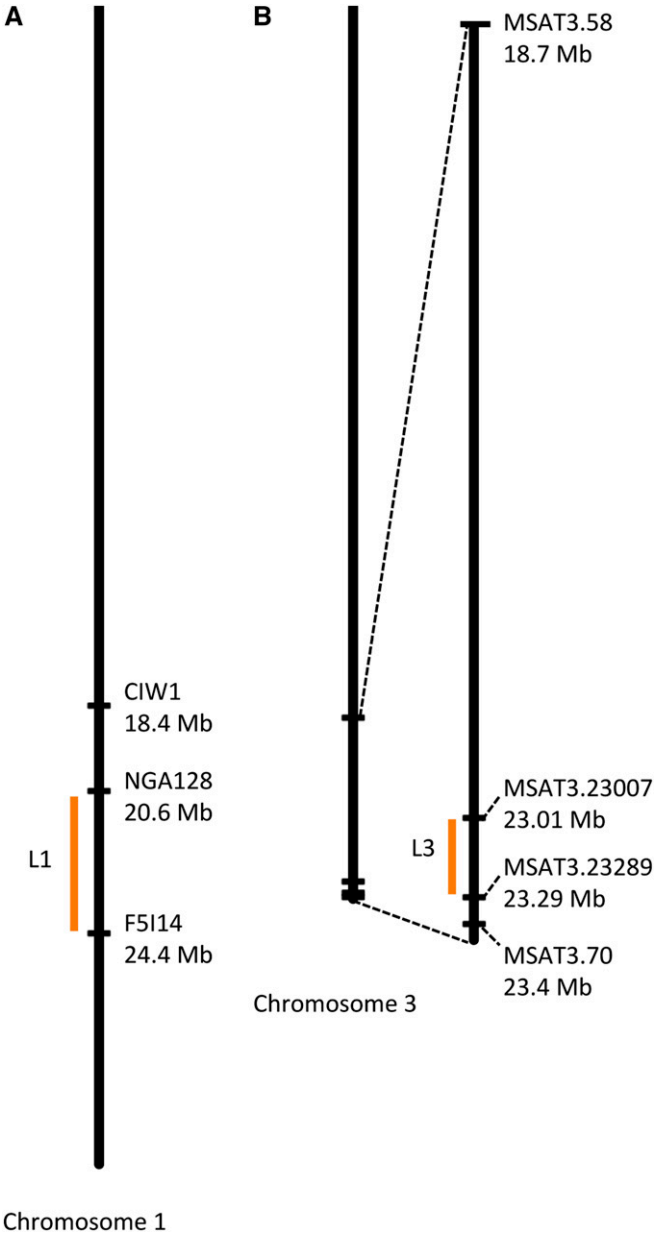

Figure 2 Mapping of the L1 and L3 loci involved in male sterility. (A) The L1 locus was narrowed down to the interval between the NGA128 and the F5I14 markers. (B) The L3 locus was narrowed down to the interval between the MSAT3.23007 and the MSAT3.23289 markers.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Cytolines confirm the Sha/Mr-0 CMS

Our previous work (Gobron *et al.* 2013) showed that the Sha × Mr-0 hybrid is completely sterile, in contrast to the Mr-0 × Sha hybrid, which has full seed set. From each of

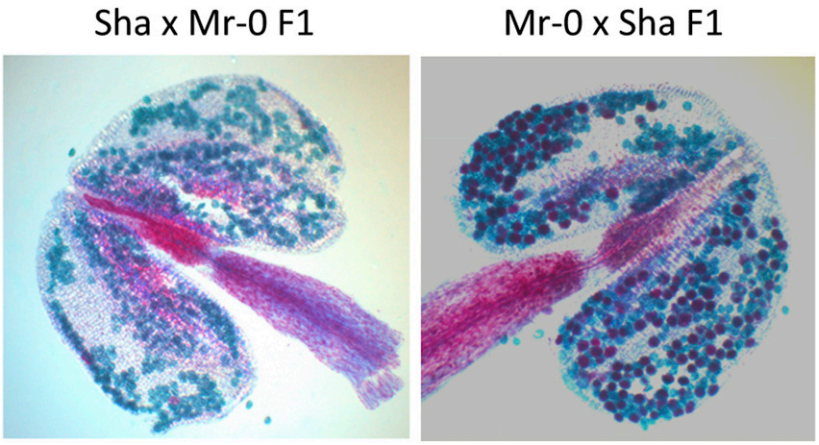

Figure 3 Pollen viability of reciprocal Sha/Mr-0 F_1 plants. In the Sha × Mr-0 F_1, all pollen grains are aborted (green), whereas the reciprocal presents a mixture of aborted (green) and viable (red) pollen grains.

these two F_1 hybrids, we created a line combining the nuclear genome of one accession with the cytoplasmic genomes of the other via a series of recurrent backcrosses with the male parent. Such genotypes are hereafter referred to as cytolines (Grace *et al.* 1994). In the greenhouse, the cytolines [Sha] Mr-0 and [Mr-0]Sha did not show any growth or developmental defects, but [Sha]Mr-0 was sterile, producing no seeds, whereas [Mr-0]Sha was fertile (Figure 1A). Seed production was restored in [Sha]Mr-0 after manual pollination with Mr-0 pollen, indicating a male dysfunction in [Sha]Mr-0 (Figure 1B). Cytological observations of anthers showed that the [Sha]Mr-0 cytoline produced almost no viable pollen, while all pollen grains were viable in [Mr-0]Sha (Figure 1C). This pattern confirmed that the Sha cytoplasm is responsible for a male sterility, which can be suppressed by factors that are present in the Sha nuclear genome (so-called Rfs) but are absent or nonfunctional in the Mr-0 nuclear genome.

The two nuclear loci L1 and L3 are required for Sha × Mr-0 hybrid male sterility

A preliminary study suggested that two regions on the southern arms of chromosome 1 and chromosome 3 carried major genetic factors for hybrid sterility (Gobron *et al.* 2013). To map these two loci independently, we created two mapping populations in the Sha cytoplasm, PopL1 and PopL3. In PopL1, the L1 region segregated heterozygous and homozygous Sha genotypes, whereas the L3 region was fixed for Mr-0. Similarly, in PopL3 the L3 region segregated heterozygous and homozygous Sha genotypes whereas L1 was fixed for Mr-0 (Figure S1). In each mapping population, plants that were homozygous Sha at the segregating region had normal seed sets. Thus, we were able to reduce the size of the region of interest by excluding regions that were homozygous Sha in sterile recombinant plants. This mapping strategy confirmed that the two regions L1 and L3 carry major factors required for Sha × Mr-0 hybrid sterility, and narrowed down the causal loci to 3.8 Mb on chromosome 1 (Figure 2A) and to 0.28 Mb on chromosome 3 (Figure 2B). The L1 locus remained rather large due to the lack of sterile recombinants in this interval, suggesting that it carries more than one determinant needed for hybrid sterility.

L1 and L3 carry PKs active in both cytoplasms

In the Mr-0 × Sha F_1 plants, although they set seeds normally, more than half of pollen grains were aborted (Table 1 and Figure 3). When genotyping a Mr-0 × Sha F_2 population with genome-wide markers, significant deficits in the Sha homozygous genotype were found at three loci (Figure 4 and Table S2). Two of these loci colocalized with L1 and L3, also involved in the sterility of the Sha × Mr-0 F_1. Biases against the Sha alleles were also observed at L1 and L3 in the progeny of a cross using the Mr-0 × Sha F_1 as the male parent and the sterile [Sha]Mr-0 cytoline as the female parent (Table 2), suggesting a relationship between segregation distortion and pollen mortality in Mr-0 × Sha F_1 plants. Pollen grains produced by a heterozygous plant and that carried the Mr-0 allele at L1 and/or L3 contributed more than expected to the next generation, indicating that the nonviable pollen grains observed in the anthers of Mr-0 × Sha F_1 plants carried Sha alleles. This was validated by genotyping mature pollen of Mr-0 × Sha F_1 plants at both loci (Figure 5).

The segregation distortions against Sha alleles at L1 and L3 in the Mr-0 cytoplasmic background were confirmed in the selfing progenies of plants that were homozygous Sha apart from L1 and L3 ([Mr-0]$L1^{H}L3^{H}$) and of plants segregating only at either L1 or L3 ([Mr-0]$L1^{H}L3^{S}$ or [Mr-0]$L1^{S}L3^{H}$) (Table 3). In all of these genotypes, unviable pollen was observed after Alexander staining (Figure 6 and Figure S4), further linking segregation biases to pollen mortality.

In the Sha cytoplasmic background, we could not examine segregation biases in the male lineage because the Sha × Mr-0 F_1 produced no viable pollen. Plants heterozygous only at L1 and L3 and homozygous Sha in the rest of their nuclear genome ([Sha]$L1^{H}L3^{H}$), although mainly sterile, produced some selfing seeds in late developmental stages (Table 1), and we detected biases against plants homozygous Sha at L1 and L3 (Table 3). These biases were confirmed in the selfing progenies of plants heterozygous only at either locus ([Sha]$L1^{H}L3^{S}$ or [Sha]$L1^{S}L3^{H}$, Table 3).

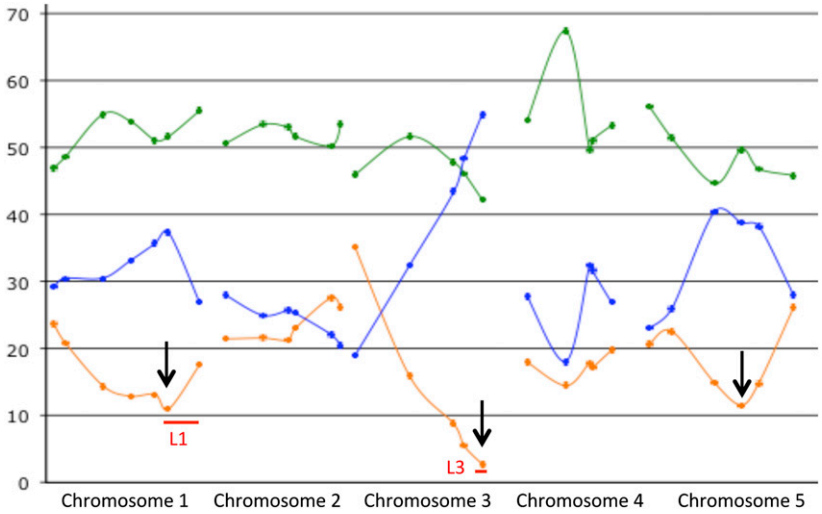

Figure 4 Genome-wide analysis of allele segregation in the Mr-0 × Sha F_2 family. The plots show the percentage of plants heterozygous (green), homozygous Mr-0 (blue), and homozygous Sha (orange) at each marker. Arrows indicate the markers where the bias against the Sha allele is maximal (see Table S2 for genotyping data). Red bars indicate the locations of the L1 and L3 loci.

Among the pollen grains produced by a plant heterozygous at L1 or L3, those carrying Sha alleles were outcompeted by those carrying Mr-0 alleles in either cytoplasm. We consistently observed pollen lethality in plants producing biased progenies (Figure 6 and Figure S4), and Sha alleles were underrepresented in mature pollen from plants heterozygous at L1 and L3 (Figure 5), indicating that dead pollen grains carried Sha alleles at L1 and/or L3. Furthermore, both the Sha accession and the [Mr-0]Sha cytoline produced only viable pollen (Figure 1C); therefore, pollen grains carrying Sha alleles at these specific loci are killed when, and only when, they are produced by a plant heterozygous at these loci, leading to a deficit in Sha alleles in the progeny. This kind of segregation distorter has previously been defined as a PK, which acts at the gametophytic stage to eliminate one parental allele from the pollen production of a hybrid (Cameron and Moav 1957). A PK relies on the interaction of two types of actors: a "killer," carried by one genotype, has a lethal effect on a "target," carried by the other genotype that does not contain the killer; in the present case, the Mr-0 alleles at L1 and L3 are killers, whereas Sha alleles are targets. We analyzed the F_2 progenies of crosses between Mr-0 and Cvi-0, known to be devoid of Rfs for the CMS (Gobron *et al.* 2013). These progenies showed significant biases against Cvi-0 alleles both at L1 and L3 (Table 4), indicating that the presence of Mr-0 alleles induces elimination of alleles from other natural accessions independently of their restorer function.

PKs and CMS jointly participate in the Sha × Mr-0 F_1 male sterility

The loss of pollen due to the PKs in Mr-0 × Sha F_1 plants was not sufficient to alter their overall fertility (Table 1). However, Sha × Mr-0 F_1 plants produced no viable pollen and no seeds. This contrast indicates that, in addition to PKs, other sterilizing factors act in the hybrid with the Sha cytoplasm and corroborates the involvement of the [Sha]Mr-0 CMS in hybrid sterility, leading to the death of pollen grains carrying Mr-0 alleles in the Sha cytoplasm. Further, we observed a difference in fertility between [Mr-0]$L1^{H}L3^{H}$ plants, which set seeds normally, despite their loss of pollen due to PKs, and [Sha]$L1^{H}L3^{H}$ plants, which were much less fertile (Table 1). PKs were active in all of these plants; therefore, their fertility difference, due to the CMS in the Sha cytoplasm, indicated that Sha had specific restorer genes at L1 and L3. We concluded that the two main loci involved in hybrid sterility, L1 and L3, carry both cytoplasm-independent PKs that lead to the death of Sha pollen grains and restorers of CMS that cause Mr-0 pollen to abort in the Sha cytoplasm. These two kinds of factors contribute together to the hybrid sterility of the Sha × Mr-0 F_1.

L1 and L3 are not sufficient to produce full F_1 male sterility

The sterility of the plants [Sha]$L1^{H}L3^{H}$ changed during their development: they produced only sterile flowers during the first 2–3 weeks after flowering, but subsequently developed some siliques with seeds (Figure 6). Heterozygosity at L1 and L3 was thus not sufficient to maintain complete sterility when the rest of the genome is homozygous Sha. In addition, surprisingly, [Sha]$L1^{M}L3^{M}$ plants, homozygous Mr-0 at both L1 and L3, were fertile (Table 1). These results strongly suggested the existence of additional Rf and potentially PKs in the genome, outside L1 and L3.

We used a quantitative genetic approach to look for additional loci involved in the fertility phenotype. To do so, we generated a mapping population (named Sha × Mr-0 F_2*, see *Materials and Methods*) in the Sha cytoplasm, where plants were homozygous Mr-0 at L1 and L3, to eliminate previously identified PK effects and fix the nonrestorer alleles at these loci, and segregating elsewhere. The Sha × Mr-0 F_2* family showed low overall fertility. We scored the overall fertility of 184 plants throughout their reproductive development (see *Materials and Methods*), and genotyped them with 33 markers scattered across the genome (Table S3). The QTL analysis identified six QTL, and one QTL–QTL interaction,

Table 2 Genotype segregation at L1 and L3 in the [Sha]Mr-0 × (Mr-0 × Sha) family

Locus	Chromosome	Marker	Position (Mb)	Number of Hz	Number of Mr-0	Number of plants	P (χ^2)	Observed Hz frequency (%)	Expected Hz frequency (%)
L1	1	NGA128	20.6	52	125	177	4×10^{-8}	29	50
L3	3	MSAT3.23007	23.1	2	179	181	2×10^{-39}	1	50

The segregation biases at L1 and L3 were measured in the male descent of a heterozygous plant to verify the male origin of the biases observed in its selfing progeny. Hz, heterozygote.

together explaining 65.5% of the phenotypic variation (Figure 7 and Table S4). For every QTL, the Sha allele was associated with higher fertility. Interestingly, Q5b is located in a region at around 17 Mb on chromosome 5, hereafter named L5, where a strong bias against the Sha allele was observed in the Mr-0 cytoplasmic background (Figure 4, Table S2, and Table S5). We also observed a strong bias against the Sha allele at this locus in the progeny of a plant [Sha]$L1^{M}L3^{M}L5^{H}$ (Table S6). We suspect that this locus is also associated with an Rf (in Sha) and a PK (whose killer element is in the Mr-0 genome).

We conclude that the pollen lethality observed in the progeny of crosses between Sha and Mr-0 results from a complex genetic determinism involving different genomic regions. Moreover, pollen lethality is caused by a plurality of factors that act concurrently: gametophytic CMS relying on several nuclear loci in interaction with the Sha cytoplasm, plus PKs acting in either cytoplasm. Remarkably, our analyses indicated that both kinds of factors colocalized at several loci.

The separation of the PK and the CMS factors suppresses hybrid sterility

Studying the fertility of reciprocal crosses between Mr-0 and a panel of 21 accessions, Gobron *et al.* (2013) observed that the 11 accessions that do not carry the mitochondrial gene *orf117Sha* give rise to fertile F_1s, whereas the 10 accessions carrying the *orf117Sha* produce male sterile F_1s when crossed with Mr-0 as the male parent. In the present work, we performed 25 additional two-way crosses between accessions and Mr-0. Among the 46 accessions studied in total, the 28 accessions that do not carry the mitochondrial *orf117Sha* produced fertile F_1s. In contrast, 14 out of the 18 crosses between Mr-0 as a male parent and accessions carrying the *orf117Sha* as females behaved like the Sha × Mr-0 cross: they produced completely male-sterile F_1 hybrids, whereas reciprocal crosses were fertile (Figure 8). Four accessions, although carrying *orf117Sha*, gave fertile F_1s when crossed to Mr-0 (Figure 8). They all belonged to the same subgroup of nuclear diversity, and we chose for further study the representative Rak-2 × Mr-0 cross, which was fertile although Rak-2 carries the same cytoplasm as Sha at all markers analyzed. We performed a paternal backcross (BCp1) of Rak-2 × Mr-0 F_1 with Mr-0, and observed that 9 out of 27 BCp1 plants were completely male sterile (Table S7). The Rak-2 cytoplasm thus induces male sterility, as expected from its similarity to the Sha cytoplasm, and the difference in fertility between the hybrids Sha × Mr-0 and Rak-2 × Mr-0 is likely due to different nuclear factors controlling pollen viability in these two crosses.

We genotyped 182 plants of each reciprocal Rak-2/Mr-0 F_2 at the loci L1 and L3 (Table 5). L3 showed a strong bias against the Rak-2 allele in both cytoplasms, as observed for the Sha allele in Sha/Mr-0 crosses, and thus most likely resulting from a similar PK effect. L1 showed a strong segregation distortion against the Mr-0 allele in the Rak-2 × Mr-0 family only, which implied an interaction with the Rak-2 cytoplasm. Among 182 F_2 plants of this family, all producing normal seed sets, there were no plant Mr-0 at markers ind1.22788 (22.8 Mb) and F5I14, an interval of 158 Kb included in L1, hereafter called L1Rak. This genetic behavior is expected for a unique Rf locus in gametophytic CMS. In addition, all sterile BCp1 plants were homozygous Mr-0 between ind1.22788 and F5I14 (Table S6), indicating that the pollen grains Mr-0 at L1Rak had died. Accordingly, we observed no viable pollen in the anthers of these BCp1 plants (Figure 9A). The anthers of the Rak-2 × Mr-0 F_1 plants carried more aborted pollen grains than those of the reciprocal F_1 (Figure 9B), which is consistent with a combination of two deleterious effects in the former (*i.e.*, PK at L3 plus CMS at L1Rak) compared to the sole action of a PK at L3 in the latter. Furthermore, among BCp1 plants

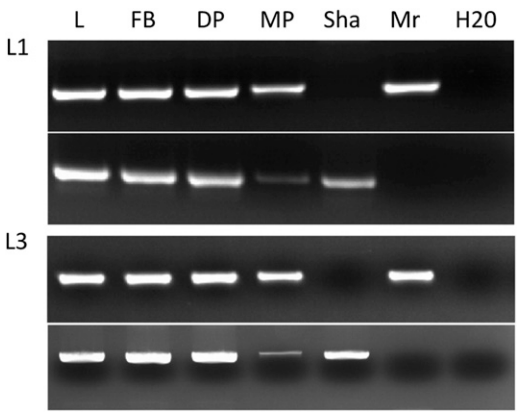

Figure 5 Genotyping at L1 and L3 of pollen from Mr-0 × Sha hybrid plants. At each locus, two different pairs of primers, respectively specific to the Mr-0 (top) or the Sha (bottom) allele, were used to amplify genomic DNA isolated from leaves (L), floral buds (FB), developing pollen (DP), and mature pollen (MP) of heterozygous plants. Sha and Mr, DNA from Sha and Mr-0 plantlets, respectively; H2O, negative control without DNA. The Sha alleles are barely detectable in mature pollen but amplified together with the Mr-0 alleles in immature pollen and vegetative tissues. Most of the pollen grains carrying the Sha alleles were thus eliminated at the mature pollen stage.

Table 3 Genotype segregation at L1 and L3 in the progeny of plants heterozygous at L1 and/or L3

Family	Locus	Chromosome	Marker	Position (Mb)	Number of Sha	Number of Hz	Number of Mr-0	Number of plants	P (χ^2)	Observed Sha frequency (%)	Expected Sha frequency (%)
[Mr-0]$L1^HL3^H$	L1	1	NGA128	20.6	15	85	70	170	2×10^{-8}	9	25
[Mr-0]$L1^HL3^H$	L3	3	MSAT3.23007	23.0	31	79	61	171	3×10^{-3}	18	25
[Mr-0]$L1^HL3^S$	L1	1	NGA128	20.6	5	100	78	183	1×10^{-13}	3	25
[Mr-0]$L1^SL3^H$	L3	3	MSAT3.23007	23.0	18	87	73	178	4×10^{-8}	10	25
[Sha]$L1^HL3^H$	L1	1	NGA128	20.6	4	29	21	54	4×10^{-3}	7	25
[Sha]$L1^HL3^H$	L3	3	MSAT3.23007	23.0	2	25	29	56	2×10^{-6}	4	25
[Sha]$L1^HL3^S$	L1	1	NGA128	20.6	10	91	83	184	2.6×10^{-13}	5	25
[Sha]$L1^SL3^H$	L3	3	MSAT3.23007	23.0	0	91	87	178	3.3×10^{-19}	0	25

The biases at L1 and L3 were observed in both cytoplasmic backgrounds and independently of each other. The observed biases are compatible with a pollen lethal effect (closer to 1:1 Hz:Mr-0 segregation). Alexander staining of the pollen from these plants is presented in Figure 6 and Figure S4. Hz, heterozygote.

heterozygous at L1Rak, we observed more aborted pollen grains in the plants that were also heterozygous at L3 than in those that were Mr-0 at L3 (Table S7), although in every case the amount of dead pollen was not sufficient to alter the overall fertility of the plants.

We conclude that in Rak-2 × Mr-0, as in Sha × Mr-0, pollen lethality results from a combination of both CMS and cytoplasm-independent PKs. L1 and L3, the two main loci involved in pollen mortality in the crosses between Sha and Mr-0, also participate in the sterility in the crosses between Rak-2 and Mr-0. However, remarkably, the plants heterozygous Rak-2/Mr-0 at L1 did not present any PK effect at this locus. Inversely, the progeny of plants heterozygous at L3 presented a strong bias against the Rak-2 allele (Table 5), typical of the PK observed at L3 in Sha/Mr-0 hybrids, whereas the Rak-2 allele at L3 had no restorer effect (Table S7). The dissociation of PK and CMS effects very likely contributes to the residual production of pollen in Rak2 × Mr-0 F_1 plants, allowing normal seed set, unlike in Sha × Mr-0 F_1 plants.

Discussion

The Sha × Mr-0 F_1 accumulates different causes of pollen sterility

We explored the genetic determinants of male sterility observed in the offspring of two distant *A. thaliana* accessions. We showed that the absence of seed set in the Sha × Mr-0 hybrid results from the combination of two different kinds of causes, both acting on male gametophyte development: gametophytic CMS leading to the death of pollen carrying Mr-0 nonrestorer alleles in a Sha cytoplasmic background,

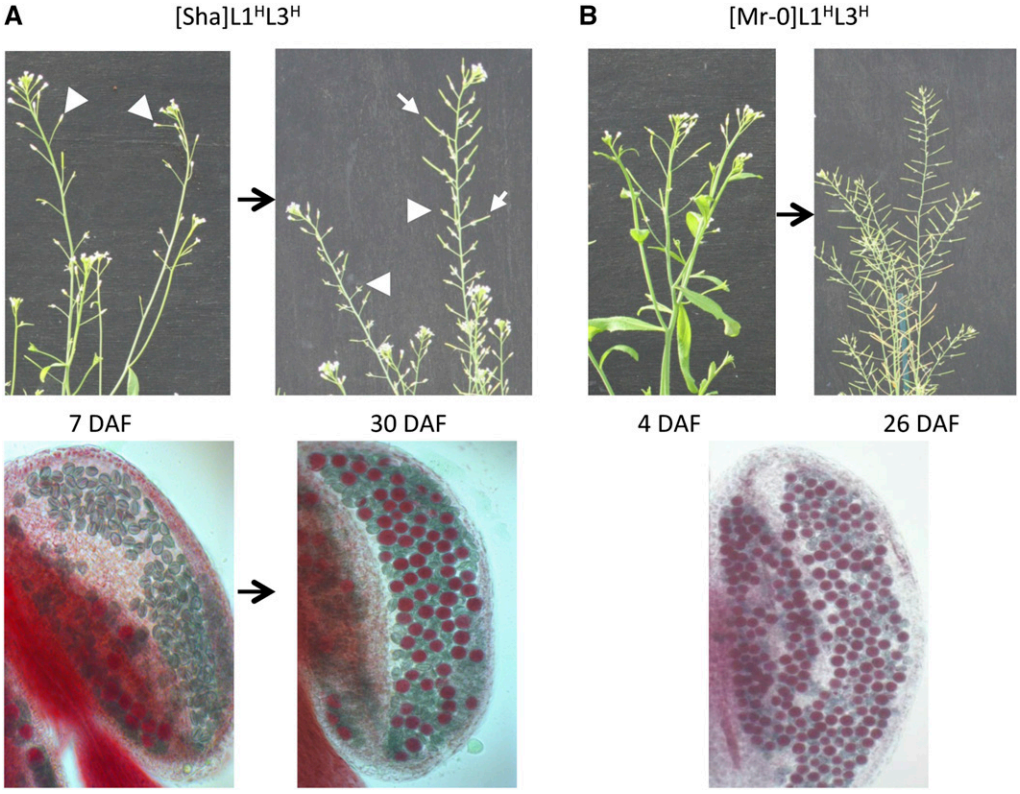

Figure 6 Reproductive phenotypes in [Sha]$L1^HL3^H$ and [Mr-0] $L1^HL3^H$ plants. Overall fertility phenotypes at two developmental stages (top panels) and pollen viability in close-up views of typical anthers after Alexander staining (bottom panels). DAF, day after flowering. (A) The [Sha] $L1^HL3^H$ plants are initially completely sterile (left panel, top), with anthers harboring only aborted (green) pollen grains (left panel, bottom). Later on, the anthers also contain viable (red) pollen grains (right panel, bottom) and plants produce fertile flowers (right panel, top). White arrows indicate developed siliques that will produce seeds; white arrowheads indicate aborted fruits. (B) [Mr-0]$L1^HL3^H$ plants are fully fertile throughout their development (top), and their anthers present a mixture of aborted and viable pollen grains throughout the reproductive phase (bottom).

Table 4 Genotype segregation at L1 and L3 in the Cvi-0 × Mr-0 and Mr-0 × Cvi-0 F_2 families

Family	Locus	Chromosome	Marker	Position (Mb)	Number of Cvi-0	Number of Hz	Number of Mr-0	Number of plants	*P* (χ^2)	Observed Cvi-0 frequency (%)	Expected Cvi-0 frequency (%)
Cvi-0 × Mr-0 F_2	L1	1	NGA128	20.6	18	98	62	178	8×10^{-6}	10	25
Mr-0 × Cvi-0 F_2	L1	1	NGA128	20.6	20	101	60	181	4×10^{-5}	11	25
Cvi-0 × Mr-0 F2	L3	3	MSAT3.23007	23.0	13	81	85	179	1×10^{-13}	7	25
Mr-0 × Cvi-0 F2	L3	3	MSAT3.23007	23.0	11	98	69	178	2×10^{-9}	6	25

and at least two PK loci leading to the specific abortion of pollen grains carrying the Sha alleles, independently of the cytoplasmic background. We confirmed the coexistence of these two mechanisms by isolating them from each other. First, the abortion of all pollen in the [Sha]Mr-0 cytoline indicates that the inability of the Mr-0 nuclear genome to restore fertility in the Sha cytoplasm is sufficient to achieve male sterility without the action of any segregation distorter. Second, heterozygotes at PK loci produce unviable pollen carrying the Sha alleles, even in a nonsterilizing cytoplasm. The normal seed set of the Mr-0 × Sha F_1, despite pollen mortality due to PKs, shows that the combination of the two mechanisms is necessary for the total sterility of the reciprocal Sha × Mr-0 F_1. L1 and L3, the two major loci involved in the Sha × Mr-0 hybrid sterility, effectively carry genetic factors for both mechanisms. In addition, in the Sha × Mr-0 F_2* population, in which these two major loci were fixed for nonrestorer Mr-0 alleles, we detected six additional QTL for fertility, indicating a complex genetic determinism for the Sha × Mr-0 hybrid sterility.

Segregation distortions result from PKs active at several loci in the same intraspecific cross

Segregation distortions were observed in Sha/Mr-0 hybrids at three loci, L1, L3, and L5 (Figure 4, Table 2, Table 3, Table S2, Table S5, and Table S6). The under-representation of the Sha allele at L1 and L3 in mature pollen (Figure 5) showed that the distortions result from allele-dependent pollen death.

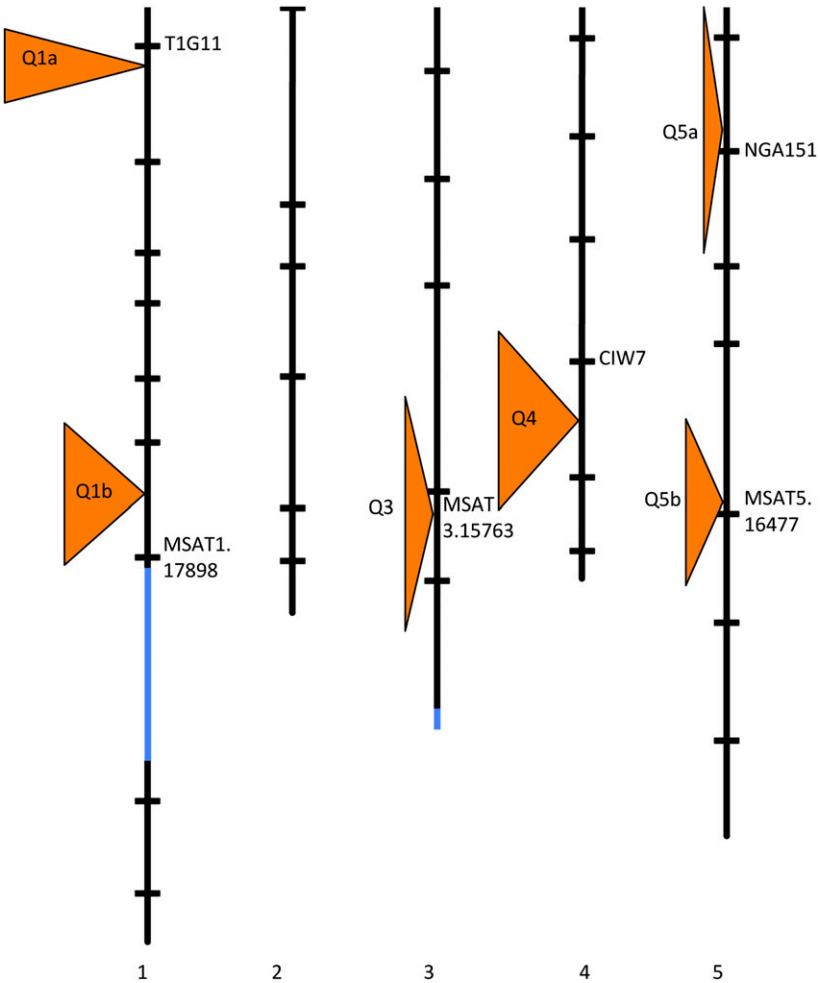

Figure 7 QTL affecting the fertility phenotype in the Sha × Mr-0 F_2* family. The regions that do not segregate in this family (homozygous for Mr-0 alleles) are indicated in blue. The orange triangles indicate the most likely positions of the QTL; their height corresponds to the 1.8 LOD support interval and their width is proportional to the LOD score of the corresponding QTL. The positions of markers are indicated as horizontal bars. The name of the marker closest to each QTL is given.

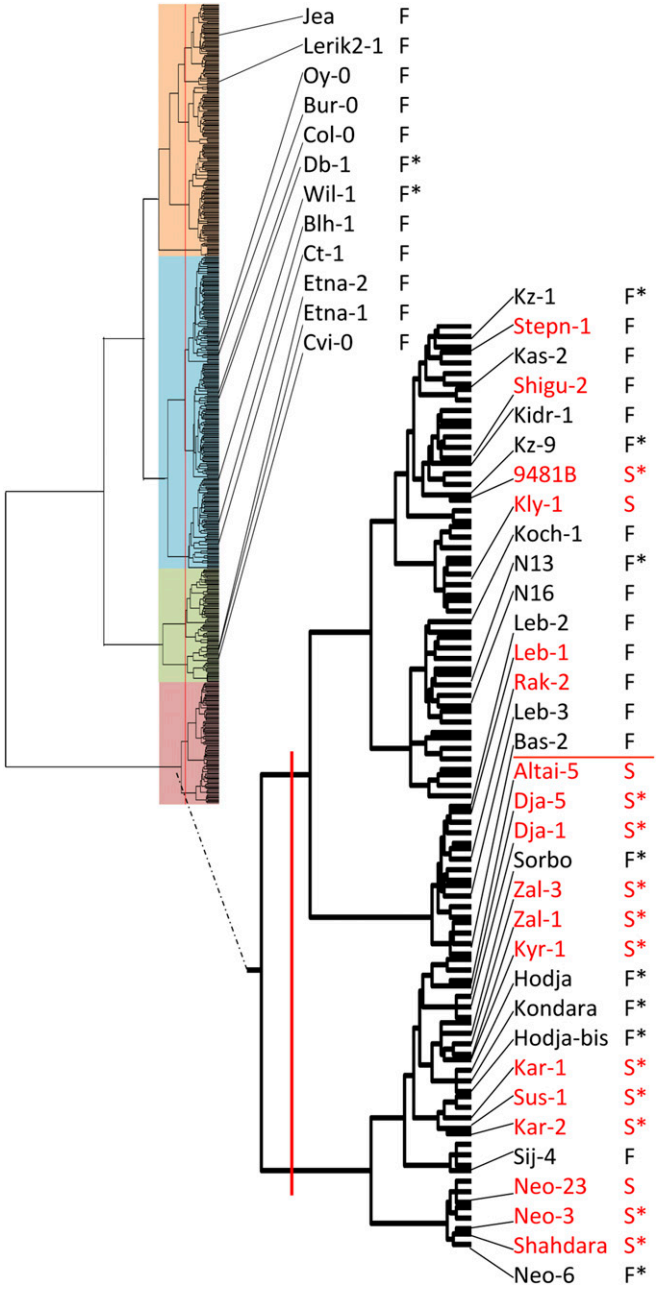

Figure 8 Fertility of 46 crosses with Mr-0 as the male parent. The accessions are organized in four main clusters (color shaded) corresponding to groups of genetic similarity obtained by clustering 598 natural accessions using 341 SNP markers (Simon *et al.* 2012). The accessions in red carry the mitochondrial sterilizing factor *orf117Sha*; they all belong to the same nuclear diversity group. The F_1 phenotype is indicated beside the name of the maternal parent by "F" for fertile F_1 or "S" for sterile F_1. A red horizontal line separates the two differentiated subgroups of nuclear diversity in this group. *F_1 phenotype from Gobron *et al.* (2013).

Such distortions may result from the fitness costs of Rf genes, expressed at the pollen viability level (Montgomery *et al.* 2014) or from the action of PKs that eliminate the Sha alleles. Here, no pollen defect was observed in plants carrying fixed Sha alleles, in either cytoplasm. Furthermore, the observation of biases against Cvi-0 alleles at L1 and L3 in Cvi-0/Mr-0 hybrids (Table 4) demonstrates that the Mr-0 alleles have killer behavior at these loci, and that the elimination of target alleles is not due to an Rf, because Cvi-0 alleles are not restorers (Gobron *et al.* 2013). We also observed the elimination of Rak-2 non-restorer alleles at L3 in the Rak-2/Mr-0 F_2s (Table 5). Taken together, these results show that PKs are the main cause of the segregation biases at L1 and L3 in the Sha/Mr-0 heterozygotes, although we cannot exclude an additional cost effect of Rfs at these loci and/or at L5.

Single-locus segregation distorters usually rely on the tight genetic linkage of several interacting genes, leading to their transmission as self-sufficient loci. Such loci have been documented in animals, especially in *Drosophila* (Orr *et al.* 2007; Larracuente and Presgraves 2012), and in *Ascomycetes* (Turner and Perkins 1979; Raju 1994). In plants, after the first description of a PK in a hybrid between two *Solanum* species (Cameron and Moav 1957), gamete killers (most often PKs) have been reported, especially in hybrids between cultivated plants and their wild relatives, where they often constitute obstacles to the breeding of desirable traits (Maan 1975; Sano 1990). In rice in particular, a number of gamete killers have been described in crosses between cultivated rice (*O. sativa*) and its wild relatives (Sano 1990; Hu *et al.* 2006; Garavito *et al.* 2010), or between *O. sativa* subspecies (Oka 1974; Zhang *et al.* 2006). The genes underlying two of the latter have been identified (Chen *et al.* 2008; Long *et al.* 2008; Yang *et al.* 2012). Gamete killers are also found in wild species, as in the interspecific cross between *Mimulus guttatus* and *M. nasutus* (Fishman and Saunders 2008), and, at the intraspecific level, in some populations of the dioecious *Silene latifolia* (Taylor and Ingvarsson 2003). Recently, several single-locus gametic transmission distorters were reported in interpopulation crosses of the *A. thaliana* relative *A. lyrata*, most of which affect pollen, although the causes of segregation distortion remain to be elucidated (Leppala *et al.* 2013).

We are not aware of any reports of gamete killers in intraspecific hybrids of *A. thaliana*, despite the large number of crosses between natural accessions available. However, we suspect that gamete killers have not been identified as such in previous studies. For example, strong segregation distortions reported in a Lov-5 × Sha F_2 (Salome *et al.* 2012) at two loci on chromosome 1 (0.5% Lov-5 and 2% Sha remaining) suggest gamete-killer effects. Strikingly, the location of the second distorted locus is consistent with it corresponding to L1, which also leads to the elimination of the Sha allele at this locus.

Sha and Rak-2 possess different Rfs

Because Rak-2 carries the same cytoplasm as Sha, in particular the mitochondrial sterility-inducing *orf117Sha*, we assumed that the Sha and Rak-2 cytoplasms belong to the same CMS-inducing system. However, the genetics of fertility restoration in Sha × Mr-0 is different from that in Rak-2 × Mr-0. Whereas restoration is complex in the Sha × Mr-0 cross, in the Rak-2 × Mr-0 CMS it is controlled by a unique Rf locus, L1Rak, included in the L1 interval: paternal backcross plants

Table 5 Genotype segregation at L1 and L3 in the Rak-2 × Mr-0 and Mr-0 × Rak-2 F_2 families

Family	Locus	Chromosome	Marker	Position (Mb)	Number of Rak-2	Number of Hz	Number of Mr-0	Number of plants	P (χ^2)	Observed Mr-0 frequency (%)	Expected Mr-0 frequency (%)	Observed Rak-2 frequency (%)	Expected Rak-2 frequency (%)
Rak-2 × Mr-0 F_2	L1	1	NGA128	20.6	69	103	10	182	1×10^{-9}	6	25	–	–
	L3	3	MSAT3.23289	23.3	3	91	88	182	6×10^{-18}	–	–	2	25
Mr-0 × Rak-2 F_2	L1	1	NGA128	20.6	50	87	45	182	0.73	25	25	–	–
	L3	3	MSAT3.23289	23.3	3	89	90	182	8×10^{-19}	–	–	2	25

were fully sterile when, and only when, homozygous Mr-0 at L1Rak (Table S7 and Figure 9A); and the Mr-0 allele at L1Rak was not transmitted through pollen in the Rak-2 cytoplasm. Therefore, the restorer genes carried by the nuclear genomes of Sha and Rak-2 are different. The presence of different sets of Rf genes in related genotypes has been reported in other species. For example, in maize, three restorers, *Rf1*, *Rf8*, or *Rf**, are able to restore fertility, in combination with *Rf2* (Wise *et al.* 1999). In radish, Ogura CMS was first described to be controlled by a unique gene in some Japanese radish cultivars (Ogura 1968), but then found to rely on two or three major restoration loci, along with minor ones, in populations from United States or European germplasms (Nieuwhof 1990; Bett 2004).

Here, the L1 locus, which encompasses L1Rak, colocalizes in Col-0 with a region carrying a cluster of no fewer than 15 mitochondria-targeted Rf-like pentatricopeptide repeat (PPR) genes (O'Toole *et al.* 2008; Fujii *et al.* 2011). The Rf-like PPR genes, defined on the basis of their homology to restorer genes identified in several species, obviously provide excellent candidates for the restoration of fertility. In addition, the Rf-like PPR genes are known to evolve more rapidly than other PPR genes, mostly by duplication events, and possibly under diversifying selection (O'Toole *et al.* 2008; Fujii *et al.* 2011). Evidence for diversifying selection under cytonuclear conflict has been reported for some PPR genes in *A. lyrata* (Foxe and Wright 2009). If PPR genes do underlie the fertility restoration at L1 and L1Rak, their rapid evolving behavior may explain the difference in the genetics of restoration between the two accessions. In rice, the *Rf-1* restoration locus of the BT-CMS line is a large cluster of highly similar PPR genes containing two related functional restorers, *Rf-1A* and *Rf-1B* (Wang *et al.* 2006), and different rice cultivars, subspecies, and species exhibit different structural organizations and copy numbers of the Rf-1 complex locus (Kato *et al.* 2007). The identification of Rf genes in Rak-2 and Sha will undoubtedly shed light on their evolutionary relationship.

Genetically linked PK and Rf loci concurrently contribute to Sha × Mr-0 hybrid sterility

Our results show that the complete male sterility of the Sha × Mr-0 F_1 hybrid results from the following features: PKs eliminate pollen carrying Sha alleles, CMS eliminates pollen carrying Mr-0 alleles, and PK and Rf loci are genetically linked, so that all pollen grains eventually abort (Figure 10). Because PKs act in both cytoplasms, whereas the CMS is active only in the Sha cytoplasm, all pollen grains produced by the Sha × Mr-0 hybrid die, but the Mr-0 pollen produced by the Mr-0 × Sha hybrid remains viable. Based on these observations, the fertility of the Rak-2 × Mr-0 F_1 plants is the result of the lack of PKs at L1 and the lack of a major restorer at L3: the absence of genetic linkage between the major Rf locus (L1Rak) and the PK at L3 permits enough residual pollen production to allow normal seed set in the hybrid. Furthermore, this model also provides an explanation for the absence of sterile

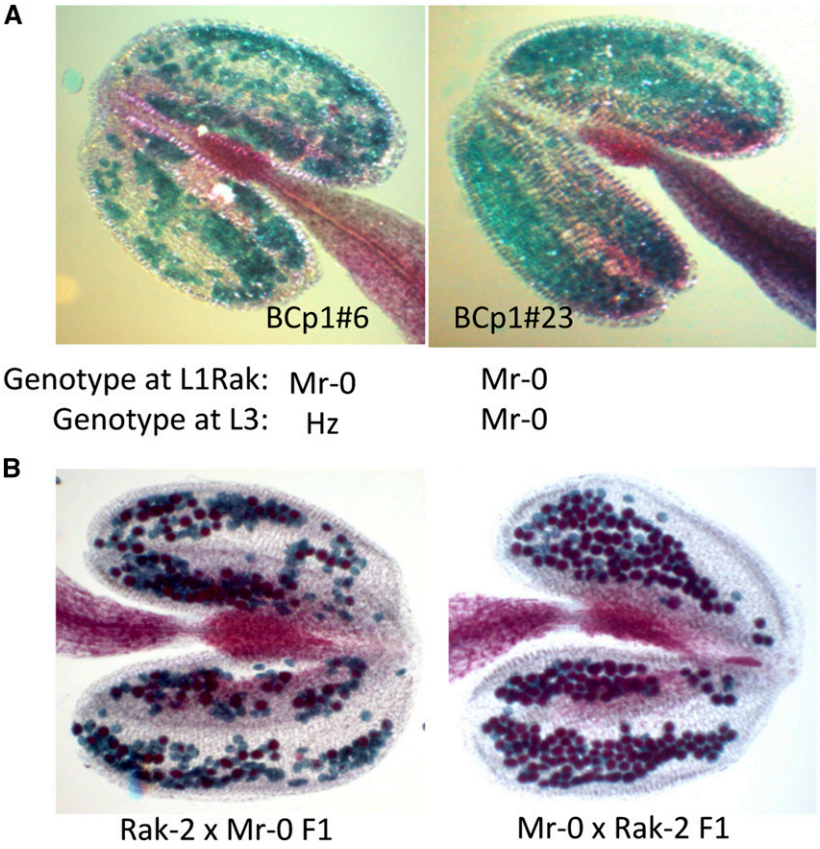

Figure 9 Pollen viability of plants from crosses between Rak-2 and Mr-0. Alexander staining (observation under a light microscope, ×10) of anthers. Aborted pollen grains are stained green, while viable pollen grains are stained red. Hz, heterozygote. (A) Sterile Rak-2 × Mr-0 BCp1 plants (see also Table S6). (B) Rak-2/Mr-0 reciprocal F_1 hybrids.

recombinants at L1 in the PopL1 mapping population: recombinants in this interval likely separated PK and Rf factors, by either loss of the Mr-0 killer factor of the PK or its linkage with a restorer allele, allowing the rescue of pollen grains.

The degree of genetic linkage between PKs and Rfs probably varies among the different loci. We suspect that PK and Rf at L1 have become dissociated by recombination between NGA128 and F5I14, which are 3.8 Mb apart in Col-0, whereas both PK and Rf factors at L3 reside in a 280 kb interval (Figure 2). In crosses between *A. lyrata* ssp. *lyrata* and *petraea*, in which transmission ratio distorters are observed at male fertility QTL (Leppala and Savolainen 2011), a major Rf locus and a segregation distorter have been mapped 2 cM apart. It is noteworthy that this distorter eliminates the allele from the sterilizing cytoplasm donor, *i.e.*, the allele linked to the Rf (Aalto *et al.* 2013).

We consider it unlikely that the genetic linkage of Rf and PK factors at the two loci L1 and L3 in a single cross is fortuitous. The corresponding genomic regions may be prone to carrying factors affecting pollen viability, for example by concentrating genes involved in pollen development. Colocalization also suggests the possibility that restoration of CMS and PKs shares a common determinant. This could be an expression signal (*e.g.*, a pollen-specific promoter), or a protein-coding sequence. For example, the *Drosophila ovd* gene has been identified as the cause of both male sterility and segregation distortion in crosses between *bogota* and *USA* subspecies of *D. pseudoobscura* (Phadnis and Orr 2009). Male sterility is visible only when *bogota* females are crossed with *USA* males, revealing asymmetrical behavior reminiscent of the asymmetrical hybrid sterility in Sha/Mr-0 *A. thaliana* crosses.

One of the loci described here colocalizes with a cluster of Rf-like PPR genes on chromosome 1. As a result of the conflict between cytoplasm and nuclear genomes in CMS (Touzet and Budar 2004; Hernandez Mora *et al.* 2010; Fujii *et al.* 2011), clusters of Rf-like PPR genes are prone to major structural rearrangements between related species or subspecies (Geddy and Brown 2007; Kato *et al.* 2007) or within species (Hernandez Mora *et al.* 2010). These allelic structural variations likely hinder meiotic recombination in these regions, a feature that may favor the stabilization of segregation distorter loci (Schwander *et al.* 2014).

Genomic conflicts potentially underlie reproductive barriers between Sha and Mr-0 accessions

Sha and Mr-0 have accumulated genetic differences at two PK loci at least, at which intragenomic conflicts are suspected, in addition to a cryptic CMS that very likely drove the selection of eight identified restorer loci, at least two of which are genetically linked to PKs. Our results show that these loci concurrently create reproductive barriers between these two accessions. The contribution of genomic conflicts to reproductive isolation has recently been emphasized (Crespi and Nosil 2013). In that regard, a recent study of hybrid male sterility between *Drosophila* species proposed that

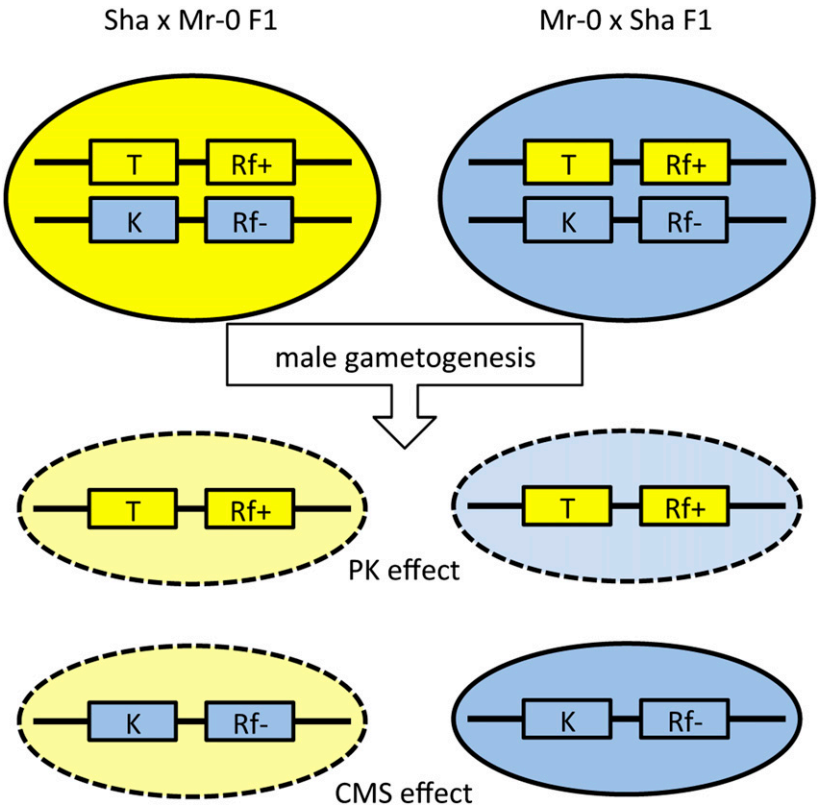

Figure 10 Genetic model of hybrid male sterility resulting from the combination of CMS and PKs. For clarity, we assumed only one nuclear locus in the genome with PK and gametophytic Rf, represented by boxes, tightly linked. The color of the boxes, and that of the ovals for cytoplasmic backgrounds, represents the parent of origin: yellow, Sha; blue, Mr-0. At the PK locus, the Sha allele is the target (T), and Mr-0 is the killer (K). For the CMS locus, the Sha allele restores fertility (Rf+), Mr-0 does not (Rf–). Heterozygotes produce only two pollen types, due to the tight genetic linkage between the two factors. The abortion of pollen is represented by a light background color and a dashed line. PK effect: the presence of the K (Mr-0) factor in the mother plant induces the abortion of pollen with the T (Sha) allele in either cytoplasm. The CMS kills pollen carrying the Rf– (Mr-0) allele only in the Sha (sterilizing) cytoplasm. Therefore, all pollen grains produced by the Sha × Mr-0 F_1 die, but from different causes, resulting in the sterility of the hybrid. Conversely, the Mr-0 × Sha F_1 produces enough viable pollen (carrying the Mr-0 allele) to ensure normal seed set.

intragenomic conflicts at segregation distorter loci drive genome divergence, leading to reproductive barriers (Zhang *et al.* 2015). These *Drosophila* loci controlling male fertility are tightly linked to sex-ratio distortion at six genomic regions, reminiscent of our results for *A. thaliana* divergent strains.

Information on the level of divergence between *A. thaliana* accessions is provided by diversity and genetic structure analyses conducted using natural variants from around the world. Sha and Mr-0 originate from geographically distant allopatric populations (Tajikistan and Sicily, respectively). Both areas were putative glacial refugia during the Pleistocene (Beck *et al.* 2008). Geographic isolation in distinct refugia is predicted to be a major factor favoring the divergence between accessions, corroborated by the highly significant genetic differentiation (F_{ST}) values between Italian and Central Asian accessions (Beck *et al.* 2008). Sha groups with other accessions from Central Asia in a highly differentiated cluster (Nordborg *et al.* 2005; Platt *et al.* 2010; Simon *et al.* 2012; Brennan *et al.* 2014). Mr-0 clusters with some Mediterranean and Cape Verde accessions (Simon *et al.* 2012), and its genetic structure is very different from all other accessions (Nordborg *et al.* 2005). Therefore, Sha and Mr-0 may come from lineages that experienced different genomic conflicts: CMS in the Sha lineage and PKs in the Mr-0 lineage.

It is also conceivable that PKs and Rf coevolved in the ancestral lineage of Sha and Mr-0. In this case, their genetic linkage may have influenced their evolutionary trajectories. For instance, the presence of a nearby killer allele may protect a nonrestorer allele from being swept out. In gametophytic CMS, heterozygotes do not transmit the nonrestorer allele through pollen. Hence, gametophytic restorer alleles are likely easily fixed, sweeping out nonrestorer ones. The presence of a linked PK favoring the CMS nonrestorer allele may slow down the fixation of restorer alleles, allowing longer survival of nonrestorer ones. The genetic variability that we observe at the locus would then be a remnant of the evolutionary history of intertwined intragenomic conflicts.

Geographically widespread selfing taxa, such as *A. thaliana*, are expected to give rise to cryptic species complexes, as has been observed in the related genus *Draba* in which similar genetic mechanisms appear to be contributing to the evolution of reproductive barriers (Skrede *et al.* 2008). Our results make it plausible that Sha and Mr-0 are currently becoming cryptic species. The identification and evolutionary analysis of the genes responsible for the Sha × Mr-0 hybrid sterility will provide clues on their functions as well as valuable information on the evolutionary dynamics of reproductive isolation in this species. This type of analysis will undoubtedly contribute to our understanding of some of significant mechanisms involved in speciation.

Acknowledgments

We gratefully acknowledge J. Jimenez-Gomez, O. Loudet, R. Mercier, G. Pelletier, and E. Téoulé for their critical reading of the manuscript. The Institut Jean-Pierre Bourgin benefits from the support of the Labex SPS (Saclay Plant Science) (ANR-10-LABX-0040-SPS).

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Communicating editor: L. C. Moyle

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.183707/-/DC1

Genomic Conflicts that Cause Pollen Mortality and Raise Reproductive Barriers in *Arabidopsis thaliana*

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DOI: 10.1534/genetics.115.183707

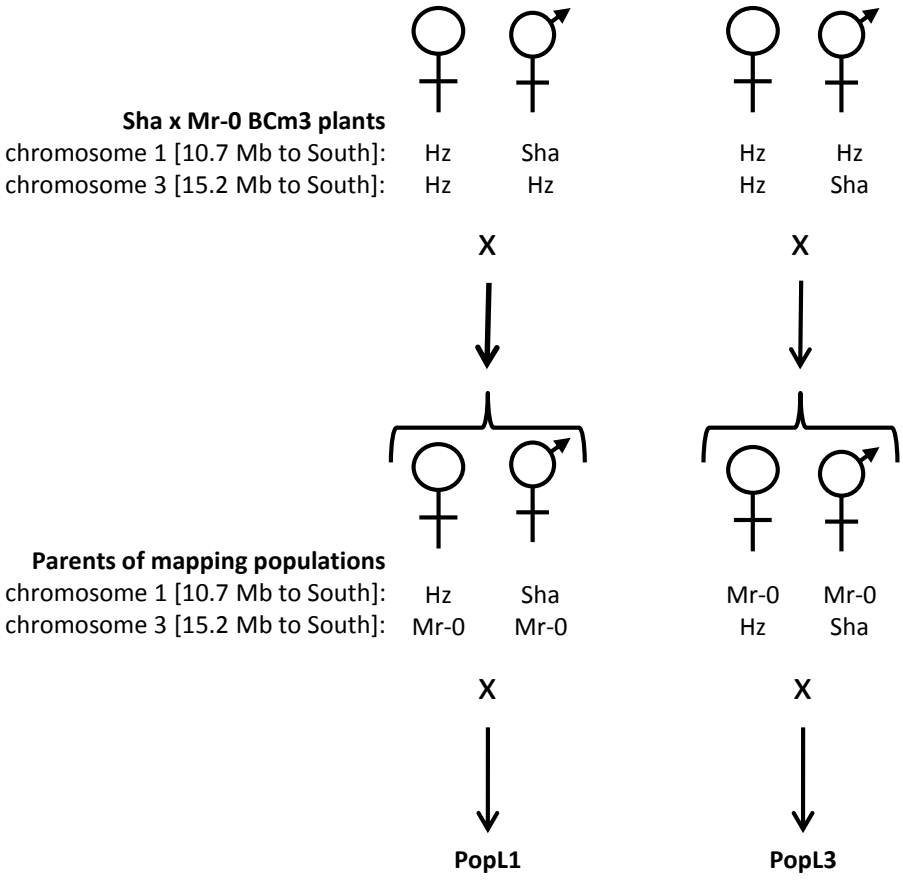

Figure S1: Construction of the PopL1 and PopL3 populations. We started from plants obtained by three recurrent maternal backcrosses (BCm3) of the Sha x Mr-0 F1 with Sha. We then crossed BCm3 plants with the indicated heterozygous (Hz) genotypes on chromosome 1 and chromosome 3. Among the progeny of these crosses, we selected plants with appropriate genotypes to be the parents of the mapping populations. Parents of PopL1 were Hz on chromosome 1 and Mr-0 on chromosome 3 for the mother, and Sha on chromosome 1 and Mr-0 on chromosome 3 for the father. Parents of PopL3 were Mr-0 on chromosome 1 and Hz on chromosome 3 for the mother, and Mr-0 on chromosome 1 and Sha on chromosome 3 for the father. Genotyping was performed with markers CIW1, NGA128, ATHGENEA and F5I14 (at 18.4, 20.6, 22.4 and 24.4 Mb respectively) for chromosome 1, and with markers MSAT3.58, MSAT3.20435, ATHFUS6 and MSAT3.70 (at 18.7, 20.43, 22.6 and 23.45 Mb respectively) for chromosome 3.

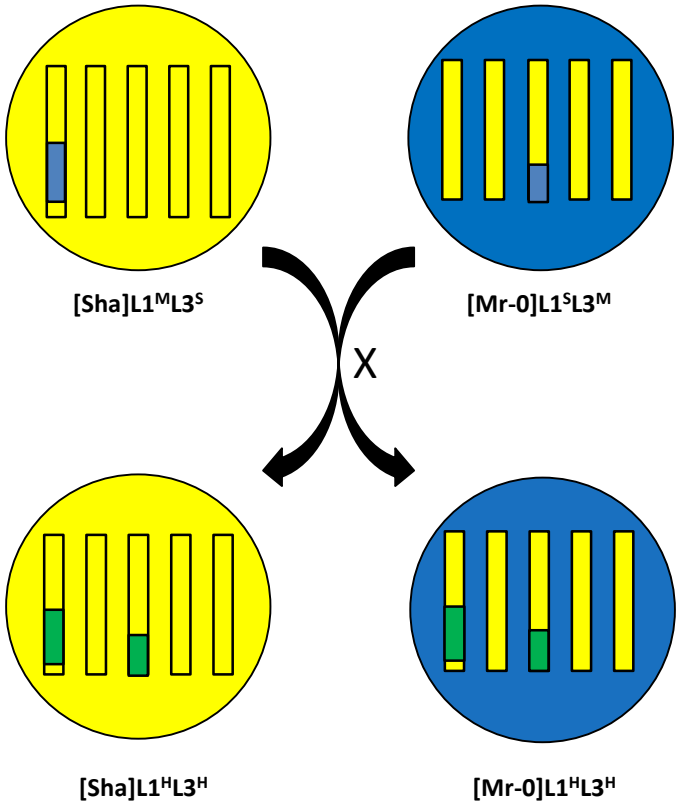

Figure S2: Construction of [Sha]L1^{H}L3^{H} and [Mr-0]L1^{H}L3^{H} families. The parents, [Sha]L1^{M}L3^{S} and [Mr-0]L1^{S}L3^{M}, were selected from Sha backcross progenies of the two reciprocal F1s after dense genotyping (see Materials and Methods). They are homozygous Mr-0 at L1 (between 18.7 and 24.4 Mb) or at L3 (from 22.6 Mb to the South telomere) respectively, in a Sha nuclear background. Their reciprocal crosses produced [Sha]L1^{H}L3^{H} and [Mr-0]L1^{H}L3^{H}, both heterozygous at L1 and L3, in either cytoplasm. The five chromosomes are represented by vertical bars in specific cytoplasmic backgrounds. Yellow, Sha; blue, Mr-0; green, heterozygous.

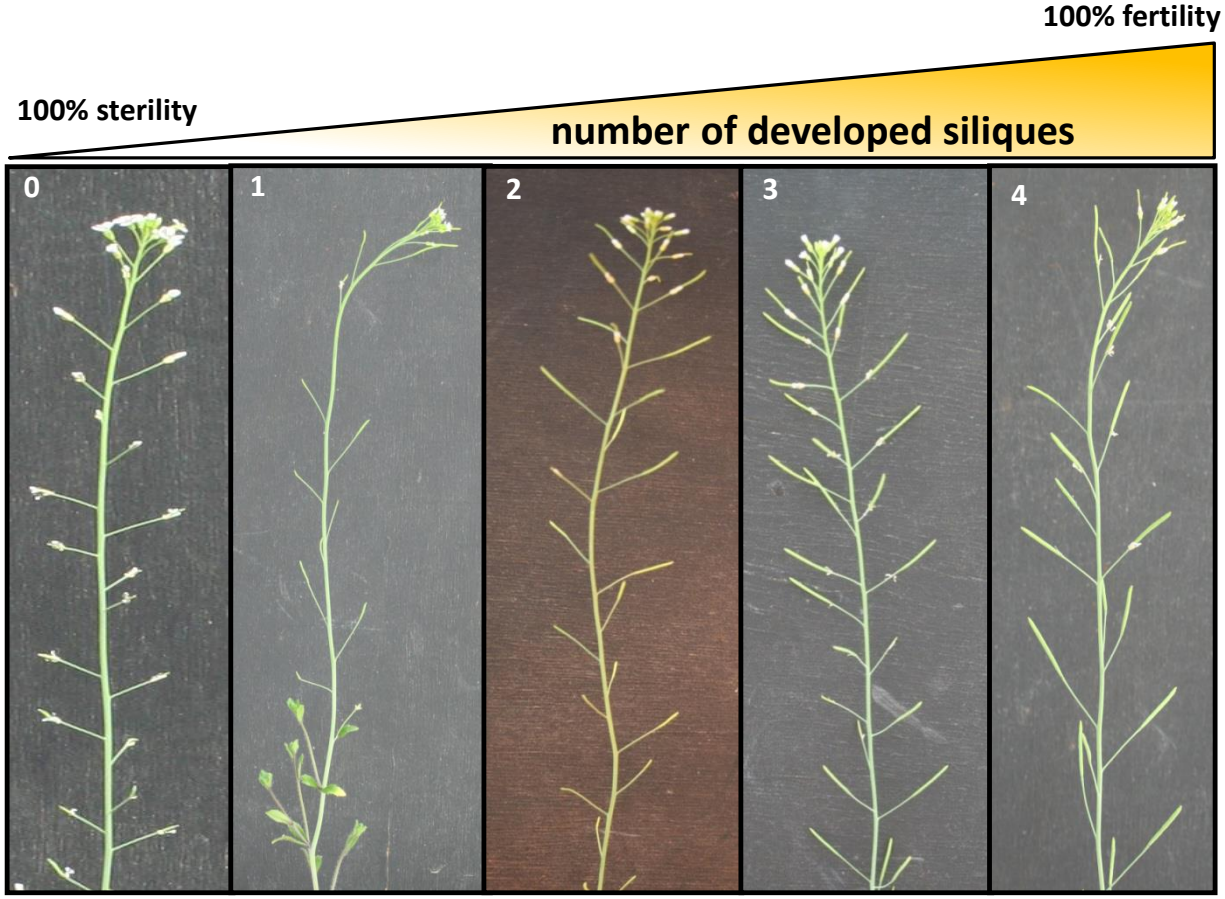

Figure S3: Scoring scale of fertility phenotypes. Plant fertility (illustrated here with individuals from the [Sha]Mr-0 x (Mr-0 x Sha) family) is scored as a visual estimate of the number and length of developed siliques per plant.
0: plant completely sterile
1: a few developed siliques
2: half of siliques are developed
3: a few aborted siliques
4: plant fully fertile

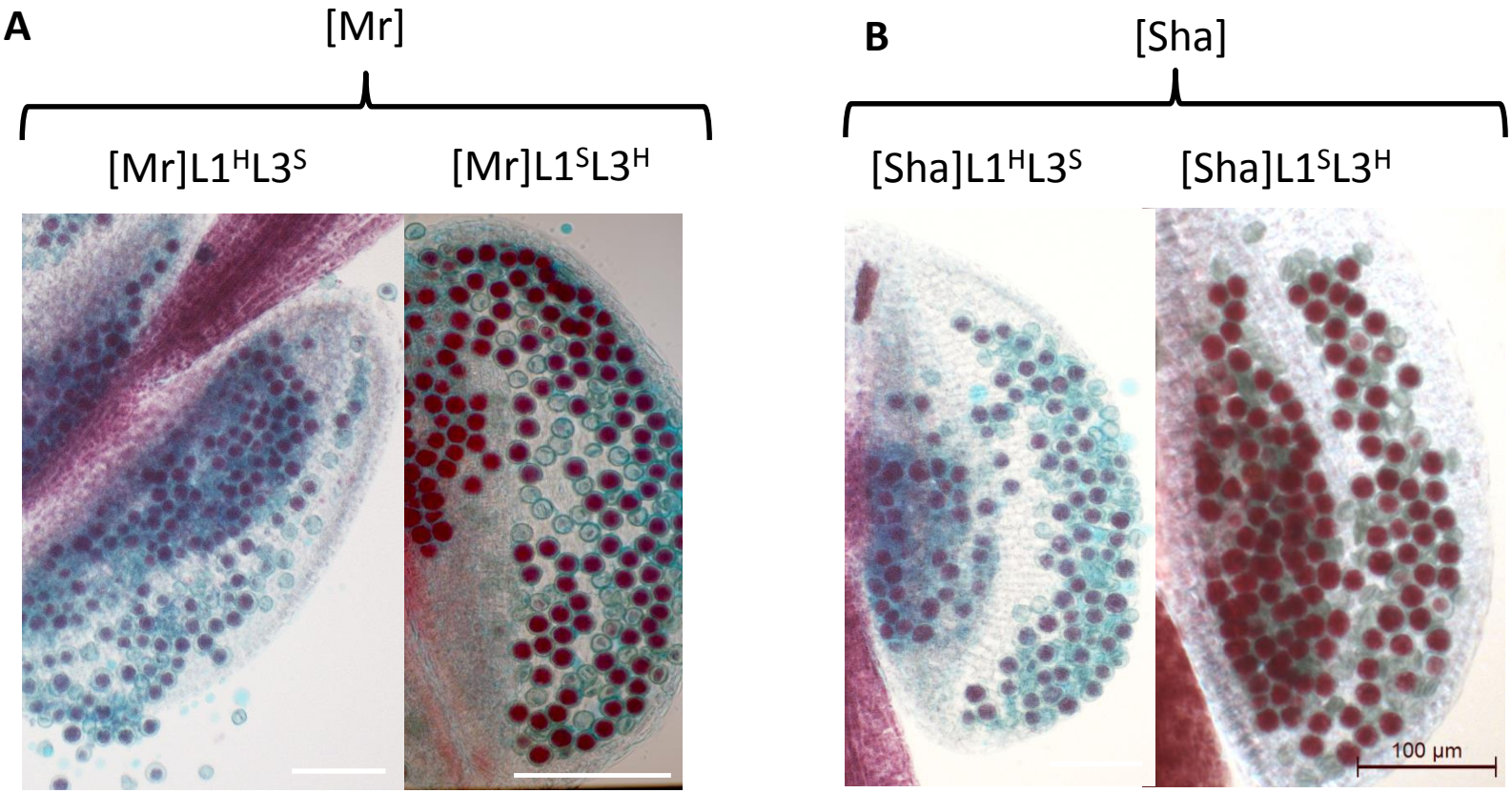

Figure S4: Pollen viability of plants heterozygous either at L1 or L3 in both cytoplasms. Alexander staining (observation under a light microscope, x10) of anthers. Aborted pollen grains are stained green, viable pollen grains are stained red.
A. Plants with Mr-0 cytoplasm. B. Plants with Sha cytoplasm.

Table S1: Genotyping markers used

Marker name	Chromosome	Position (Mb)	Forward Primer (F) 5'-3'	Reverse Primer (R) 5'-3'
T1G11	1	1.24	GAAGACAAAGCTCTGCAGTAATG	AATTGCATAAGGCACTTGAAAG
MSAT1.14	1	5.02	GCAAACAAAGAAAAAGTAT	GGTGGAAACGTCCC
MSAT1.07964	1	7.96	GTCGTTGCTTCCTCTCCTTG	GCCCCTGTTGTTCAATCAAA
CIW12	1	9.62	AGGTTTTATTGCTTTTCACA	CTTTCAAAAGCACATCACA
MSAT1.12091	1	12.1	GTGTCCCCATGTGCTCTTCT	AGATCGAGTGGGGAAACCTT
MSAT1.4	1	14.16	CTAAACTAGAACCAGGGGTAA	ACAAAAATCGTGGTGATAATA
MSAT1.17898	1	17.9	ATTCCGGTGAAATCATCTCG	TCCGCTTCCTTCTCACACTT
CIW1	1	18.4	ACATTTTCTCAATCCTTACTC	GAGAGCTTCTTTATTTGTGAT
NGA128	1	20.63	GGTCTGTTGATGTCGTAAGTCG	ATCTTGAAACCTTTAGGGAGGG
ATHGENEA	1	22.4	ACCATGCATAGCTTAAACTTCTT	ACATAACCACAAATAGGGGTG
ind1.22788	1	22.8	CTGAACTTAACAAACCAAGGAGA	TTTCCAAGCATTCAGTGAGTT
F5I14	1	24.37	CTGCCTGAAATTGTCGAAAC	GGCATCACAGTTCTGATTCC
MSAT1.13	1	25.83	CAACCACCAGGCTC	GTCAAACCAGTTCAATCA
NGA692	1	28.84	TTTAGAGAGAGAGAGCGCGG	AGCGTTTAGCTCAACCCTAGG
MSAT2.5	2	0.21	TGAGAGGGACAGATAGGAA	ATCAAAAGGGATACTGACAA
MSAT2.28	2	6.41	AATAGAAATGGAGTTCGACG	TGAACTTGTTGTGAGCTTTG
F3P11.6b	2	8.43	TTCAATCTTCTCTACTGTCTTCG	AGCAGGAAGTAGTAAGTGGAATA
CZSOD2	2	12.02	GAATCTCAATATGTGTCAAC	GCATTACTCCGGTGTCGTC
NGA168	2	16.3	TCGTCTACTGCACTGCCG	GAGGACATGTATAGGAGCCTCG
MSAT2.10	2	18.02	ACAAACATGTTCTGGGTTA	ATTCTTCATTATCTGCTGCT
MSAT3.02052	3	2.05	AAGGTTACTCTTATCGTTTAT	GGTTTCAACTTAGACGTTT
NT204	3	5.57	TGGAAGCTCTAGAAACGATCG	ACCACCTAAACCGAGAATTGG
MSAT3.2	3	9.06	AAGGTACGGCGGTGGATATTG	CGGGGATTTCTTCTTCCTGTG
MSAT3.15763	3	15.76	TGGAAAACGAAATACATCAAATCA	CGCGGATGTATAGACGGGTA
MSAT3.58	3	18.66	GGTTGAAACTCTATGGTAT	CGCATTTCTTGATTTACTA
MSAT3.20435	3	20.46	ATCGATCGGTCTCGTGTTCT	CACCATTGGATGTGGCTTTA
ATHFUS6	3	22.64	TCGTTACACTGGCTTGCTTG	TTCCTTGATCAGATTTGGTCG
MSAT3.23007	3	23.01	AAAAGCGTGATTGTGTTGTG	TGGAATGTTCCATCTTCAACT
MSAT3.23289	3	23.29	ACGTCCCATCATATCCCTCT	ACGGAGGACTTTGTAAGCAA
MSAT3.70	3	23.45	CACCCATAGACACACAACC	TCAAAACCTCCAACCATAC
MSAT4.00986	4	0.98	TTCTGAATTGGATTCCGAGAG	CCGTCGCACACATACACA
IND4.04197	4	4.2	CTGGCTTCGACTTGATTCT	GCCAGAGTTGATCTGATGC
MSAT4.35	4	7.55	CCCATGTCTCCGATGA	GGCGTTTAATTTGCATTCT
CIW7	4	11.52	AATTTGGAGATTAGCTGGAAT	CCATGTTGATGATAAGCACAA
MSAT4.13	4	15.3	GGAACAAGAACACAGTGAA	ATAAATCTAGGCAGGACAAG
MSAT4.21	4	17.69	TTATGCTATGGCTGTTTGGT	CGAAATCTGTTCTTGCATTC
ATHCTR1A	5	0.98	TATCAACAGAAACGCACCGAG	CCACTTGTTTCTCTCTCTAG
NGA151	5	4.67	GTTTTGGGAAGTTTTGCTGG	CAGTCTAAAAGCGAGAGTATGATG
NGA139	5	8.43	GGTTTCGTTTCACTATCCAGG	AGAGCTACCAGATCCGATGG
MSAT5.10960	5	10.96	GCGCAATTTCGAACATATCA	CACGGTGATGGTGCATTTAG
MSAT5.16477	5	16.48	AAACTGGTGGCTTTCCCATT	CATTGAAAGCTAACTGTGAAGCA
MSAT5.20037	5	20.03	CTTTCGGTGGATAAATCATGTTA	CGCATACAAATTTTCTGCTATGT
JV75.76	5	23.88	CACAATCAGAGGGGGTTGAT	AAATTTTGGGGGAAATGAAA
L1Mr	1	20.01	CAGAAAACAGATAATAGCTAAG	GAGATAGGGAGAGTCGACGC
L1Sha	1	20.01	GACCAGAAAACAGATAATGAGT	GAGATAGGGAGAGTCGACGC
L3Mr	3	23.14	TCTTCGTTTTAGGGTCGTGA	TTCATGCTTCTGAACTACTATGTT
L3Sha	3	23.14	TCAATCATCAATAACCCTGCGG	TGGTCACTAAATAGCGAGAGAGA

Table S2: Genome-wide allele segregation in the Mr-0 x Sha F2 family

Marker name	Chromosome	Position (Mb)	Nb Sha	Nb Hz	Nb Mr-0	Total	P (χ^2)
T1G11	1	1.24	43	85	53	181	4.1E-02
NGA63	1	3.22	37	86	54	177	1.8E-02
CIW12	1	9.62	26	99	55	180	3.8E-04
MSAT 1.4	1	14.2	23	96	59	178	0.4E-04
CIW 1	1	18.37	24	93	65	182	9.3E-06
NGA128	**1**	**20.63**	**20**	**94**	**68**	**182**	**0.3E-06**
MSAT1.13	1	25.83	32	101	49	182	0.7E-02
MSAT 2.5	2	0.21	39	92	51	182	4.5E-02
MSAT 2.28	2	6.41	38	94	44	176	5.4E-02
MSAT 2.17	2	10.73	39	97	47	183	5.1E-02
CZSOD 2	2	12.02	42	94	46	182	8.3E-02
MSAT 2.10	2	18.02	50	91	40	181	5.7E-02
MSAT 2.22	2	19.63	46	94	36	176	3.8E-02
MSAT 3.02052	3	2.05	61	80	33	174	6.3E-04
MSAT 3.32	3	11.21	29	94	59	182	6.4E-04
MSAT 3.58	3	18.66	16	87	79	182	0.3E-10
MSAT 3.20455	3	20.43	10	84	88	182	1.8E-16
MSAT 3.70	**3**	**23.45**	**5**	**76**	**99**	**180**	**5.4E-24**
MSAT 4.00986	4	0.98	33	99	51	183	0.9E-02
MSAT 4.35	4	7.55	20	93	25	138	0.2E-04
C1W7	4	11.52	32	89	58	179	0.2E-02
MSAT 4.18	4	11.97	31	92	57	180	0.2E-02
MSAT 4.14	4	15.21	36	97	49	182	2.7E-02
NGA 158	5	1.7	35	95	39	169	2.5E-02
ICE1	5	5.4	40	91	46	177	7.6E-02
MSAT 5.12611	5	12.61	25	75	68	168	0.6E-06
MSAT 5.8	**5**	**17.16**	**20**	**86**	**67**	**173**	**0.3E-06**
MSAT 5.20037	5	20.03	26	82	67	175	4.8E-06
MSAT 5.19	5	25.92	44	77	47	168	5.3E-02

Hz: heterozygote

Table S3: Phenotyping and genotyping data for the Sha x Mr-0 F2* family. (.xlsx, 76 KB)

Available for download as a .xlsx file at:

http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.183707/-/DC1/TableS3.xlsx

Table S4: QTLs for fertility in the Sha x Mr-0 F2* family

QTL	Chromosome	position (cM)	LOD score	% variance explained	Closest marker
Q1a[§]	1	3.2	46.2	19.7	T1G11
Q1b[§]	1	73.2	25.1	11.9	MSAT1.17898
Q3	3	57.0	9.23	4.8	MSAT3.15763
Q4	4	52.0	26.6	12.5	CIW7
Q5a	5	9	6.2	3.3	NGA151
Q5b	5	54	10.5	5.4	MSAT5.16477

§: interacting QTLs (LOD score: 13.4)

Table S5: Genotype segregation at L5 in the [Sha]Mr-0 x (Mr-0 x Sha) family

Locus	Chr	Marker	Position (Mb)	Number of Hz	Number of Mr-0	Number of plants	P (χ^2)	Observed Hz frequency	Expected Hz frequency
L5	5	MSAT5.16477	16.5	25	157	182	1.3E-22	14%	50%

Hz: heterozygote

Table S6: Genotype segregation at L5 in the progeny of a [Sha]L1^{M}L3^{M}L5^{H} plant

Locus	Chr	Marker	Position (Mb)	Number of Sha	Number of Hz	Number of Mr-0	Number of plants	P (χ^2)	Observed Sha frequency	Expected Sha frequency
L5	5	MSAT5.16477	16.5	6	92	79	177	7.3E-14	3%	25%

Hz: heterozygote

Table S7: Genotypes and phenotypes of the Rak-2 x Mr-0 BCp1 plants

Chromosome	1	1	1	3		
Locus	L1	L1 (L1Rak)*	L1 (L1Rak)*	L3	Plant fertility	Pollen viability
Marker	NGA128	IND1.22788	F5I14	MSAT 3.23289		
Position (Mb)	20.63	22.79	24.37	23.23		
Rak-2 x Mr-0 BCp1#8	Mr-0	Mr-0	Mr-0	Mr-0	0	
Rak-2 x Mr-0 BCp1#16	Mr-0	Mr-0	Mr-0	Mr-0	0	
Rak-2 x Mr-0 BCp1#23	Mr-0	Mr-0	Mr-0	Mr-0	0	0.1
Rak-2 x Mr-0 BCp1#12	Mr-0	Mr-0	Mr-0	Mr-0	0	0.8
Rak-2 x Mr-0 BCp1#27	Mr-0	Mr-0	Mr-0	Hz	0	0
Rak-2 x Mr-0 BCp1#6	Mr-0	Mr-0	Mr-0	Hz	0	0
Rak-2 x Mr-0 BCp1#11	Mr-0	Mr-0	Mr-0	Hz	0	0
Rak-2 x Mr-0 BCp1#14	Mr-0	Mr-0	Mr-0	Hz	0	
Rak-2 x Mr-0 BCp1#15	Mr-0	Mr-0	Mr-0	Hz	0	0
Rak-2 x Mr-0 BCp1#3	Mr-0	Hz	Hz	Hz	3	1.6
Rak-2 x Mr-0 BCp1#13	Mr-0	Hz	Hz	Hz	3.8	
Rak-2 x Mr-0 BCp1#1	Mr-0	Hz	Hz	Hz	4	1.5
Rak-2 x Mr-0 BCp1#5	Hz	Hz	Hz	Mr-0	4	
Rak-2 x Mr-0 BCp1#18	Hz	Hz	Hz	Mr-0	4	
Rak-2 x Mr-0 BCp1#22	Hz	Hz	Hz	Mr-0	4	
Rak-2 x Mr-0 BCp1#24	Hz	Hz	Hz	Mr-0	4	2.1
Rak-2 x Mr-0 BCp1#9	Hz	Hz	Hz	Mr-0	4	2.8
Rak-2 x Mr-0 BCp1#10	Hz	Hz	Hz	Mr-0	4	
Rak-2 x Mr-0 BCp1#21	Hz	Hz	Hz	Mr-0	4	
Rak-2 x Mr-0 BCp1#25	Hz	Hz	Hz	Mr-0	4	
Rak-2 x Mr-0 BCp1#2	Hz	Hz	Hz	Hz	4	
Rak-2 x Mr-0 BCp1#4	Hz	Hz	Hz	Hz	4	
Rak-2 x Mr-0 BCp1#7	Hz	Hz	Hz	Hz	4	1.4
Rak-2 x Mr-0 BCp1#17	Hz	Hz	Hz	Hz	3.8	
Rak-2 x Mr-0 BCp1#19	Hz	Hz	Hz	Hz	3.5	
Rak-2 x Mr-0 BCp1#20	Hz	na	Hz	Hz	3.5	1.6
Rak-2 x Mr-0 BCp1#26	Hz	Hz	Hz	Hz	4	

Hz: heterozygote

* the L1Rak locus is included in the L1 interval

Supplementary information on plant material

Production of the [Sha]Mr-0 and [Mr-0]Sha cytolines: The [Sha]Mr-0 and [Mr-0]Sha cytolines were obtained after recurrent paternal backcrosses of Sha x Mr-0 and Mr-0 x Sha F1s. For each cross 15 BCp3 plants were genotyped with 384 SNP markers distributed throughout the genome (SIMON *et al.* 2012). The list and positions of the informative SNPs (polymorphic between Sha and Mr-0) are available at https://www.versailles.inra.fr/ijpb/crb/anatool/. One Mr-0 x Sha BCp3 plant homozygous for Sha alleles at all markers was selected to found the [Mr-0]Sha cytoline. For the Sha x Mr-0 cross, no BCp3 plant was homozygous for Mr-0 alleles at all markers, so a fourth paternal backcross (BCp4) was performed, whose progeny was genotyped with microsatellite markers at the heterozygous regions remaining in the BCp3 plant. One Sha x Mr-0 BCp4 plant homozygous for Mr-0 alleles at all markers was selected to found the [Sha]Mr-0 cytoline.

Production of near isogenic lines: We first selected, in the Sha x Mr-0 BCm3 progeny (Figure S1), a plant with a Sha nuclear background but heterozygous at L1 in Sha cytoplasm ([Sha]$L1^{H}L3^{S}$) and, in the Mr-0 x Sha BCp3 progeny, a plant with a Sha nuclear background, but heterozygous at L3 in Mr-0 cytoplasm ([Mr-0]$L1^{S}L3^{H}$). These plants were identified by dense genotyping with 384 genome-wide SNP markers (SIMON *et al.* 2012). In their selfed progenies, we selected the genotype [Sha]$L1^{M}L3^{S}$, in which L1 (from 18.7 to 24.4 Mb) was homozygous Mr-0, and the genotype [Mr-0]$L1^{S}L3^{M}$, in which L3 (from 22.6 Mb to the South telomere) was homozygous Mr-0. These two plants were then crossed both ways to obtain [Sha]$L1^{H}L3^{H}$ and [Mr-0]$L1^{H}L3^{H}$ whose progenies segregated both L1 and L3 in a Sha nuclear background, but in Sha and Mr-0 cytoplasms, respectively (Figure S2).