Modifiers of genetic dominance at the Arabidopsis self-incompatibility locus retain proto-miRNA features and act through non-canonical gene silencing pathways

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Main Text

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Abstract

Self-incompatibility in flowering plants is a common mechanism that prevents self-fertilization and promotes outcrossing. In Brassicaceae, there is high genetic diversity at the locus controlling self-incompatibility, and dozens of distinct alleles are organized in a complex dominance hierarchy: the gene controlling self-incompatibility specificity in pollen shows monoallelic expression in heterozygote individuals, and this is achieved through the action of sRNA precursors that resemble miRNAs, although the underlying molecular mechanisms remain elusive. Here, we engineered *Arabidopsis thaliana* lines expressing components of the *Arabidopsis halleri* self-incompatibility system, and we then used a reverse genetics approach to pinpoint the pathways underlying the function of these sRNA precursors. We showed that they trigger a robust decrease in transcript abundance of the recessive pollen self-incompatibility genes, but not through the canonical transcriptional or post-transcriptional gene silencing pathways. Furthermore, we observed that single sRNA precursors are typically processed into hundreds of sRNA molecules of distinct sizes, abundance levels and ARGONAUTE loading preferences. This heterogeneity closely resembles that of proto-miRNAs, the evolutionary ancestors of miRNAs. Our results suggest that these apparently arbitrary features, which are often associated with lack of effects on gene expression, are crucial in the context of the self-incompatibility dominance hierarchy since they allow for one sRNA precursor of a given allele to repress multiple other recessive alleles. This study not only provides an in-depth characterization of the molecular features underlying complex dominance interactions, but also constitutes a unique example of how specific evolutionary constraints shape the progression of sRNA precursors along the proto-miRNA – miRNA evolutionary continuum.

Significance statement

miRNAs are genetically encoded small non-coding RNA molecules that control gene expression. Here, we characterized a set of regulatory loci present in the self-incompatibility locus of *Arabidopsis halleri* that show features of proto-miRNAs, the evolutionary ancestors of miRNA genes. We report that despite the absence of the classical miRNA features thought to be essential for regulation of gene expression, the proto-miRNAs generated by these loci robustly repress the activity of self-incompatibility genes through non-canonical silencing pathways, and thus function to establish a complex dominance hierarchy between self-incompatibility alleles. We show that the evolutionary forces promoting diversity of self-incompatibility alleles have likely favored the maintenance of proto-miRNA features in order to mediate dominance interactions between numerous alleles, thus preventing the transition of self-incompatibility proto-miRNAs into canonical miRNAs genes.

Main text

Introduction

Genetic dominance is the widespread property by which one allele can mask the phenotypic effect of another in a heterozygous genotype **(1)**. Dominance introduces non-linearity in the genotype-to-phenotype map and influences the exposure of mutations to natural selection, thus altering the probability and dynamics of their fixation, elimination, or maintenance in natural populations **(2)**. The evolution of dominance has been subject to a fierce debate in the field of genetics throughout the 20th century **(3)**, specifically around the existence of genetic elements dedicated to altering the dominance of other alleles in *trans* **(4)**, and the possibility for natural selection to act effectively on these elements **(5)**. The existence of these dominance modifiers has remained elusive for many years; nevertheless, theoretical studies have predicted that dominance modifiers could exist in cases where heterozygosity is high, such as in Batesian and Müllerian mimicry in butterflies, or at the self-incompatibility locus of Brassicaceae plants **(3)**. More recently, dominance modifiers similar to those envisioned by Fisher **(4)** were empirically identified at the self-incompatibility locus of Brassica and Arabidopsis **(6–8)**. However, their mode of action and evolutionary properties remain elusive.

Self-incompatibility is a widespread self-recognition mechanism in hermaphroditic flowering plants that prevents selfing and enforces outcrossing. In Brassicaceae, self-pollen recognition is controlled at the sporophytic level by a single non-recombining self-incompatibility locus (*S*-locus) containing two protein-coding genes: one encoding a trans-membrane receptor (SRK) expressed in papillae cells as the female component, and the male component consisting of an SCR peptide produced in the tapetum of the anther and deposited on the surfaces of the pollen grains. The SRK and SCR proteins function as a receptor-ligand system, such that pollen germination is stopped when a pair of cognate SRK and SCR proteins interact upon pollen deposition on the pistil, ultimately preventing fertilization **(9–13)**. The *S*-locus is subject to an intense long-term balancing selection, which favors the maintenance of a high number of distinct *S*-alleles **(14–17)**. Although heterozygosity is typically very high at the *S*-locus, monoallelic expression of the male *SCR* gene is the rule, following a transitive and mostly linear dominance hierarchy among *S*-alleles **(18–20)**. These dominance interactions maximize reproductive fitness, since repressing one of the two *S*-alleles carried by a diploid heterozygous male individual avoids rejection by pistils expressing the same allelic specificity, and thus maximizes the number of available female partners **(21)**. In *Arabidopsis lyrata* and *A. halleri*, respectively, 56 and 43 *S*-alleles have been documented so far **(15, 22)**, and are divided in four main dominance classes, with class IV being the most dominant and class I the most recessive **(23, 24)**.

Underlying these dominance interactions are *S*-locus dominance modifiers, which take the form of sRNA generating loci. Similarly to miRNAs genes, these loci are transcribed into an RNA molecule that folds into a hairpin structure, which is subsequently cleaved into sRNAs. In *A. halleri,* eight distinct families of sRNA precursors exist, and they are predicted to control dominance interactions between and within the four *S*-allele dominance classes **(6)**. Interestingly, in Brassica where only two dominance classes exist, only two sRNA precursors genes have been identified **(7, 8, 20)**, suggesting that there is an association between the complexity of the dominance network and the total number

of dominance modifiers. The two sRNA precursors of Brassica (*Smi* and *Smi2*) have each been suggested to form a hairpin and produce a 24-nt sRNA with sequence similarity to the 5' region of *SCR* (referred to *SP11* in the Brassica nomenclature). The presence of this sRNA is associated with decreased expression of the recessive *SCR* allele as well as deposition of DNA methylation at the targeted region in the tapetum cells **(7, 8, 20)**. This is reminiscent of what is observed in several plant species where 21/24-nt sRNAs derived from miRNAs genes or inverted repeats can be loaded onto proteins of the ARGONAUTE4 (AGO4) clade and subsequently elicit *de novo* DNA methylation at target loci, such as transposable elements or protein-coding genes, through the RNA-directed DNA Methylation (RdDM) pathway **(25–29)**. Similar mechanisms could be utilized by *Smi* and *Smi2*; nevertheless, empirical tests to validate this hypothesis are missing.

In Brassica, the dominance hierarchy of *S*-alleles is proposed to rely on the combinatorial effect of single nucleotide variants at the *Smi* and *Smi2* sequences and at their respective *SCR* target sites **(8)**. In Arabidopsis, where more dominance classes exist, the dominance network is hypothesized to rely on two properties: i) recessive alleles carry more target sites and these sites are more generalist (*i.e.* they are targeted by more sRNA precursors than dominant alleles); and ii) sRNA precursors of dominant alleles are more generalist (*i.e.* they target a higher number of alleles than sRNA precursors in less dominant alleles) **(6)**. Despite this observation, the molecular features allowing sRNA precursors to target multiple alleles remain to be identified. In addition, among the eight families of sRNA precursors previously predicted to control the hierarchy in Arabidopsis, only one has been subject to formal experimental validation.

In this study, we aimed at identifying the molecular pathway underlying the sRNA-mediated dominance interactions between *S*-alleles in Arabidopsis. To achieve this, we engineered and characterized a series of *A. thaliana* lines recapitulating the self-incompatibility phenotype, and used these lines to show that two different sRNA precursors from dominant *A. halleri S*-loci trigger a decrease in abundance of recessive *SCR* transcripts. We thus provide a proof of concept that this heterologous approach can be used to validate sRNA precursor function, both at the phenotypic and at the molecular level. This system also allowed us to investigate if key components of the canonical RdDM pathway, such as the ARGONAUTE proteins AGO4 and AGO6, and subunits of the DNA-dependent RNA polymerases POLIV and POLV, are required for the repression of *SCR* transcript by *S*-locus sRNA precursors, and our analyses suggest that the two selected sRNA precursors do not act through the canonical RdDM pathway.

Additionally, and in order to better understand the role of sRNA precursors genes in the extensive and complex *S*-locus dominance network, we characterized their molecular features, revealing that despite their structural similarity to miRNAs genes, they rather resemble their evolutionary precursors: proto-miRNAs **(30, 31)**. Proto-miRNAs loci are inverted repeats present in several plant genomes that have acquired transcriptional competence, forming long hairpins which can be diced by multiple Dicer-like proteins (DCLs), leading to a complex population of sRNAs. There is little evidence that such a sRNA population could cause substantial effects in gene expression, at least through the canonical PTGS pathway **(32–36)**; therefore, these structures have been mostly regarded as substrates for selection to act on, rather than having immediate biological significance. Notwithstanding, we show that

112 *S*-locus proto-miRNA genes are able to influence the transcript abundance of *SCR* alleles, affect the
113 pollen germination rate and, consequently, mediate *S*-allele dominance interactions. *S*-locus proto-
114 miRNAs produce a large population of sRNAs with different sizes and sequences, which are important
115 to maximize the number of targeted alleles, thus allowing one allele to be dominant over many others.
116 Overall, our results provide a unique window into the function and evolution of proto-miRNAs,
117 constituting the first example of a biological system that depends on their action.

118 **Results**

119 *The sRNA precursors genes Ah04mir1887 and Ah20mirS3 control dominance interactions at the S-*
120 *locus by decreasing the transcript level of the recessive SCR01 allele*

121 To study in detail the molecular mechanisms controlling the activity of dominance modifiers in the
122 self-incompatibility system of *A. halleri*, we first recreated the self-incompatibility reaction of *A. halleri* in
123 the selfing species *A. thaliana*, since the latter is a more tractable study model. To do this, we expressed
124 the *SRK01* receptor and its cognate *SCR01* ligand in female and male parents, respectively **(6, 37, 38)**.
125 Manual crosses between these plants revealed that the germination of pollen from a fully fertile *SCR01*-
126 expressing male was abolished when deposited on pistils of an *SRK01*-expressing female, the hallmark
127 of a successful self-incompatible reaction **(Fig. 1C and D, wt panels)**. Given the challenges faced in
128 prior studies to reconstruct this reaction in the Col-0 ecotype, all *A. thaliana* plants used in this study
129 are of the C24 ecotype, where the self-incompatibility can be faithfully recreated as shown both in this,
130 and other studies **(37, 39, 40)**.

131 Among the eight known families of *A. halleri* sRNA precursors loci, only one - mirS3 - has been
132 experimentally validated to regulate dominance between two distinct *S*-alleles: specifically, the
133 dominance of allele Ah20 over Ah01, mediated by Ah20mirS3 sRNAs, which target the *SCR01* intronic
134 region **(Fig. 1B) (6)**. In this study, we set out to examine if the yet uncharacterized mir1887 family could
135 have a similar function. For this, we focused on testing the role of the sRNA precursor Ah04mir1887 in
136 controlling the dominance of allele Ah04 over Ah01, whose sRNAs are predicted to target the 5' region
137 of *SCR01* **(Fig. 1A)**. We thus generated transgenic lines expressing Ah04mir1887 and introgressed this
138 transgene into an *SCR01*-expressing male, and used these plants to pollinate *SRK01*-expressing
139 females, testing whether the presence of the Ah04mir1887 sRNA precursor locus could transform the
140 previously incompatible interaction between *SCR01* and *SRK01* into a compatible one **(Fig. 1C)**. In the
141 particular case of Ah20mirS3, the transgenic line previously generated by Durand et al. **(6)** and used
142 here expresses both Ah20mirS3 and Ah20mir1887 because of their close physical proximity within the
143 Ah20 allele **(Fig. 1B)**. To specifically isolate the impact of Ah20mirS3 in the dominance interaction of
144 Ah20 over Ah01, we developed the *SCR01*^{*mir1887} transgenic line, which contains five point mutations
145 designed to fully disrupt the slight homology observed between some Ah20mir1887 sRNAs and *SCR01*
146 **(Fig. 1B; Fig. 1 S1)**. We then used this modified *SCR01* line for all subsequent tests on the functional
147 role of Ah20mirS3.

148 We observed that combining either Ah04mir1887 (Interaction A), or Ah20mirS3 (Interaction B) with
149 *SCR01* results in a switch from an incompatible to a compatible pollen germination phenotype, which
150 is accompanied by a dramatic decrease in the abundance of the *SCR01* transcript, as measured by

151 RT-qPCR **(Fig. 1C and D, wt panels)**. This shows that both Ah04mir1887 and Ah20mirS3 act as
152 dominance modifiers in their respective allelic interactions, and that they accomplish this by decreasing
153 *SCR01* transcript abundance, thus preventing SRK-SCR cognate recognition and allowing pollen
154 germination to occur. Moreover, these results demonstrate that in Arabidopsis, *SCR* transcript
155 abundance can be modulated by multiple sRNA precursor families that use distinct target sites in the
156 *SCR* locus.

157 *Decrease in SCR01 transcript abundance does not depend on RdDM*

158 Next, we aimed at elucidating the exact molecular mechanism responsible for the reduction of
159 *SCR01* transcript abundance triggered by the two *S*-locus sRNA precursors. Given their resemblance
160 to miRNAs genes, one potential scenario is that sRNAs produced by Ah04mir1887 and Ah20mirS3
161 could trigger post-transcriptional gene silencing (PTGS) of the *SCR01* transcript. However, since these
162 sRNAs are predicted to target the 5' region and intronic region of *SCR01*, and since the substrate of
163 PTGS is generally thought to be the mature mRNA **(41)**, this does not support PTGS as the pathway
164 responsible for the decrease in *SCR01* transcript abundance. An alternative scenario is that
165 Ah04mir1887 and Ah20mirS3 sRNAs could trigger the RNA-directed DNA methylation pathway
166 (RdDM), resulting in the deposition of DNA methylation in their respective *SCR01* target sites, and
167 reduced *SCR01* transcription. Interestingly, previous studies have established that the silencing of
168 recessive alleles in the homologous self-incompatibility system of *Brassica* relies on sRNA-mediated
169 DNA methylation, although the specific pathway underlying this process remains elusive **(7, 8, 20)**.
170 Thus, we focused our attention on the RdDM pathway, and set out to test if mutating key components
171 of this pathway would prevent Ah04mir1887 and Ah20mirS3 from reducing *SCR01* transcript
172 abundance. To circumvent the absence of RdDM mutant alleles in the C24 ecotype, we generated and
173 validated C24 *ago4 ago6* CRISPR/Cas9 mutant alleles **(Fig. 1 S2)**, and used the previously published
174 C24 *polIV* and *polV* knock-out alleles **(42)**.
175 To our surprise, none of the RdDM mutants impaired the activity of the tested sRNA precursors. In
176 the case of Ah04mir1887, *SCR01* transcript abundance is reduced in all mutant backgrounds to a
177 similar level as in wild-type, which is reflected by a compatible pollen germination phenotype **(Fig 1C)**.
178 Similarly, the presence of Ah20mirS3 is associated with a decrease in *SCR01* transcript in all mutant
179 backgrounds, showing that this sRNA precursor is still functional **(Fig. 1D)**. Remarkably, this is not
180 reflected in a compatible pollen germination phenotype since the basal *SCR01* transcript level is
181 increased in both *polIV* and *polV* mutant backgrounds when compared with wild-type **(Fig. 1D, *polIV***
182 **and *polV* panels vs. wt panel)**, pointing to an effect of these mutants on the expression of the *SCR01*
183 transgene. This suggests that the *SCR01*^{*mir1887} construct might be intrinsically targeted for repression
184 by RdDM, a phenomenon often observed in transgenic lines **(43)** (note that the *SCR01* and
185 *SCR01*^{*mir1887} transgenes used in Interaction A and B, respectively, correspond to two independent
186 transgenic lines with different insertion sites; **see Methods section**). As such, and even though the
187 addition of Ah20mirS3 in these mutant backgrounds strongly reduces *SCR01* transcript abundance, it
188 does not do so sufficiently to compromise the self-incompatibility reaction. Indeed, our data shows that

189 a strong reduction of *SCR01* transcript abundance is required for a compatible pollen germination
190 phenotype, since even very low levels of transcript can trigger an incompatibility reaction **(Fig. 1 S3)**.
191 To further assess if the decrease in *SCR01* transcript abundance triggered by sRNA precursors is
192 associated with deposition of DNA methylation at *SCR01* target sites, we used the BiSulfite Amplicon
193 Sequencing technique - BSAS **(28, 44)**, which relies on PCR enrichment of regions of interest using
194 bisulfite converted DNA as a template. *SCR01* expression is restricted to the tapetum cells of immature
195 floral buds, which are challenging to manually isolate in *A. thaliana* due to their small size and
196 embedment within other floral tissues. To overcome this limitation, we performed BSAS on 8-10
197 biological replicates of *SCR01* transgenic lines both in the presence and absence of the sRNA precursor
198 transgenes **(Fig. 1 S4),** and generated an average coverage of 1400x for each cytosine located in the
199 *SCR01* target sites and in the 800 bp around them **(Table S3)**. This allows us to detect minute DNA
200 methylation differences between samples, thus compensating for the difficulty in enriching for tapetum
201 tissue. We observed strong DNA methylation levels at the *SCR01* target sites **(Fig. 1 S4)**; however, no
202 significant changes were detected in the presence of Ah04mir1887 or Ah20mirS3, suggesting that the
203 decrease in *SCR01* transcript abundance is not mediated by changes in DNA methylation at the target
204 sites **(Fig. 1 S4)**, which is in line with the absence of effects in the tested RdDM mutants. The observed
205 high methylation levels at *SCR01* target sites, coupled with the specific *SCR01* expression in tapetum
206 cells, led us to hypothesize that these methylation levels might represent the methylation status of
207 surrounding floral tissues rather than specifically reflecting tapetum methylation. To investigate this
208 further, we categorized BSAS reads based on the fraction of methylated cytosines within a single read.
209 Our analysis revealed that a significant proportion of reads exhibited complete methylation, with a
210 notable fraction showing little to no methylation **(Fig. 1 S5)**. We postulate that this subset of
211 unmethylated reads may originate from tapetum cell DNA, where *SCR01* is actively expressed.
212 Notwithstanding, no significant changes in unmethylated read abundance were detected in the
213 presence of Ah04mir1887 or Ah20mirS3, again suggesting these sRNA precursors have no impact on
214 DNA methylation.
215 Together, these results indicate that neither PTGS nor RdDM, at least in their canonical forms,
216 appear to be responsible for the reduction in *SCR01* transcript abundance triggered by *S*-locus sRNA
217 precursors. Despite this, both Ah04mir1887 and Ah20mirS3 strongly and robustly decrease *SCR01*
218 transcript levels, which we show is necessary to abolish the self-incompatibility reaction.

219 *Ah04mir1887 and Ah20mirS3 loci strongly resemble proto-miRNAs genes*

220 *S*-locus sRNA precursors, including those of the mir1887 and mirS3 families studied here, consist
221 of inverted repeats that are transcribed and predicted to form a RNA hairpin structures containing a 5'
222 arm, a terminal loop and a complementary 3' arm **(Fig. 2A) (6)**, akin to the structures formed by
223 precursor miRNA (pri-miRNA) molecules. To delve deeper into the molecular properties of
224 Ah04mir1887 and Ah20mirS3, and to explore potential non-canonical pathways through which these
225 precursors may act, we conducted sRNA sequencing on *A. thaliana* plants harboring the Ah04mir1887
226 and Ah20mirS3 transgenes. We observed that dicing of *S*-locus sRNA precursor hairpins leads to
227 numerous sRNAs – at least 106 in the case of Ah04mir867, and 224 in the case of Ah20mirS3 **(Fig.**

228 **2B-D)**. These sRNAs range from 18 to 25 nt in length, with no clear predominance of a specific length
229 **(Fig. 2 S1 A-B)**, and their abundances vary by several orders of magnitude **(Fig. 2B, Fig. 2 S1 C-D)**.
230 Given the large population of sRNAs produced by a single precursor, we aimed at evaluating the
231 functional relevance of each sRNA in controlling the abundance of the *SCR01* transcript. For this, we
232 used a previously published algorithm **(6)**, which attributes a targeting score based on the homology
233 between a single sRNA and the *SCR* genomic region. A previous study has demonstrated that in
234 multiple *A. halleri S*-locus heterozygotes, a targeting score equal to or above 18 is associated with
235 phenotypic dominance and a sharp decrease of the recessive *SCR* transcript level **(18)**. Using this
236 algorithm, we found that 12 out of the total 106 sRNAs produced by Ah04mir1887, and 33 out of the
237 total 224 sRNAs produced by Ah20mirS3, have a base-pairing score of ≥ 18 and are thus predicted to
238 target *SCR01*. This subset of putatively functional sRNAs displays a variety of sizes and expression
239 levels **(Fig. 2B, see grey box)**.
240 We additionally performed ARGONAUTE immunoprecipitation (IP) assays, followed by sRNA
241 sequencing in the Ah04mir1887 and Ah20mirS3 *A. thaliana* transgenic lines, which showed that
242 functional sRNAs produced from either precursor can be loaded into AGO1 (characteristic of the PTGS
243 pathway) and AGO4 (characteristic of the RdDM pathway) **(Fig. 2 C-D)**. Notwithstanding this, a large
244 part of these functional sRNAs (75% in Ah04mir1887 and 48% in Ah20mirS3) are not found to be loaded
245 in either of these two AGO proteins, again consistent with our observations that both the canonical
246 PTGS and RdDM pathways are unlikely to underlie the activity of these sRNA precursors. We also
247 noted that the majority of sRNAs loaded in AGO1 have a 5' terminal uracil, reflecting the known AGO1
248 preference for binding these sRNAs **(45)**. However, sRNAs loaded into AGO4, or with an unknown
249 loading pattern, do not show a significant bias towards a specific 5' nucleotide **(Fig. 2 S1 E-F)**. Overall,
250 these results show that the predicted functionality of a given sRNA does not seem to be tied to a specific
251 set of molecular features.
252 Despite the structural similarity between *S*-locus sRNA precursors and miRNAs, and the existence
253 of shared features, such as the presence of mature 21-nt sRNAs and loading of sRNAs into AGO1, it
254 is evident that *S*-locus sRNA precursors deviate from the typical characteristics associated with
255 canonical miRNAs, and cannot be characterized as such. Instead, our data show that Ah04mir1887
256 and Ah20mirS3 highly resemble proto-miRNAs, the evolutionary predecessors of miRNAs: they have
257 an unusually staggered hairpin processing pattern with numerous sRNAs derived from both the 5' and
258 3' arms, and differ in size, abundance and association with AGO proteins. Proto-miRNAs are thought
259 to have no target genes and/or to cause no significant effect in transcript abundance **(30, 31, 33–36)**.
260 This is not the case for Ah04mir1887 and Ah20mirsS3 since their effect on *SCR01* transcript levels is
261 evident and necessary to modulate the self-incompatibility phenotype **(Fig. 1C and D, wt panels)**.

262 *proto-miRNA features allow S-locus sRNA precursors to target multiple S alleles*

263 We hypothesized that this heterogeneity in hairpin processing pattern could play an important role
264 in the context of the complex *S*-locus dominance hierarchy by allowing a single sRNA precursor to exert
265 dominance over multiple other alleles. To test this, we expanded our analysis of the function of

266 Ah04mir1887 and Ah20mirS3 beyond the interaction with allele Ah01, and looked at their role within
267 the *A. halleri* dominance network **(Fig. 3)**.
268 We compiled previously published phenotypic data obtained by controlled reciprocal crosses
269 between different *A. halleri S*-locus heterozygotes to determine the position of allele Ah20 and Ah04 in
270 the dominance hierarchy of a total of 15 *S*-alleles **(Fig. 3) (6, 18, 46)**. From this, we could infer that
271 Ah20 is dominant over 13 other *S*-alleles, with 10 of these interactions relying on the action of
272 Ah20mirS3 **(Fig. 3)**, as determined by the sRNA-*SCR* targeting algorithm described above. Thus,
273 Ah20mirS3 is not only able to mediate the repression of *SCR01*, but also the repression of at least nine
274 other distinct *SCR* alleles. Similarly, Ah04mir1887 mediates the repression of at least five different *SCR*
275 alleles that are recessive to Ah04 **(Fig. 3)**.
276 To test if the large population of sRNAs produced by these *S*-locus precursors is important in
277 repressing multiple target alleles, we examined the role of each individual sRNA produced by both
278 Ah04mir1887 and Ah20mirS3 **(Fig. 4)**: 11% of Ah04mir1887 sRNAs **(Fig. 4A, cluster 1)**, and 26% of
279 Ah20mirS3 sRNAs **(Fig. 4B, cluster 1)** are predicted to target at least one *SCR* allele within the inferred
280 dominance network presented in **Figure 3**. As is the case in Interaction A and B, there does not seem
281 to be a clear correlation between abundance and sRNA functionality in any of the analyzed interactions
282 **(Fig. 2B and Fig. 4A-B, see cluster 1 vs. cluster 2)**. Nevertheless, sRNAs that are predicted to be
283 functional have a larger size than those that are not (median of 23 nt vs. 21 nt), and in the case of
284 Ah20mirS3, this is also associated with a larger number of sRNAs being loaded in AGO proteins **(Fig.
285 4A-B; see cluster 1 vs. cluster 2).** Additionally, we can observe that most *S*-alleles are redundantly
286 targeted by multiple sRNAs **(Fig. 4)**.
287 When investigating in more detail the positional correspondence between sRNAs and the
288 Ah04mir1887/Ah20mirS3 precursor hairpin structure we noticed that sRNAs are produced in similar
289 amounts from both the 5' and the 3' arms of the hairpin **(Fig. 5)**. Each hairpin arm produces numerous
290 sRNAs that show a staggered pattern, suggesting imprecise dicing and resulting in a population of
291 sRNAs that show small base-pair differences between them. In the case of Ah04mir1887, only the
292 sRNAs produced from the 5' hairpin arm are predicted to be functional against the *S*-alleles tested here
293 **(Fig. 5A)**. Remarkably, in the case of Ah20mirS3, each hairpin arm shows functional specialization
294 since sRNAs from the 5' arm target all allele classes, except Class IV; while sRNAs from the 3' arm
295 exclusively target Class IV and Class III alleles. Together, these observations suggest that the high
296 number and heterogeneous processing of sRNAs is a conserved and important feature of *S*-locus sRNA
297 precursors which allows targeting of multiple alleles and thus underlies their generalism.

298 Discussion

299 *S-locus sRNA precursors act through non-canonical gene silencing pathways*

300 Our characterization of self-incompatibility transgenic lines of *A. thaliana* contributes additional
301 evidence to the work of Durand et al. **(6)**: collectively, these studies establish *A. thaliana* as a tractable
302 system to study dominance interactions among *A. halleri S*-alleles and validate the role of two distinct
303 sRNA precursor families in these interactions, yet the precise mechanisms underlying their action
304 remain elusive. Our study reveals that some of the sRNAs generated in the processing of the *S*-locus

305 sRNA precursors have a length of 24 nt and are loaded into AGO4, potentially indicating a role for
306 RdDM. Nevertheless, *ago4 ago6*, *polIV* and *polV* mutations did not significantly affect the action of *A.*
307 *halleri S*-locus sRNA precursors, suggesting that canonical RdDM is not the primary mechanism
308 through which Ah20mirS3 and Ah04mir1887 mediate the reduction in *SCR01* transcript levels.
309 Considering their hairpin structure and their ability to also generate AGO1-loaded 21-nt sRNAs, it could
310 also be envisioned that these precursors might act through PTGS. Still, the location of target sites in
311 the 5' region and intronic regions of *SCR01* poses a conundrum, since PTGS is reported to occur in the
312 cytoplasm, using mature mRNA as a cleavage substrate **(41)**. Notably, in animals, several lines of
313 evidence point to miRNAs acting both in the cytoplasm and in the nucleus, with effects at the
314 transcriptional and post-transcriptional levels **(47–49)**. In plants, it has been observed that an inverted
315 repeat targeting the intron of the soybean gene *FAD12-1A* triggers cleavage of its precursor mRNA
316 (pre-mRNA) in the nucleus, which is accompanied by accumulation of siRNAs of the cleaved pre-mRNA
317 **(50)**. Additionally, all main miRNA processing components, including AGO1, are present in the nucleus
318 **(51–53)**. Thus, given the independent slicer activity of AGO1 **(54)**, it could be hypothesized that this
319 ARGONAUTE uses sRNAs to target the *SCR01* pre-mRNA in the nucleus, promoting its cleavage co-
320 transcriptionally. Alternatively, the interaction between AGO1 and the *SCR* pre-mRNA could promote
321 disassembly of the PolI II complex leading to the arrest of transcription, as has been suggested to occur
322 in some miRNA genes upon salt stress **(55)**. Even though we could not detect any siRNAs
323 corresponding to the cleavage of the *SCR01* pre-mRNA in any of our sRNA sequencing experiments
324 **(data not shown)**, future experiments should investigate the possibility that the sRNA precursors at the
325 *S*-locus could act through nuclear PTGS or through transcriptional arrest.

326 Our results that Ah20mirS3 and Ah04mir1887 work through an RdDM-independent pathway contrast
327 with previous observations in Brassica: in this latter system, the sRNA precursors *Smi* and *Smi2* are
328 associated with deposition of DNA methylation at *SCR* target sites and a decrease in *SCR* expression
329 **(7, 8, 20)**. DNA methylation may result directly from the incorporation of *Smi* and *Smi2* sRNAs into
330 AGO4-clade proteins, activating RdDM. Alternatively, as suggested by Finnegan et al. **(56)**, it could
331 depend on *Smi*-induced cleavage of an antisense transcript at the *SCR* locus, generating sRNAs that
332 could subsequently be co-opted into the RdDM pathway. Even though empirical investigations are still
333 necessary to test these hypotheses, the cumulative evidence from these and our study suggests that
334 different sRNA precursors within the Brassicaceae self-incompatibility system can elicit distinct effector
335 pathways, that nevertheless converge into the same molecular phenotype: reduction of *SCR* transcript
336 abundance. This would imply that these gene regulatory elements have an outstanding molecular
337 flexibility, despite functioning within the specific evolutionary constraints of the self-incompatibility
338 system. Thus, it cannot be excluded that different *A. halleri* sRNA precursors, as well as precursors
339 from other self-incompatibility species such as *A. lyrata* and *Capsella grandiflora* could co-opt different
340 gene regulatory pathways. Future investigations in these different species, using heterologous
341 expression systems as done in this study, or in the native context whenever genetic transformation is
342 feasible, will hopefully answer this outstanding question.

343 *The complex S-allele dominance network relies on the unique features of S-locus sRNA precursors*

344 Durand et al. **(6)** previously showed that the *S*-locus sRNA precursors of *A. halleri* have high
345 generalism, and suggested that dominant *S*-alleles in particular, rely on this ability to target multiple
346 recessive alleles, rather than on carrying an increased number of sRNA precursors. Our study uncovers
347 the molecular basis for this generalism, and shows that the apparent haphazardous processing of *S*-
348 locus sRNA precursors is required to generate numerous functional sRNAs that target multiple alleles
349 cooperatively: remarkably, all *SCR* alleles analyzed in this study are targeted by multiple sRNAs of a
350 single sRNA precursor, and/or are targeted by distinct sRNA precursors. This contrasts with the less
351 complex dominance hierarchy of Brassica, where fewer allelic interactions are present, and a single
352 functional sRNA molecule *per* precursor has been suggested to mediate all dominance relationships
353 **(7, 8)**. It is important to note that while we investigated a total of 15 *S*-alleles in this study, at least 43
354 are known to exist in *A. halleri* **(15)**, and > 80 in the closely related *Capsella grandiflora* **(22)**. Therefore,
355 in this study we most likely underestimated the fraction of functional sRNAs in each precursor, since
356 those that are non-functional in the allelic interactions explored here could potentially have a role in
357 interactions that have not yet been characterized.

358 Overall, the evidence suggests that collaborative targeting, either by multiple sRNAs of a single
359 precursor, or by sRNAs from distinct sRNA precursors, is an essential feature of this system: firstly, it
360 is essential for granting an *S*-allele its complete targeting spectrum across the many *S*-alleles
361 segregating in the population; and secondly, the use of multiple sRNAs with distinct molecular features
362 could trigger diverse effector pathways acting on the same target allele, which could perhaps underlie
363 the robust reduction of recessive *SCR* transcripts observed in all allelic interactions studied thus far
364 (this study and **(18)**).

365 Intriguingly, this mirrors the collaborative non-self recognition model observed in Solanaceae, where
366 each self-incompatibility allele contains multiple *SLF* genes. SLF proteins play a crucial role in
367 recognizing and removing the toxic non-self female S-RNase, which otherwise inhibits pollen
368 germination. In this system, the presence of multiple *SLF* genes within a single S-locus allele, each
369 capable of recognizing a distinct S-RNase allele, coupled with the ability of some *SLF* genes to
370 recognize and detoxify multiple S-RNases (generalism), is crucial. This collaborative functionality
371 among *SLF* genes ensures a comprehensive recognition spectrum for a given self-incompatibility allele,
372 thereby preserving the intricate inter-allelic interaction network within Solanaceae **(57)**. While in
373 Solanaceae, the generalism of SLF proteins likely depends on their ability to establish protein-protein
374 interactions with distinct S-RNases **(58)**, in *A. halleri*, sRNA precursor generalism relies on their unique
375 molecular features, specifically their ability to produce numerous sRNAs with distinct targeting spectra.

376 *S-locus sRNA precursors provide a window into the miRNA evolution continuum*

377 Although sharing some structural similarities with miRNAs, the features of *S*-locus sRNA precursors
378 more closely resemble those of proto-miRNAs. We hypothesize that the specific selective constraints
379 of the *S*-locus dominance network have likely contributed to the persistence of proto-miRNA features
380 over extended evolutionary times, and prevented their progression towards canonical miRNA features,
381 as this would likely render them unable to effectively target multiple *S*-alleles. It would be interesting to

test if apparently stabilized proto-miRNAs are also used in other biological systems where the expression of multiple genes/alleles needs to be coordinated by a single regulator and under similar evolutionary constraints as those acting on the *S*-locus. The mimicry of wing patterns in *Heliconius* butterflies would be an interesting model system to test this, since balancing selection has favored the appearance of numerous alleles of the supergene controlling wing pattern, and dominance relationships between them are prevalent **(59, 60)**.

To our knowledge, the *A. halleri S*-locus sRNA precursors represent the first instance where proto-miRNA-like features are biologically relevant, and actively selected for. And despite not conforming to canonical gene regulatory pathways, our data shows that *S*-locus proto-miRNAs exert a considerable impact on gene expression. Prior investigations of proto-miRNA function have primarily evaluated the functionality of these structures in the light of canonical miRNA pathways, often concluding that their functional impact is negligible **(32–36)**. Based on our findings, we propose that further exploration is necessary to determine whether proto-miRNAs can employ non-canonical pathways to regulate gene expression in diverse biological contexts beyond the *S*-locus. Investigating the function and mode of action of proto-miRNAs in different organisms will contribute not only to better understand their potential roles, but also to uncover the key steps in the emergence of proto-miRNAs and their transition into canonical miRNAs.

Materials and Methods

Plant material & growing conditions

Wild-type (wt) and transgenic *Arabidopsis thaliana* plants used in this study were grown in a greenhouse, using standard growth conditions (16 h light/8 h dark; 110 µmol/s/m2; 21°C; 70% humidity). Seeds were germinated directly on soil, or in MS-medium (0.43% MS-salts, 0.8% Bacto agar, 0.19% MES hydrate, and 1% sucrose) when antibiotic selection was necessary for transgene selection.

Because the self-incompatibility reaction cannot be faithfully reconstructed in the *A. thaliana* Col-0 ecotype **(37–40)** all wild-type and mutant plants used in this study were of the C24 ecotype. *polIV* and *polV* correspond to the previously published and validated *rdm5 (nrpd1a)* and *rdm6 (nrpd1b*) mutant alleles **(42)**. These two mutants were generated in the *ros1* mutant background, which was removed in our study by backcrossing to wt C24 plants. The *ago4 ago6* double mutant was generated in the C24 background using CRISPR/Cas9 **(see section *Generation and validation of CRISPR/Cas9 mutants* for more details)**.

To obtain the *S*-allele sequences of Ah65, Ah60, Ah03, Ah33 and Ah19, five *Arabidopsis halleri* individuals containing a combination of these alleles were collected from diverse natural European populations **(61)**, and grown under standard greenhouse conditions in order to obtain plant material for DNA extraction and sequencing **(see the section *DNA extraction, Nanopore sequencing and assembly of S-alleles* for more details)**. Following Goubet et al. **(23)**, we further obtained the S-allele sequence of Ah01, Ah02 and Ah25 by constructing and screening BAC libraries from fresh leaf samples, and fully sequencing the positive clones using PACBIO.

419 *Cloning*

420 Several transgenic lines were used in this study: lines carrying SRK01, SCR01, and the sRNA
421 precursor Ah20mirS3 were cloned as detailed previously **(6)**.
422 The precursors Ah20mir1887 and Ah20mirS3 are in very close physical proximity within the Ah20
423 allele, and the previously published Ah20mirS3 line contains both sRNA precursors **(6)**. While
424 performing sRNA sequencing of AGO-IP experiments **(see section *ARGONAUTE*
425 *immunoprecipitation and sRNA sequencing*)**, we noticed that some Ah20mir1887 sRNAs show
426 homology to the 5' region of *SCR01*. The base-pairing score of these predicted interactions varies
427 between 12.5 and 18.5, depending on the sRNAs **(Fig. 1 S1, see the section *Target site inference*
428 *for Ah04mir1887 and Ah20mirS3* for more details on these scores)**. To confidently isolate the effect
429 of Ah20mirS3 in the dominance interaction of Ah20 over Ah01 (Interaction B), we introduced five point
430 mutations in the SCR01^{*mir1887} sequence that disrupt the homology with the Ah20mir1887 sRNAs,
431 rendering the Ah20mir1887 target site non-functional. The SCR01^{*mir1887} transgene was generated by
432 performing site-directed mutagenesis on the already published SCR01 clone **(6)**, using the primers
433 detailed in **table S1**. The resulting amplicon was then recombined into the entry vector pDONR-Zeo,
434 and subsequently recombined into the pB7m34GW destination vector using the 3-fragment Gateway
435 Cloning System (Invitrogen), including 5' and 3' mock sequences, as detailed previously **(6)**.
436 Ah04mir1887 was cloned by amplifying a region spanning this sRNAs precursor and containing 2
437 kb of upstream and downstream sequences from a BAC clone carrying allele Ah04, using the primers
438 detailed in **table S1**. The resulting amplicon was then recombined into the entry vector pDONR-Zeo,
439 and subsequently recombined into the pH7m34GW destination vector using the 3-fragment Gateway
440 Cloning System (Invitrogen), including 5' and 3' mock sequences, as detailed previously **(6)**.
441 Arabidopsis thaliana C24 plants were transformed using the floral dip method **(62)**, and transgenic
442 lines were selected using the appropriate antibiotics.

443 *Generation and validation of CRISPR/Cas9 mutants*

444 Given the aforementioned limitation that the self-incompatibility reaction of *A. halleri* can only be
445 reconstituted in the *A. thaliana* C24 ecotype **(37–40)**, we created the *ago4 ago6* RdDM mutants in this
446 specific ecotype using CRISPR/Cas9. The expression cassette of pCBC-DT1T2 **(63)** was amplified
447 using two overlapping forward and reverse primers that contained two gene-specific sgRNA sequences
448 (Fw1: 5'-ATATATGGTCTCGATTGNNNNNNNNNNNNNNNNNNNGTT-3'; Fw2: 5'-
449 TGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC-3'; Rv1: 5'-
450 ATTATTGGTCTCGAAACNNNNNNNNNNNNNNNNNNNCAA-3'; Rv2: 5'-
451 AACNNNNNNNNNNNNNNNNNNCAATCTCTTAGTCGACTCTAC-3', where N corresponds to the
452 gene-specific gRNAs **(table S1)**, and underlined regions correspond to BsaI restriction sites). The
453 amplicons obtained from this PCR reaction were gel-isolated and inserted into pHEE401E **(64)** using
454 BsaI and T4 ligase (Thermo Fisher Scientific). The pHEE401E vectors containing gRNAs against *AGO4*
455 and *AGO6* were transformed into the *Agrobacterium tumefaciens* strain GV3101. The floral-dip method
456 **(62)** was then used to transform wt C24 *A. thaliana* plants.

457 To screen for T1 mutant plants, the regions targeted by the gRNAs were Sanger sequenced to
458 identify *ago4 ago6* mutants with premature stop codons in both genes **(Fig. 1 S2)**. In the T2 generation,
459 double homozygous mutants that did not carry the pHEE401E were selected by genotyping **(see table**
460 **1 for primers)**.
461 To confirm that *ago4 ago6* mutations impair DNA methylation, a Chop-PCR assay was performed
462 **(65)**. Briefly, this assay uses genomic DNA treated with the methylation-sensitive enzyme HaeIII as a
463 template for PCR. Primers targeting regions known to have AGO4/AGO6-dependent DNA methylation,
464 as well as control unmethylated regions are amplified **(see table S1 for primers)**. Using this method,
465 we could determine that the *ago4 ago6* mutant generated in this study shows reduced DNA methylation
466 levels, as has been previously published for other mutant alleles of these genes **(Fig. 1 S2 C) (66)**.

467 *Pollen germination assays*

468 All lines used for the reconstruction of the self-incompatibility reaction in *A. thaliana* were used in
469 hemizygous state to mimic the abundance of sRNA precursors, and *SCR/SRK* in *A. halleri* individuals
470 heterozygous at the *S*-locus. Genotyping of each transgenic line/mutant was done by classical PCR, or
471 using the KASPar assay **(67) (table S1)**. Paternal plants hemizygous for *SCR01* and sRNA precursors
472 were obtained by crossing *SCR01* to Ah04mir1887 homozygous lines or by crossing *SCR01*^{*mir1887} to
473 Ah20mirS3 homozygous lines.
474 To assess the self-incompatibility reaction at the phenotypic level, pollen germination assays were
475 performed on manually crossed plants. Maternal plants hemizygous for *SRK01* were emasculated one
476 day before anthesis, and manually pollinated 24h later with paternal plants hemizygous for *SCR01,*
477 *SCR01*^{*mir1887}, *SCR01* Ah04mir1887 or *SCR01*^{*mir1887} Ah20mirS3. Pollinated pistils were transferred to
478 fixing solution 6h after pollination and stained with aniline blue, as described before **(6)**. These pistils
479 were then mounted on microscope slides and imaged using a Zeiss AX10 fluorescence microscope,
480 where we counted the number of germinated pollen grains present in each pistil.
481 To evaluate the effect of RdDM mutants on the activity of *S*-locus sRNA precursors we crossed the
482 RdDM mutants *ago4 ago6*, *polIV* and *polV* with the previously described paternal plants carrying *SCR01*
483 and sRNA precursors (*SCR01, SCR01*^{*mir1887}, *SCR01* Ah04mir1887 and *SCR01*^{*mir1887} Ah20mirS3). We
484 then obtained plants hemizygous for the *SCR01* and sRNA precursor transgenes and homozygous for
485 the mutations of interest and used these as paternal plants in crosses with maternal hemizygous *SRK01*
486 plants, as described above.
487 As a control, all maternal and paternal lines used in this study were crossed to wt C24. Germination
488 and seed set for all these genotypes were comparable to that of wt x wt crosses **(data not shown)**,
489 confirming that the transgenes and mutations had no detectable effect on plant fertility.

490 *RT-qPCR*

491 To measure the abundance of *SCR01* transcripts, we isolated immature buds of plants hemizygous
492 for *SCR01, SCR01*^{*mir1887}, *SCR01* Ah04mir1887 or *SCR01*^{*mir1887} Ah20mirS3. RNA was extracted using
493 the NucleoSpin RNA Plus kit (Macherey-Nagel) and cDNA was synthesized with the RevertAid First
494 Strand cDNA Synthesis Kit (Thermo Scientific). qPCR was performed using the iTaq Universal SYBR

495 Green Supermix (BioRad) in a Lightcycler 480 instrument (Roche), with the primers found in **table S1**.
496 *ACT8* was used as a reference gene. Transcript abundance was quantified using the Pfaffl method
497 **(68)**.

498 *ARGONAUTE immunoprecipitation and sRNA sequencing*

499 AGO1 and AGO4 immunoprecipitation (IP) assays were performed on immature buds of plants
500 carrying a homozygous Ah04mir1887 or Ah20mirS3 transgenes, using a previously published protocol
501 **(69)**, with the following modifications: IPs were performed on 1.4 g of finely ground powder of frozen
502 immature buds. The anti-AGO1 (AS09 527, Agrisera) and anti-AGO4 (AS09 617, Agrisera) antibodies
503 were added at a 1/500 dilution for the incubation step and their immobilization was performed using
504 Dynabeads Protein G (Invitrogen). The IP products were washed six times with PBS prior to sRNA
505 extraction using the Trizol reagent (Ambion). In parallel, an aliquot of each input extract was used for
506 total sRNA isolation using the Trizol LS reagent (Invitrogen), according to the supplier instructions. For
507 each genotype three sRNA fractions were obtained: AGO1-IP, AGO4-IP and input.
508 These three sRNA fractions were subjected to an acrylamide gel-based size selection for sRNAs.
509 These sRNAs were then used for TruSeq Small RNA (Illumina) library preparation and sequenced on
510 a NextSeq 500 platform (Illumina), using 75 bp single-reads. Reads were trimmed, quality filtered, and
511 size-selected using TrimGalore **(70)**. We followed a two-step mapping procedure with ShortStack
512 (allowing for one mismatch) **(71)**, where we mapped sRNAs from each sample to the *A. thaliana* TAIR10
513 genome (masking the *S*-locus region), and to the sequence of the corresponding *A. halleri S*-allele
514 (Ah04, for samples carrying Ah04mir1887 and Ah20 for samples carrying Ah20mirS3). Only reads that
515 mapped exclusively to the *S*-locus were kept for further analysis. sRNA read counts were normalized
516 to rpm to allow comparison of abundance levels across samples.
517 AGO loading was inferred based on the ratio of reads found in the AGO1-IP fraction and the AGO4-
518 IP fraction: a sRNA was considered to be loaded in AGO1 or AGO4 when more than 50% of its total
519 reads were derived from either the AGO1-IP fraction or the AGO4-IP fraction, respectively. A given
520 sRNA was classified as having an unknown loading when no reads were found in both AGO-IP
521 fractions, or when the amount of reads found in the input fraction was superior to that found in any of
522 the two AGO-IP fractions.

523 *Target site inference for Ah04mir1887 and Ah20mirS3*

524 To identify sRNA target sites on *SCR* genomic regions, we first generated a database containing all
525 sRNAs sequenced in the AGO-IP experiments described above. In parallel, a database containing *SCR*
526 genomic regions of different *A. halleri S*-loci was compiled. This included alleles from all four dominance
527 classes, and comprised previously published sequencing data **(see *Data Availability* section for a**
528 **complete list of data sources)**. In addition to this, long read Nanopore sequences newly obtained
529 from individuals carrying alleles Ah65, Ah60, Ah03, Ah33 and Ah19 were also added to this database
530 **(see section *Nanopore sequencing and assembly of S-alleles* for more details)**.
531 Using the databases of sRNA sequences and *SCR* alleles, we applied a previously published
532 homology detection algorithm **(6)** to find target sites. Functional sRNA are defined as those with a

533 homology score of ≥ 18, since this score has been consistently shown to be associated with a decrease
534 in *SCR* transcript abundance of the targeted allele (6, 18). The identified sRNA - *SCR* interactions were
535 then represented in a heatmap **(Fig. 4)** using ComplexHeatmap **(72)**, and in a circos plot **(Fig. 5)** using
536 circlize **(73)**.

537 *DNA extraction, Nanopore sequencing and assembly of S-alleles*

538 To obtain the *S*-locus sequences of alleles Ah65, Ah60, Ah03, Ah33 and Ah19, 2 g of fresh leaves
539 were collected from five different individuals carrying these alleles and flash-frozen. High molecular
540 weight genomic DNA was extracted as described before **(74)**. Long read sequences were obtained by
541 Oxford Nanopore Technologies (ONT) flowcells v9. To polish the contigs obtained by long read
542 sequencing Illumina data were also generated (with no step of PCR amplification to minimize
543 sequencing bias).

544 For Nanopore library preparation, the smallest genomic DNA fragments were first eliminated using
545 the Short Read Eliminator Kit (Pacific Biosciences). Libraries were then prepared according to the
546 protocol 1D Native barcoding genomic DNA (with EXP-NBD104 and SQK-LSK109) provided by Oxford
547 Nanopore Technologies. Depending on how many samples were pooled, 250 ng (pool of 9 samples) to
548 1 µg (pool of 4 samples) of genomic DNA fragments were repaired and end-prepped with the NEBNext
549 FFPE DNA Repair Mix and the NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs).
550 Barcodes provided by ONT were then ligated using the Blunt/TA Ligase Master Mix (NEB). Barcoded
551 fragments were purified with AMPure XP beads (Beckmann Coulter), then pooled and ONT adapters
552 were added using the NEBNext Quick Ligation Module (NEB). After purification with AMPure XP beads
553 (Beckmann Coulter), each library was mixed with the sequencing buffer (ONT) and the loading beads
554 (ONT) and loaded on a PromethION R9.4.1 flow cell. In order to maintain the translocation speed, flow
555 cells were refueled with 250 µl Flush Buffer when necessary. Reads were basecalled using Guppy
556 version 3.2.10, 4.0.11, 5.0.12, 5.0.13, 5.0.16 or 5.1.12. The Nanopore long reads were not cleaned and
557 raw reads were used for genome assembly.

558 For Illumina PCR-free library preparation, 1.5 µg of genomic DNA was sonicated to a 100–1500-bp
559 size range using a Covaris E220 sonicator (Covaris). The fragments (1µg) were end-prepped, and
560 Illumina adapters (NEXTFLEX Unique Dual Index Barcodes, Perkin Elmer) were added using the Kapa
561 Hyper Prep Kit (Roche). The ligation products were purified twice with 1X AMPure XP beads (Beckman
562 Coulter). The libraries were then quantified by qPCR using the KAPA Library Quantification Kit for
563 Illumina Libraries (Roche), and their profiles were assessed on an Agilent Bioanalyzer (Agilent
564 Technologies). The libraries were sequenced on an Illumina NovaSeq 6000 instrument (Illumina) using
565 150 base-length read chemistry in a paired-end mode.

566 Nanopore sequencing data were assembled using Necat **(75)** with a genome size of 240 Mbp and
567 the remaining parameters left as default. Contigs produced by Necat were polished one time using
568 Racon **(76)** with Nanopore reads, then one time with Medaka
569 (https://github.com/nanoporetech/medaka, model r941_prom_hac_g507) and Nanopore reads, and two
570 times with Hapo-G v1.3.4 **(77)** and Illumina short reads. However, the cumulative sizes of our long-read
571 assemblies were larger than the estimated 240 Mb, suggesting that the assembly size was currently

572 inflated by the presence of allelic duplications. Thus, HaploMerger2 **(78)** was run (Batch A twice to
573 remove major misjoin and one Batch B) to generate a haploid version of each assembly.
574 To detect the *S*-locus region in the generated assemblies, blast searches were performed using the
575 genes flanking the *S*-locus (*ARK3* and *U-box* **(23)**) as queries. This allowed us to precisely narrow down
576 the genomic regions corresponding to the full Ah65, Ah60, Ah03, Ah33 and Ah19 *S*-alleles. We
577 annotated *SRK* using blast searches, and following Goubet et al. **(23)**, we used Fgenesh+ **(79)** to predict
578 the genomic sequence of *SCR* based on a database of SCR protein sequences from already known S-
579 alleles.

580 *Phenotypic determination of the S-loci dominance hierarchy*

581 The *S*-locus dominance hierarchy in **Fig. 3** is based on a compilation of controlled reciprocal crosses
582 between different *A. halleri* individuals **(6, 18, 46, 80)**. In cases where interactions between alleles of
583 two different dominance classes had not been tested phenotypically, dominance was inferred based on
584 the phylogenetic class of each allele, since interclass dominance interactions are predictable **(15, 24)**.
585 In the case of intraclass relationships, such as for alleles Ah65, Ah60 and Ah33 (all belonging to class
586 III), phenotypic dominance has not been determined yet, and thus the ordering of these alleles along
587 the hierarchy remains arbitrary at this stage.

588 *BSAS sequencing*

589 To measure DNA methylation in *SCR01* target sites we used the BiSulfite Amplicon Sequencing
590 Technique - BSAS **(28, 44)** which relies on PCR enrichment of regions of interest using bisulfite
591 converted DNA as a template. To assess if the presence of *S*-locus sRNA precursors is associated with
592 DNA methylation changes in the respective *SCR01* target regions, we isolated immature buds of
593 *SCR01, SCR01*^{*mir1887}, *SCR01* Ah04mir1887 and *SCR01*^{*mir1887} Ah20mirS3 plants. Genomic DNA was
594 extracted using the NucleoSpin Plant II kit (Macherey-Nagel), and converted with the EZ DNA
595 Methylation Kit (Zymo Research), including the modifications A and B, as described by the supplier, to
596 optimize the conversion efficiency. Eight to ten biological replicates were extracted and converted for
597 each genotype. Converted DNA was then amplified using primers flanking the Ah04mir1887 or
598 Ah20mirS3 target sites in the intron and 5' region regions of *SCR01*, respectively **(table S1)**. For each
599 sRNA precursor, seven amplicons were generated, which amplify the top and bottom strands of the
600 ~800 bp that flank the target site. As a negative control, we also amplified *AT2G20610*, which has been
601 previously described to be an unmethylated negative control in *A. thaliana* Col-0 plants **(28)**.
602 Amplification of bisulfite converted DNA was performed using the TaKaRa EpiTaqTM HS (Takara), and
603 the resulting PCR reactions were cleaned-up using the NucleoSpin Gel and PCR Clean-up XS kit
604 (Macherey-Nagel). All amplicons obtained from a single biological replicate were pooled in equimolar
605 amounts and a total of 100 ng were used for library preparation following the modified Illumina Nextera
606 Flex library preparation protocol described before (28, 81). Libraries were sequenced in a MiSeq
607 (Illumina) platform using 250 bp paired-end reads. Reads were trimmed, quality-filtered, and mapped
608 to the Ah01 allele using methlypy **(82)**, following the paired-end pipeline and specifying *AT2G20610* as
609 the unmethylated control. Conversion efficiencies for each sample were estimated based on the

methylation levels detected in the *AT2G20610* locus, and were between 97% and 83% **(table S2)**. The number of methylated reads covering each *SCR01* cytosine position, as outputted by methylpy, can be found in **table S3**. Average DNA methylation levels within the top and bottom strands of the ~800 bp regions flanking Ah04mir1887 and Ah20mirS3 target sites were summarized in R **(83)** and plotted with ggplot2 **(84)**.

To identify DNA methylation heterogeneity at the single-molecule level, the methylation rate over each single read aligned to the *SCR01* sequence in three different cytosine contexts (CG, CHG and CHH) was computed using the Bismark methylation call output **(85)**. For each sample, reads were classified in three distinct categories: highly methylated reads with 90% or more methylated cytosines, weakly methylated reads with a methylation rate inferior to 10%, and everything in between. Statistical differences in number of highly and weakly methylated reads between the *SCR01* and *SCR01* Ah04mir1887 or *SCR01*^{*mir1887} and *SCR01*^{*mir1887} Ah20mirS3 genotypes were assessed using a two-tailed Mann-Whitney test with continuity correction.

Data availability

The AGO-IP and sRNA sequencing data is deposited in the Gene Expression Omnibus database, under the SubSeries reference GSE249507.

The Illumina and Oxford Nanopore sequencing data of the five *A. halleri* individuals carrying alleles Ah65, Ah60, Ah03, Ah33 and Ah19 are available in the European Nucleotide Archive, under the project number PRJEB70880.

The *S*-locus sequences used in this study are a combination of previously published data (6, 23) and data generated in this study. All allele sequences can be found in GenBank, under the following references: Ah01 - this study, PP396894; Ah02 - this study, PP396895; Ah03 - this study, PP537897; Ah04 - KJ461484; Ah10 - KM592810 and KM592817; Ah12 - KJ772373 and KJ772377; Ah13 - KJ461479 and KJ461483; Ah19 - this study, PP537898; Ah20 - FO203486; Ah25 - this study, PP396896; Ah28 - KJ461478; Ah29 - KM592803; Ah33 - this study, PP537899; Ah60 - this study, PP537900; Ah65 - this study, PP537901.

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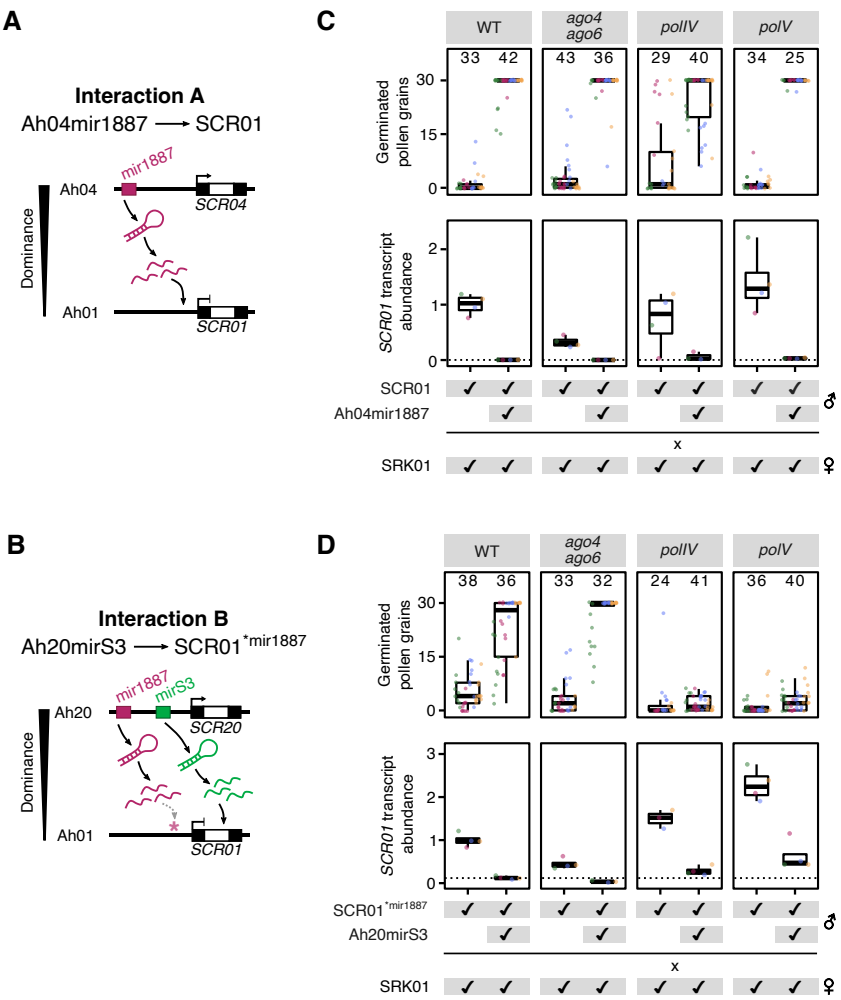


Figure 1. The RdDM pathway is not required for the activity of Ah04mir1887 and Ah20mirS3.

(A,B) Dominance interactions studied in this paper. The black and white regions within *SCR* genes correspond to exons and introns, respectively. **(A)** Interaction A focuses on the role of the sRNA precursor Ah04mir1887 in mediating the dominance of Ah04 over Ah01. **(B)** Interaction B focuses on the role of Ah20mirS3 in mediating the dominance of Ah20 over Ah01. *S*-locus sRNA precursors consist of inverted repeats that form hairpins which are processed into multiple sRNAs showing sequence homology to specific *SCR* regions: Ah04mir1887 sRNAs show homology to the 5' region region of *SCR01*, while Ah20mirS3 sRNAs show homology to the intronic region (white) of *SCR01*. Because sRNAs produced from the Ah20mir1887 precursor show homology to the wt *SCR01* 5' region region, and to study the specific effect of Ah20mirS3 in Interaction B, the *SCR01*^{*mir1887} line containing five point mutations in the *SCR01* 5' region was created, which disrupts the homology with Ah20mir1887 sRNAs. **(C,D)** Phenotypic and expression assay evaluating the role of key RdDM components on the action of Ah04mir1887 **(C)**, and Ah20mirS3 **(D)**. Crosses between *SRK01*-expressing mothers and *SCR01*-expressing fathers were performed in wt and RdDM mutant backgrounds in the absence or presence of the respective sRNA precursor. The number of germinated pollen grains of the indicated genotype was counted on pistils of SRK01 mothers. *SCR01* expression in immature buds of the indicated genotype was measured by RT-qPCR and normalized to the wt level. Five biological replicates

846 (represented by the differently colored dots) were analyzed for pollen germination and *SCR01*
847 expression. Numbers on top of columns indicate the total number of siliques sampled for pollen
848 germination assays.

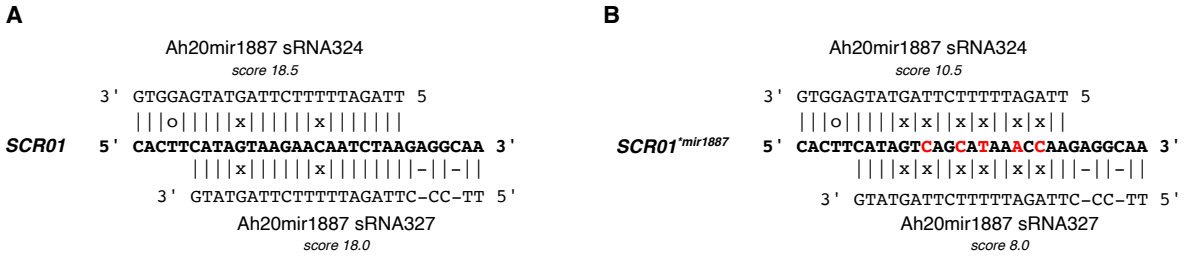
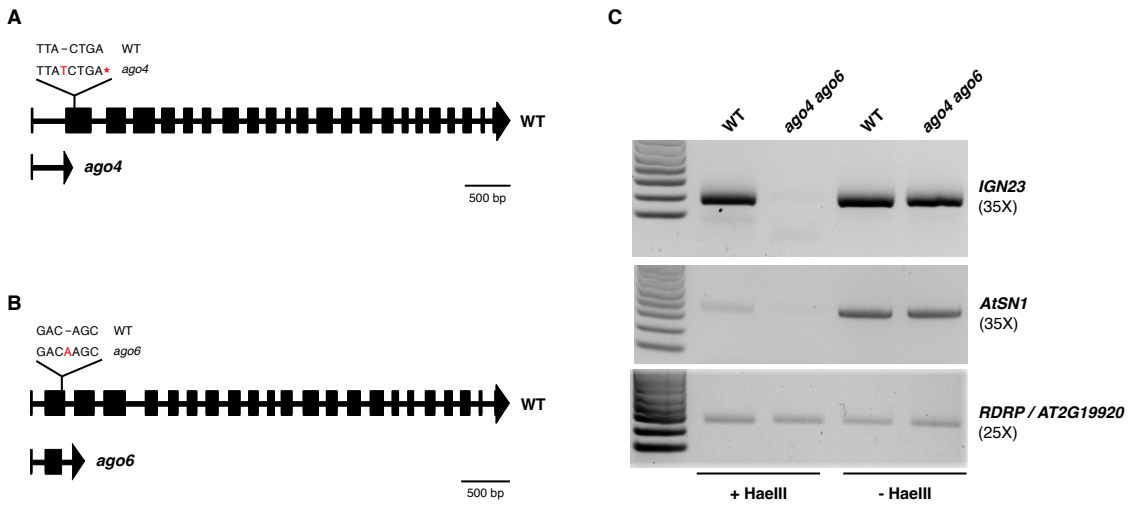


Figure 1 S1. *SCR01*^{*mir1887} transgenic line

(A) Alignment between the native mir1887 target site on *SCR01* and Ah20mir1887 sRNAs with a targeting score ≥ 18. **(B)** Alignment between the same sRNAs and the mutated mir1887 target site of the transgenic line *SCR01*^{*mir1887}. Note that the mutations decrease the homology score below 18, suggesting that these sRNAs are not able to target the mutated site. Mutated sites are in red.



854 **Figure 1 S2. Validation of the RdDM CRISPR/Cas9 mutants generated in this study.**

855 **(A-B)** Schematic representation of CRISPR/Cas9 induced mutations in the *AGO4* and *AGO6* genes.
856 Sequence changes are shown in red, premature stop codons are represented by the asterisk. **(C)** Chop-
857 PCR assay on wt C24 plants and *ago4 ago6* double mutant plants. *IGN23* and *AtSN1* correspond to
858 RdDM-dependent methylated regions, while *RDRP* corresponds to a non-methylated region.

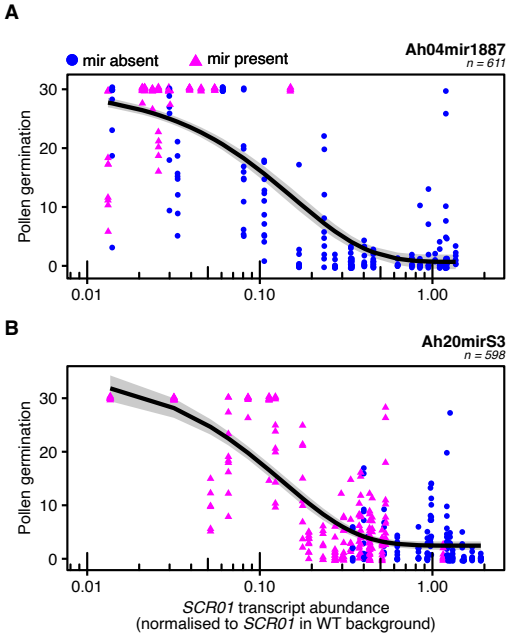


Figure 1 S3. A compatible pollen germination phenotype requires a low *SCR01* transcript abundance.

Non-linear regression analysis modeling the relationship between *SCR01* transcript abundance levels and pollen germination. For both Ah04mir1887 **(A)** and Ah20mirS3 **(B)** sRNA precursors a compatible pollen germination phenotype (~20-30 pollen grains) requires a 10-fold decrease in *SCR01* transcript abundance (compared to the level observed in the wt). Analysis was performed using the phenotypic and expression data presented in **Fig. 1 C-D**. The black line represents the predicted model and the gray area corresponds to the 95% confidence interval.

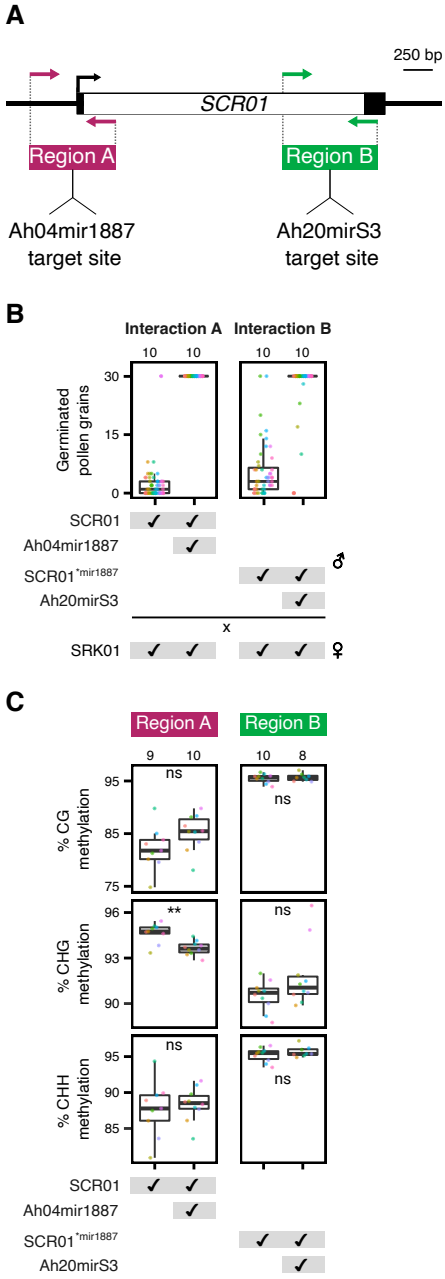


Figure 1 S4. Ah04mir1887 and Ah20mirS3 are not associated with detectable DNA methylation changes at *SCR01* target sites.

(A) Schematic representation of the BSAS assay performed to measure DNA methylation at the Ah04mir1887 target site (*SCR01* 5' region - Region A), and at the Ah20mirS3 target site (*SCR01* intron - Region B). Region A and Region B correspond to fragments of 794 and 871 bp, respectively, centered in each sRNA precursor's target site. **(B)** Phenotypic validation of lines used for the BSAS assay. Colored dots correspond to values obtained for each biological replicate. Number of biological replicates is indicated at the top of the panel. For each biological replicate pollen germination on a total of 5 *SRK01* pistils was counted. **(C)** Average DNA methylation levels on Region A (Interaction A) and Region B (Interaction B) of *SCR01*. DNA methylation levels correspond to the average of methylation in all

877 cytosines in the top and bottom DNA strands of sampled regions. Number of biological replicates is
878 indicated at the top of the panel. Statistical differences were assessed using a two-tailed Mann-Whitney
879 test with continuity correction (ns - non significant, p-value > 0.05; ** - p-value ≤ 0.01).

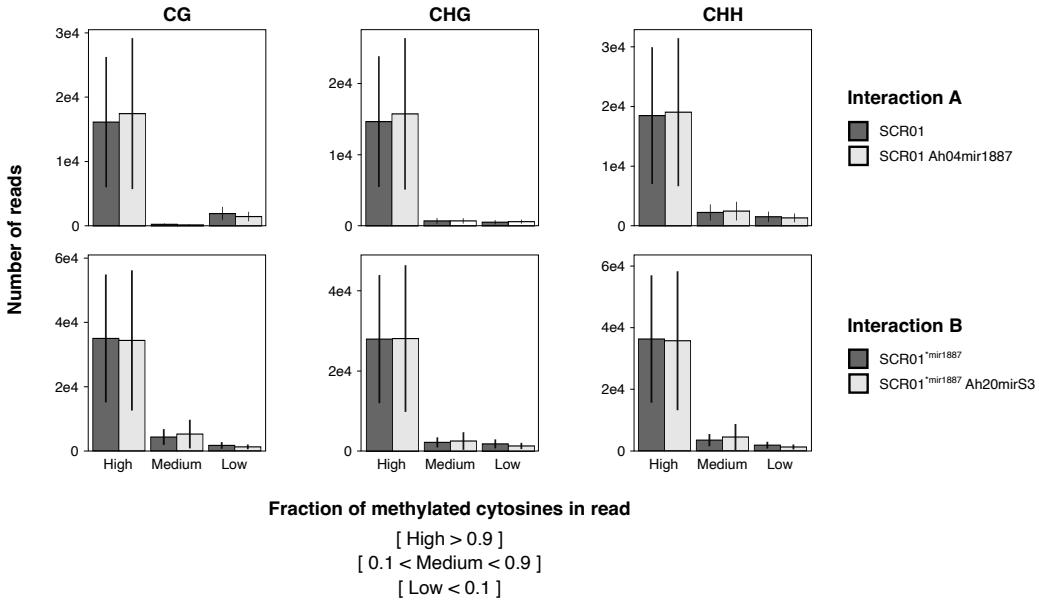


Figure 1 S5. Ah04mir1887 and Ah20mirS3 target sites are heavily methylated.

BSAS reads were grouped according to the fraction of methylated cytosines within each read. High: reads where nearly all cytosine positions are methylated (> 90% cytosines); Low: reads where very few cytosine positions are methylated (< 10% cytosines); Medium: all reads with more than 10% and less that 90% cytosines methylated. Error bars represent the standard deviation between replicates. No statistically significant differences were detected in the number of highly and lowly methylated reads between the *SCR01* and *SCR01* Ah04mir1887 or *SCR01*mir1887* and *SCR01*mir1887* Ah20mirS3 genotypes (p-value > 0.05, two-tailed Mann-Whitney test with continuity correction).

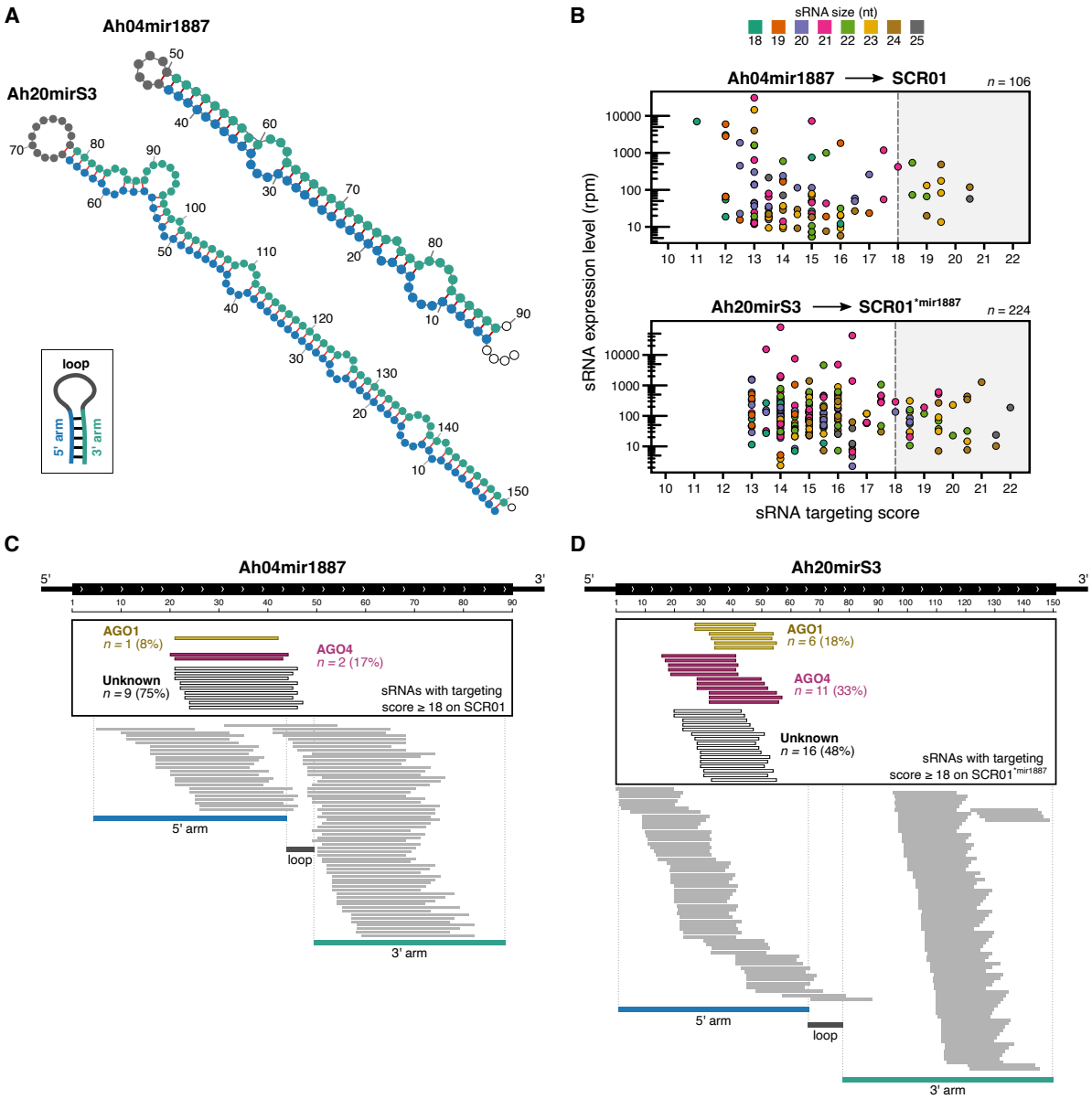


Figure 2. Ah04mir1887 and Ah20mirS3 hairpins are processed into numerous sRNAs.

(A) Predicted RNA hairpin structures of the *S*-locus sRNA precursors Ah04mir1887 and Ah20mirS3. **(B)** Expression of Ah04mir1887 and Ah20mirS3 sRNAs as a function of their targeting score against *SCR01*. The color of the dots corresponds to the size of the sRNA. The grey box highlights sRNAs predicted to induce a reduction in *SCR01* transcript abundance (score ≥ 18). *n* corresponds to the total number of unique sRNAs identified upon sequencing for each sRNA precursor. **(C-D)** Genomic representation of the Ah04mir1887 and Ah20mirS3 loci, and their respective sRNAs. The black rectangle highlights sRNAs predicted to induce a reduction in *SCR01* transcript abundance (score ≥ 18). AGO loading information for each functional sRNA is indicated by the sRNA color (yellow – sRNA loaded predominantly in AGO1; magenta – sRNA loaded predominantly in AGO4; white – sRNAs with unknown loading profile), **see section *ARGONAUTE immunoprecipitation and sRNA sequencing*** for more details.

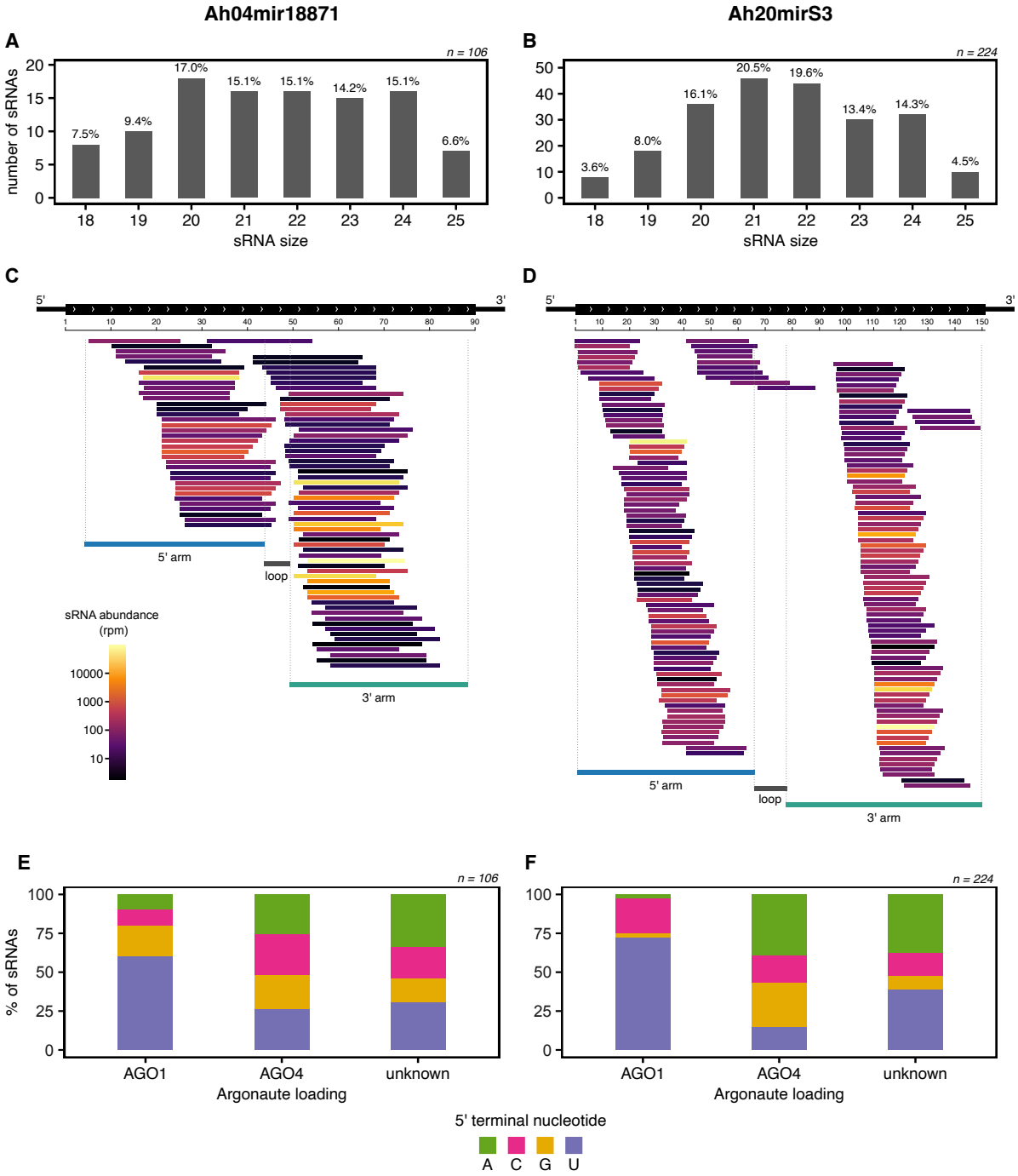


Figure 2 S1. sRNAs produced from Ah04mir1887 and Ah20mirS3 do not show a specific size bias but vary in their abundance levels.

Size distribution of sRNAs derived from Ah04mir1887 **(A)** and Ah20mirS3 **(B)**. **(C-D)** Genomic representation of the Ah04mir1887 and Ah20mirS3 loci, and their respective sRNAs. sRNAs are colored according to their abundance level. **(E-F)** 5' terminal nucleotide frequencies in Ah04mir1887 and Ah20mirS3 sRNAs, related to their inferred AGO loading pattern.

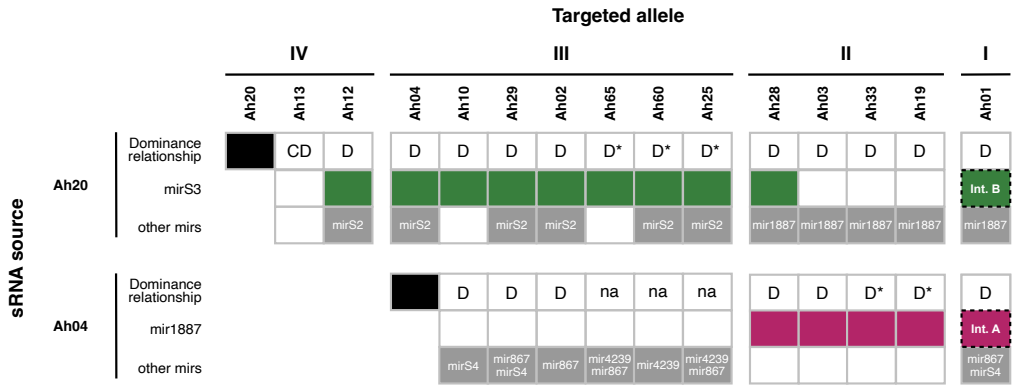


Figure 3. Ah04mir1887 and Ah20mirS3 underlie numerous *S*-allele dominance interactions.

Schematic representation of the dominance relationship between alleles Ah20, Ah04 and other *S*-alleles in the current *A. halleri* dominance network. The dominance phenotypes observed are represented by D (dominant, as determined by phenotypic assays), CD (codominant, as determined by phenotypic assays), and na (no phenotypic data available). D* refers to a predicted dominance relationship, based on the difference between the dominance class of the sRNA source and the targeted allele (15, 24). The sRNA precursors underlying each dominance relationship are highlighted in green (mirS3), magenta (mir1887), or grey (other mirs). The specific interactions studied earlier in this manuscript – Interaction A and Interaction B - are highlighted by a dashed box.

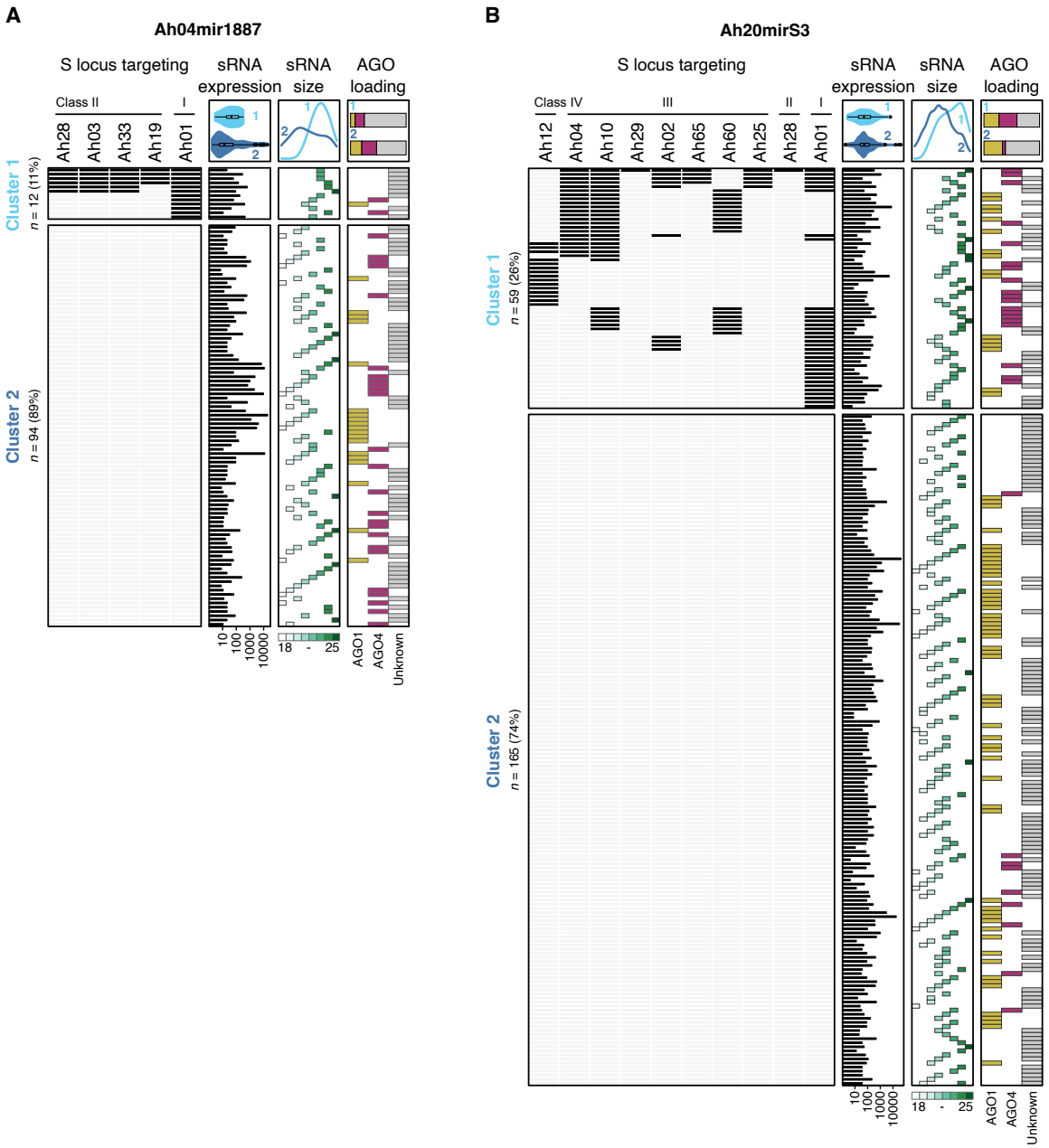


Figure 4. Ah04mir1887 and Ah20mirS3 produce multiple sRNAs targeting one or more *S*-alleles.

Heatmap representing the *S*-loci targeted by Ah04mir1887-derived sRNAs **(A)** and Ah20mirS3-derived sRNAs **(B)**. A black rectangle indicates that a specific sRNA has a targeting score of ≥ 18 against the corresponding *S*-allele. Cluster 1 groups sRNAs that target at least one *S*-allele, while Cluster 2 groups sRNAs that have no targets in the represented *S*-alleles. sRNA expression levels, sRNA size and AGO loading patterns are indicated for each sRNA. Summary plots comparing these features in Cluster 1 and Cluster 2 are shown in the top panel. *n* corresponds to the number of sRNAs produced by each sRNA precursor.

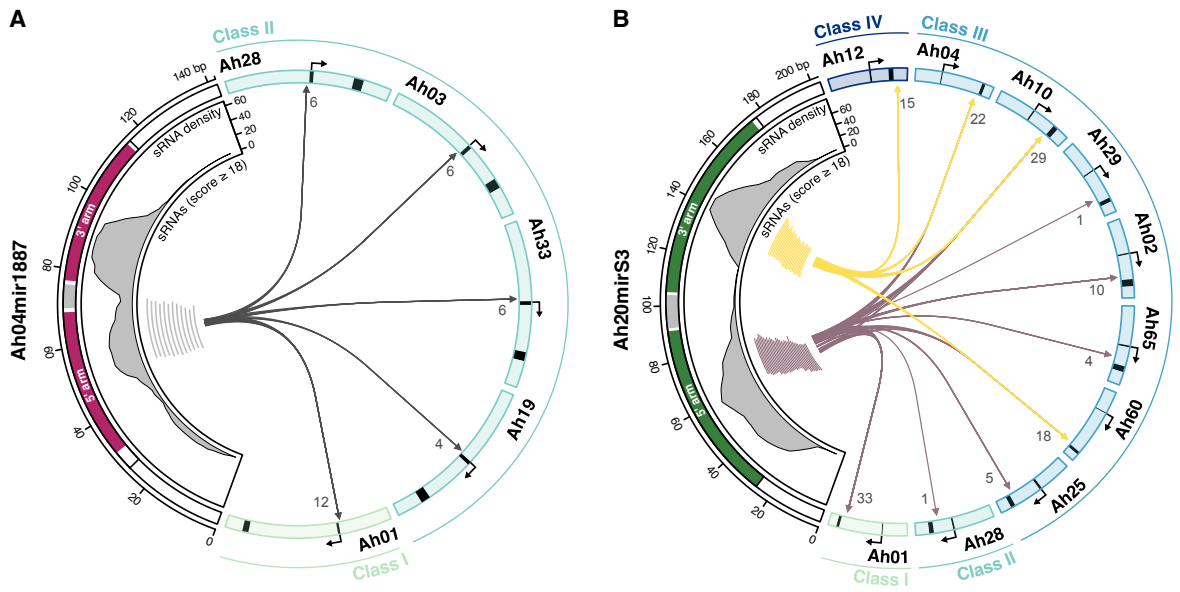


Figure 5. The targeting repertoire of *S*-locus sRNA precursors is enhanced by their heterogeneous processing pattern.

Circos plot representing the positional relationship between sRNAs produced by Ah04mir1887 **(A)**, Ah20mirS3 **(B)** and the corresponding targeted *S*-alleles. The genomic regions harboring Ah04mir1887 and Ah20mirS3 are represented on the left side of the plots. The predicted 5' and 3' hairpin arms are represented in magenta (Ah04mir1887) and green (Ah20mirS3), and the sRNA density (number of sRNAs) is plotted along the sRNA precursor region. Individual sRNAs with a score of ≥ 18 are represented by the grey, yellow or dark purple segments. Arrowed segments represent the sRNA source - target relationship. The numbers at the end of these arrowed segments indicate the total number of sRNAs targeting that respective allele. The *SCR* genomic region of targeted *S*-alleles is represented on the right side and includes 1500 bp upstream of the TSS and 500 bp downstream of the TES. *SCR* exons 1 and 2 are colored in black.