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## Base-pairing requirements for small RNA-mediated gene silencing of recessive self-incompatibility alleles in *Arabidopsis halleri*

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1 **Base-pairing requirements for small RNA-mediated gene**  
2 **silencing of recessive self-incompatibility alleles in *Arabidopsis***  
3 ***halleri*.**

4

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16

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## Abstract

33 Small non-coding RNAs are central regulators of genome activity and stability. Their  
34 regulatory function typically involves sequence similarity with their target sites, but  
35 understanding the criteria by which they specifically recognize and regulate their targets  
36 across the genome remains a major challenge in the field, especially in the face of the  
37 diversity of silencing pathways involved. The dominance hierarchy among self-  
38 incompatibility alleles in Brassicaceae is controlled by interactions between a highly  
39 diversified set of small non-coding RNAs produced by dominant S-alleles and their  
40 corresponding target sites on recessive S-alleles. By controlled crosses, we created  
41 numerous heterozygous combinations of S-alleles in *Arabidopsis halleri* and developed an  
42 RT-qPCR assay to compare allele-specific transcript levels for the pollen determinant of  
43 self-incompatibility (*SCR*). This provides the unique opportunity to evaluate the precise  
44 base-pairing requirements for effective transcriptional regulation of this target gene. We  
45 found strong transcriptional silencing of recessive *SCR* alleles in all heterozygote  
46 combinations examined. A simple threshold model of base-pairing for the sRNA-target  
47 interaction captures most of the variation in *SCR* transcript levels. For a subset of S-alleles,  
48 we also measured allele-specific transcript levels of the determinant of pistil specificity  
49 (*SRK*) and found sharply distinct expression dynamics throughout flower development  
50 between *SCR* and *SRK*. In contrast to *SCR*, both *SRK* alleles were expressed at similar  
51 levels in the heterozygote genotypes examined, suggesting no transcriptional control of  
52 dominance for this gene. We discuss the implications for the evolutionary processes  
53 associated with the origin and maintenance of the dominance hierarchy among self-  
54 incompatibility alleles.

55

## Introduction

56 Small non-coding RNAs are short RNA molecules (20-25nt) with a range of regulatory  
57 functions (Vazquez *et al.*, 2010; Aalto & Pasquinelli, 2012). The best-known members of  
58 this class of molecules are microRNAs, which are typically involved in post-transcriptional  
59 gene silencing and regulate the activity of their target gene in *trans* by either mRNA  
60 cleavage (quickly followed by degradation) or by blocking translation (Li *et al.*, 2014). In  
61 some cases, the action of microRNAs leads to the production of secondary phased short  
62 interfering RNAs (pha-siRNAs) by their target coding or non-coding sequence, which in  
63 turn can regulate other downstream targets (Fei *et al.*, 2013). Another major set of small  
64 RNAs is heterochromatic short interfering RNAs (hc-siRNAs) which are mediating  
65 transcriptional silencing of repeat sequences in the genome through epigenetic  
66 modification by the RNA-dependent DNA methylation pathway (RdDM, Matzke *et al.*,  
67 2009).

68 Both microRNAs and siRNAs guide their effector molecules (members of the  
69 ARGONAUTE gene family: AGO1 and AGO4, respectively) to their target sites by  
70 sequence similarity through base-pairing. For plant microRNAs, sequence similarity with  
71 the target sequence is typically very high and appears to be a shared feature of all  
72 functionally verified interactions (Wang *et al.*, 2015). High base-pairing complementarity,  
73 however, is not the sole determinant of target specificity, and the position of the  
74 mismatches along the microRNA:target duplex is also important. Indeed, expression assays  
75 showed that while individual mismatches typically have limited functional consequences,  
76 they can also entirely inactivate the interaction when they hit specific positions such as, for  
77 example, the 10<sup>th</sup> and 11<sup>th</sup> nucleotide, corresponding to the site of cleavage (Jones-Rhoades

78 *et al.*, 2006). Furthermore, the position of mismatches along the microRNA:target duplex  
79 also seems to be crucial, with a greater tolerance in the 3' than the 5' region of the  
80 microRNA (up to four mismatches generally have limited functional consequences in the  
81 3' region, while only two mismatches in the 5' region seem sufficient to abolish the target  
82 recognition capability; Liu *et al.*, 2014, Mallory *et al.*, 2004; Parizotto *et al.*, 2004; Schwab  
83 *et al.*, 2005). These observations have led to the formulation of general “rules” for  
84 microRNA targeting (Axtell & Meyers, 2018), but at the same time they also revealed a  
85 large number of exceptions. As a result, *in silico* prediction of microRNA target sites  
86 currently remains a difficult challenge (Ding *et al.*, 2012; Axtell & Meyers, 2018). For  
87 other types of small RNAs (pha-siRNAs and hc-siRNAs), even less is known about the  
88 base-pairing requirements for targeting, mostly because of the absence of experimentally  
89 confirmed examples of discrete, single siRNA target sites either in *cis* or in *trans* (Wang *et*  
90 *al.*, 2015).

91 In this context, the recent discovery by Tarutani *et al.* (2010), Durand *et al.* (2014) and  
92 Yasuda *et al.*, (2016) of a highly diversified set of small non-coding RNAs at the gene  
93 cluster controlling self-incompatibility (SI) in Brassicaceae, provides an experimentally  
94 tractable model to evaluate the base-pairing requirements for silencing by a set of sRNAs  
95 that are regulating expression of a single gene. Sporophytic SI is a genetic system that  
96 evolved in several hermaphroditic plant lineages to enforce outcrossing by preventing self-  
97 fertilization, hence avoiding inbreeding depression (De Nettancourt, 2001). In the  
98 Brassicaceae family, SI is controlled by a single genomic region called the “S-locus”,  
99 which contains two tightly linked genes, namely *SCR* and *SRK*, that encode the pollen S-  
100 locus cysteine-rich and the stigma S-locus receptor kinase recognition proteins,

101 respectively. This system involves a polymorphism in which multiple deeply diverged  
102 allelic lines are maintained, and accordingly a large number of S-alleles is typically found  
103 in natural populations of self-incompatible species (Castric & Vekemans, 2004). With such  
104 a large allelic diversity and the very process of self-rejection, most individual plants are  
105 heterozygotes at the S-locus. Yet in most cases, only one of the two S-alleles in a  
106 heterozygous genotype is expressed at the phenotypic level in either pollen or pistil, as can  
107 be revealed by controlled pollination assays on pollen or pistil tester lines (Llaurens *et al.*,  
108 2008; Durand *et al.*, 2014). Which of the two alleles is expressed is determined by their  
109 relative position along a dominance hierarchy, whose molecular basis for the pollen  
110 phenotype has been initially studied in the genus Brassica. In this genus, dominance is  
111 controlled at the transcriptional level in pollen (Schopfer 1999, Kakizaki *et al.* 2003).  
112 Transcriptional silencing of recessive alleles by dominant alleles is caused by 24nt-long  
113 trans-acting small RNAs produced by dominant S-alleles and capable of targeting a DNA  
114 sequence in the promoter sequence of the *SCR* gene of recessive S-alleles, provoking DNA  
115 methylation (Shiba *et al.* 2006). Details of how these sRNAs achieve their silencing  
116 function remain incompletely understood (Finnegan *et al.*, 2011), but it is clear that their  
117 biogenesis is similar to that of microRNAs (*i.e.*, they are produced by a short hairpin  
118 structure), while their mode of action is rather reminiscent of that of siRNAs (*i.e.*, the  
119 transcriptional gene silencing functions through recruitment of the methylation machinery).  
120 Strikingly, the full dominance hierarchy in the Brassica genus seems to be controlled by  
121 just two small RNAs called *Smi* and *Smi2* (Tarutani *et al.*, 2010, Yasuda *et al.* 2016). *Smi*  
122 and *Smi2* target distinct DNA sequences, but both are located in the promoter region of  
123 *SCR*, and both seem to involve DNA methylation and 24-nt active RNA molecules.

124 The dominance hierarchy in Brassica is, however, peculiar in that only two ancestral allelic  
125 lineages segregate in that genus (the class I and class II alleles referred to above, see *e.g.*  
126 Leducq *et al.*, 2014), whereas self-incompatible species in Brassicaceae typically retain  
127 dozens of highly divergent ancestral allelic lineages (Castric & Vekemans, 2004). A recent  
128 study showed that in *Arabidopsis halleri*, a Brassicaceae species with multiple allelic  
129 lineages at the S-locus, the dominance hierarchy among S-alleles in pollen is controlled by  
130 not just two but as many as eight different sRNA precursor families and their target sites,  
131 whose interactions collectively determine the position of the alleles along the hierarchy  
132 (Durand *et al.*, 2014). In that genus, much less is known about the mechanisms by which  
133 the predicted sRNA-target interactions translate into the dominance phenotypes. First, the  
134 expression dynamics of the *SCR* gene across flower development stages is poorly known.  
135 Indeed, Kusaba *et al.* (2002) measured expression of *SCR* alleles in *A. lyrata*, but focused  
136 on only two S-alleles (*SCRa* and *SCRb*, also known as *AlSCR13* and *AlSCR20*,  
137 respectively, in Mable *et al.* 2003) and showed striking differences in their expression  
138 dynamics in anthers. Hence, the developmental stage at which the transcriptional control of  
139 dominance in pollen should be tested is not precisely known. Second, while they did  
140 confirm monoallelic expression, consistent with the observed dominance relationship  
141 between the two alleles (*SCRb* > *SCRa*, Kusaba *et al.* 2002), the fact that only a single  
142 heterozygote combination was measured among the myriad possible combinations given  
143 the large number of S-alleles segregating in that species (at least 43 S-alleles: Genete *et al.*,  
144 2020) prevents generalization at this step. Hence, a proper experimental validation of the  
145 transcriptional control of dominance among S-alleles in the *Arabidopsis* genus is still  
146 lacking. Third, Durand *et al.*, (2014) observed rare sRNA-target interaction predictions that



147 did not agree with the observed dominance phenotype. In particular, they identified pairs  
148 of S-alleles where no sRNA observed as being produced by the dominant allele was  
149 predicted to target the *SCR* gene of the recessive one, while the dominance phenotype had  
150 been well established phenotypically by controlled crosses (*e.g.* Ah04>Ah03) suggesting  
151 the possibility that mechanisms other than transcriptional control may be acting.  
152 Conversely, in other rare cases, sRNAs produced by a recessive S-allele were predicted to  
153 target the *SCR* gene of a more dominant allele, suggesting exceptions to the set of base-  
154 pairing rules used to predict target sites. Fourth, the target sites for the two sRNAs in  
155 Brassica were both located in the promoter sequence (Tarutani *et al.*, 2010, Yasuda *et al.*  
156 2016), and can thus reasonably be expected to prevent transcriptional initiation through  
157 local modification of the chromatin structure associated with DNA methylation. Many of  
158 the predicted sRNA target sites in *A. halleri*, however, are rather mapped to the *SCR* intron  
159 or the intron-exon boundary (beside some in the promoter as well, Durand *et al.* 2014),  
160 which suggests that distinct silencing pathways might be acting (Cuerda-Gil & Slotkin,  
161 2016). It thus remains to be determined whether transcriptional control is also valid when  
162 the targets are at other locations along the *SCR* gene structure. Finally, the dominance  
163 hierarchy at the female determinant *SRK* differs from that at *SCR*, co-dominance being  
164 more frequent than on the pollen side both in Brassica (Hatakeyama *et al.*, 2001) and in *A.*  
165 *halleri* (Llaurens *et al.*, 2008). Limited transcriptional analysis in Brassica and Arabidopsis  
166 suggests that dominance in pistils is not associated with *SRK* expression differences, but  
167 again the number of allelic pairs tested has remained limited (Suzuki *et al.* 1999; Kusaba *et*  
168 *al.* 2002).

169 Here, we take advantage of the fact that dominance interactions in *Arabidopsis* SI are  
170 controlled in pollen by a diversity of sRNAs and the diversity of their target sites to  
171 determine the base-pairing requirements for successful small-RNA mediated  
172 transcriptional silencing of recessive *SCR* alleles. We first used controlled crosses to obtain  
173 a large collection of *A. halleri* plants in which S-alleles were placed in various  
174 homozygous and heterozygote combinations for which pairwise dominance interactions  
175 had been determined. We then developed and validated a qPCR protocol for allele-specific  
176 expression of a set of nine *SCR* and five *SRK* alleles in *A. halleri*. This enabled us to  
177 analyse the expression dynamics across four flower developmental stages of each of these  
178 alleles and test the transcriptional control of dominance for both genes in many  
179 heterozygote combinations. We quantified the strength of silencing of recessive *SCR*  
180 alleles and propose a quantitative threshold model for how sequence identity between the  
181 small non-coding RNAs and their target sites results in silencing. We discuss the  
182 implications of this model on the evolutionary processes associated with the origin and  
183 maintenance of the S-locus dominance hierarchy in Brassicaceae.

184

## 185 **Material & Methods**

### 186 **Plant material**

187 We used controlled crosses to create a collection of 88 *A. halleri* plants containing nine  
188 different S-alleles (S1, S2, S3, S4, S10, S12, S13, S20, and S29) in a total of 37 of all 45  
189 possible homozygous and heterozygous combinations. Some S-locus genotypes were  
190 obtained independently by different controlled crosses and were considered below as

191 “biological replicates” (different genetic backgrounds, on average  $n= 2.05$  biological  
192 replicates per S-locus genotype, Table S1 & S2). Three plants were cloned by cuttings and  
193 considered as “clone replicates” (identical genetic background, Table S1) that we used to  
194 evaluate the expression variance associated with different genetic backgrounds.

195 Each plant was genotyped at the S-locus using the PCR-based protocol described in  
196 Llaurens *et al.* (2008). Pairwise dominance interactions between S-alleles of the  
197 heterozygote combinations were either taken from Llaurens *et al.* (2008); Durand *et al.*  
198 (2014); Leducq *et al.* (2014) or were newly determined by controlled pollination assays  
199 following the protocol of Durand *et al.*, (2014). In a few instances, relative dominance  
200 status of the two alleles had not been resolved phenotypically and were inferred from the  
201 phylogeny of *SRK* alleles, which is largely consistent with the dominance hierarchy  
202 (Durand *et al.* 2014). The pairwise dominance interactions between these alleles as  
203 determined by pollen and pistil compatibility phenotypes of heterozygote plants are  
204 reported in Table S3.

## 205 RNA extraction and reverse transcription

206 On each plant, we collected flower buds at four developmental stages: 1) five highly  
207 immature inflorescence extremities (more than 2.5 days before opening, buds below  
208 0.5mm, stages 1-10 in *A. thaliana* according to Smyth *et al.*, 1990); 2) ten immature buds  
209 (2.5 days before opening, between 0.5 and 1mm, approximately stage 11); 3) ten mature  
210 buds (one day before opening, longer than 1mm, approximately stage 12); and 4) ten open  
211 flowers (approximately stages 13-15). These stages were characterized by establishing the  
212 size distribution within each stage and measuring the time to flower opening based on ten  
213 buds. Samples collected were flash-frozen in liquid nitrogen, then stored at  $- 80^{\circ}\text{C}$  before

214 RNA extraction. Tissues were finely ground with a FastPrep-24 5G Benchtop  
215 Homogenizer (MP Biomedicals, Model #6004-500) equipped with Coolprep 24 x 2mL  
216 adapter (6002-528) and FastPrep Lysis Beads & Matrix tube D. Total RNAs were  
217 extracted with the Arcturus “Picopure RNA isolation” kit from Life Science (PN:  
218 KIT0204) according to the manufacturer’s protocol, including a step of incubation with  
219 DNase to remove gDNA contamination. We normalized samples by using 1 mg of total  
220 RNA to perform reverse-transcription (RT) using the RevertAid Fermentas enzyme  
221 following the manufacturer’s instructions.

## 222 Primer design

223 A major challenge to study expression of multiple S-alleles is the very high levels of  
224 nucleotide sequence divergence among them, precluding the possibility of designing qPCR  
225 primers that would amplify all alleles of the allelic series (both for *SRK* and *SCR*). Hence,  
226 we rather designed qPCR primers specifically targeted towards each of the *SCR* and *SRK*  
227 alleles, and for each heterozygote genotype we independently measured expression of both  
228 alleles of each gene. Primers were designed based on genomic sequences from BAC clones  
229 (Goubet *et al.* 2012; Durand *et al.* 2014; Novikova *et al.* 2017), with a length of ~20  
230 nucleotides, a GC content around 50% and a target amplicon size around 150nt (Figure  
231 S1). For *SCR*, we focused on a set of 9 S-alleles. Whenever possible, we placed primers on  
232 either side of the *SCR* intron to identify and discard amplification from residual gDNA.  
233 However, because the coding sequence of the *SCR* gene is short, the number of possible  
234 primers was limited and this was not always possible. In two cases (*SCR01* and *SCR20*),  
235 both primers were thus located within the same exon. For *SRK* alleles, the primers were  
236 also designed on either side of the first intron to avoid genomic contamination (Figure S2).

237 Because no differences in transcript levels were previously observed between dominant  
238 and recessive *SRK* alleles (Suzuki *et al.* 1999; Kusaba *et al.* 2002), and given the effort  
239 required to optimize new qPCR primers, we decided to place more effort on *SCR* and  
240 focused on a more limited number of *SRK* alleles ( $n=5$ ). To obtain relative expression  
241 levels across samples, we used *actin 8* (At1g49240) as a housekeeping gene for  
242 standardization after we verified that the *A. thaliana* and *A. halleri* sequences are identical  
243 at the primer positions (An *et al.* 1996). Primer sequences are reported in Table S4.

#### 244 Quantitative real-time PCR

245 On each cDNA sample, at least three qPCR reactions (referred to below as “technical”  
246 replicates) were performed for *actin 8* and for each of the *S*-alleles contained in the  
247 genotype (one *S*-allele for homozygotes, two *S*-alleles for heterozygotes). The runs were  
248 made on a LightCycler480 (Roche) with iTaq Universal SYBR Green Supermix (Bio-rad,  
249 ref 172-5121). Amplified cDNA was quantified by the number of cycles at which the  
250 fluorescence signal was greater than a defined threshold during the logarithmic phase of  
251 amplification using the LightCycler 480 software release 1.5.0 SP3. The relative transcript  
252 levels are shown after normalisation with actin amplification through the comparative  $2^{-\Delta Ct}$   
253 method (Livak & Schmittgen, 2001). The  $Ct_{SCR}$  and  $Ct_{SRK}$  values of each technical  
254 replicate were normalized relative to the average  $Ct_{actin}$  measure across the three replicates.

#### 255 Validation of qPCR primers at the dilution limits

256 Given the very large nucleotide divergence between alleles of either *SCR* or *SRK*, cross-  
257 amplification is unlikely. However, to formally exclude that possibility, we first performed  
258 cross-amplification experiments by using each pair of *SCR* primers on a set of cDNA  
259 samples that did not contain that target *SCR* allele but instead contained two other *SCR*

260 alleles in various heterozygous genotypic combinations ( $n=7$  on average). In order to  
261 evaluate our ability to measure expression of *SCR* alleles in biological situations where  
262 they are expected to be transcriptionally silenced, we then used a series of limit dilutions to  
263 explore the loss of linearity of the relationship between  $Ct$  and the dilution factor (six to  
264 eight replicates per dilution level). Then we examined the shape of the melting curves to  
265 determine whether our measures at this limit dilution reflected proper PCR amplification or  
266 the formation of primer dimers. Finally, we used water in place of cDNA to evaluate the  
267 formation of primer dimers in complete absence of the target template DNA.

## 268 Expression dynamics and the effect of dominance

269 We used generalized linear mixed models (lme4 package in *R*; Bates *et al.*, 2014) to  
270 decompose  $Ct$  values normalized by the *actin 8* control (as the dependent variable) into the  
271 effects of five explanatory variables. Two of them were treated as fixed effects:  
272 developmental stage (4 categories) and relative dominance of the allele studied in the  
273 genotype (3 categories: recessive, dominant, homozygous). Because expression of the  
274 different *SCR* (and *SRK*) alleles was quantified by different primer pairs with inevitably  
275 different amplification efficiencies,  $Ct$  values cannot be directly compared across alleles  
276 and accordingly we included the identity of *SCR* or *SRK* alleles as random effects.  
277 Biological and clone replicates were also treated as random effects, with clones nested  
278 within biological replicates (Table S5). We visually examined normality of the residuals of  
279 the model under different distributions of  $2^{-\Delta Ct}$ , including Gaussian, Gamma and Gaussian  
280 with logarithmic transformations. We tested whether the different *S*-alleles have different  
281 expression profiles across developmental stages, as suggested by Kusaba *et al.* (2002) for  
282 *SCR* in *A. lyrata*, by using ANOVA to compare nested models in which a random effect

283 for the interaction between the “allele measured” and “stage” effects was either absent  
284 (model 1) or introduced (model 2, Table S5b) in addition to the fixed effect of stage. The  
285 existence of this interaction was tested for *SCR* and *SRK* separately.

## 286 Target features and silencing effect.

287 Expression of *SCR* in heterozygote genotypes in *A. halleri* is controlled by a small RNA-  
288 based regulatory machinery (Durand *et al.* 2014). We then sought to determine how *SCR*  
289 transcript levels were affected by specific features of the small RNA-target interactions  
290 between *S*-alleles. We retrieved sRNA sequencing data from individuals carrying eight of  
291 the nine *S*-alleles considered (S01, S03, S04, S10, S12, S13 and Ah20 from Durand *et al.*  
292 (2014) and S02 from Novikova *et al.* (2017)). No sRNA sequencing data were available  
293 for the last *S*-allele (S29). We used these sRNA sequencing data to determine the complete  
294 set of sRNA molecules uniquely produced by the annotated sRNA precursors of each of  
295 these eight *S*-alleles. To do that, we mapped the sRNA reads to the sRNA precursor  
296 sequences carried by the respective *S*-alleles after excluding those that mapped to other  
297 locations in the closely related *A. lyrata* genome (Durand *et al.* 2014). For each sRNA  
298 produced by a given *S*-allele, we then predicted putative target sites on the *SCR* gene of all  
299 other *S*-alleles including 2kb of genomic nucleotide sequence both upstream and  
300 downstream of *SCR* using a dedicated alignment algorithm and scoring matrix, as  
301 described in Durand *et al.* (2014). Briefly, alignment quality was assessed by a scoring  
302 system based on the addition of positive or negative values for matching nucleotides (+1),  
303 mismatches and gaps (-1), taking into account the non-canonical G:U interaction (-0.5).  
304 For each pair of alleles considered, only the sRNA/target combination with the highest  
305 score was selected for further analysis (Table S6). The analysis was performed regardless

306 of the dominance relationship (i.e. we predicted putative target sites of sRNAs produced by  
307 dominant S-alleles onto recessive S-alleles, and reciprocally from recessive S-alleles onto  
308 dominant S-alleles). Because the mechanisms by which silencing is achieved remain  
309 unclear at this stage, we did not filter these sRNA further in terms of length or identity of  
310 the 5' nucleotide, in line with Durand *et al.* (2014). In the cases where the target with the  
311 highest score was due to a sRNA with non-canonical size (anything but 21 or 24nt), we  
312 also reported the best target score among the set of 21 and 24nt sRNA molecules produced  
313 by the same S-allele (Table S6). We used Akaike Information Criteria (AIC) to compare  
314 how well different base-pairing scores for target site identification predicted the level of  
315 *SCR* expression (and hence the silencing phenomenon), varying the threshold from 14 to  
316 22. Lower values of AIC are associated with a best fit of the model. We then added a new  
317 fixed effect in our basal model to test whether targets at different positions along the *SCR*  
318 gene (5 categories: 5' portion, exons, intron, overlapping the exon-intron boundary or 3'  
319 portion of the gene) are associated with different strengths of silencing. For this analysis,  
320 we included only targets above the threshold identified (score  $\geq$  18).

321 Effective silencing of recessive *SCR* alleles in *Brassica rapa* is dependent upon  
322 combinations of individual sequence mismatches between the *Smi* & *Smi2* small RNAs and  
323 their target sites in the class II alleles (Yasuda *et al.*, 2016), but interaction in this study  
324 relied on raw counts of nucleotide mismatches and were thus not directly comparable to  
325 our results. To determine whether the base-pair requirements for silencing are similar, we  
326 thus reanalysed these interactions using our scoring system to compare the small RNA-  
327 target alignment scores between Brassica and Arabidopsis (Tarutani *et al.*, 2010, Yasuda *et*  
328 *al.*, 2016).



329 Finally, we used the phylogeny in Durand *et al.* (2014) to classify sRNA/target interactions  
330 into “recent” (mir867 and mirS4) and “ancient” (mirS1, mirS2 mirS3, mirS5, mir1887 and  
331 mir4239). Based on this classification, we used a linear regression to compare the  
332 alignment score for recent and ancient sRNAs and tested the hypothesis that interactions  
333 with base-pairing scores above the threshold at which silencing was complete correspond  
334 to recently emerged interactions that have not yet accumulated mismatches.

335

336

## Results

337

### Validation of the qPCR protocol and the allele-specific primers

338 The specificity test confirmed the absence of cross-amplification between alleles, as the  $C_t$   
339 measures for water control and cross amplification were comparably high (around  $C_t=34$ )  
340 and both were higher than the positive controls (median  $C_t=22$ , Figure S3). Overall, serial  
341 dilutions of the template cDNA confirmed linearity of the  $C_t$  measure within the range of  
342 values observed for a given allele across the different conditions examined (Figure S4a).  
343 Because we study a silencing phenomenon, we then explored how signal was lost at the  
344 dilution limits. As expected, linearity started to be lost at very low cDNA concentrations  
345 (in particular for alleles *SCR01*, *SCR02*, *SCR04*, *SCR13* and *SCR20*, Figure S4a), and  
346 examination of melting curves under these conditions indicated the formation of primer  
347 dimers rather than the expected transcripts. Hence, we note that comparing levels of  
348 expression for a given allele between different recessive contexts (*e.g.* when silenced by  
349 different sRNAs) should be challenging, especially for the above-mentioned alleles.

350 Linearity was good for most *SRK* alleles (Figure S4b) except for *SRK12* (data not shown),  
351 so this allele was excluded from further analyses.

### 352 *SCR* and *SRK* expression dynamics across flower development stages

353 In total, we performed 344 RNA extractions and RT-PCR from the 37 different S-locus  
354 genotypes sampled at four developmental stages. For *SCR*, we measured 1,838  $Ct_{SCR}/Ct_{actin}$   
355 expression ratios (*i.e.* an average of 26.9 expression measures per S-allele in each diploid  
356 genotype, Table S1). For *SRK*, we measured 480  $Ct_{SRK}/Ct_{actin}$  ratios (*i.e.* an average of 11.1  
357 expression measures per S-allele in each diploid genotype, Table S2). Distribution of the  
358 residuals of the generalized mixed linear model was closest to normality after log-  
359 transformation of the ratios (Figure S6). As expected, measured expression levels were  
360 more highly repeatable across clones than across biological replicates for a given S-locus  
361 genotype (deviance estimates of 0.40, 1.08, respectively, Table S5a). The deviance  
362 associated with the allele's expression dynamic was higher (deviance = 4.56), although we  
363 note that the technical error was also important (deviance = 6.08, Table S5a). We first  
364 examined the expression dynamics of the different *SCR* alleles. Because recessive *SCR*  
365 alleles were consistently silenced (see below), we isolated the effect of developmental  
366 stages by focusing only on genotypes in which each focal allele was known to be dominant  
367 at the phenotypic level (Figure 1a). Overall, we observed a strong pattern of variation  
368 among stages (F-value: 10.76, *p*-value: 5.7e-5, Table S5c) with high expression of *SCR* in  
369 buds at early developmental stages (<0.5 to 1mm), and low expression in late buds right  
370 before opening and in open flowers. This pattern is consistent with degeneration in these  
371 stages of the anther tapetum, the cellular layer where *SCR* is expected to be expressed. The  
372 expression dynamics of *SRK* was sharply different from that of *SCR*, with monotonously

373 increasing expression in the course of flower development, with lowest expression in  
374 immature buds (<0.5mm) and highest expression in open flowers (Figure 1b, F-value:  
375 4.411,  $p$ -value: 0.007, Table S5h). We found evidence that the expression dynamics varied  
376 across *S*-alleles, not only for *SCR* ( $\text{Chi}^2$ : 308.19,  $p$ -value < 2.2e-16, Table S5b) in line with  
377 Kusaba *et al.*, (2002), but also for *SRK* ( $\text{Chi}^2$ : 6.9103,  $p$ -value 0.00857, Table S5g).

378

### 379 Transcriptional control

380 Based on these results, we averaged  $2^{-\Delta C_t}$  values across <0.5mm to 1mm stages to compare  
381 expression of a given focal *SCR* allele between genotypic contexts where it was either  
382 dominant or recessive relative to the other allele present in the diploid genotype. Of the 54  
383 pairwise interactions for which the dominance phenotype had been firmly established by  
384 controlled crosses and the qPCR assay had been performed for both *SCR* alleles (Table  
385 S3), as many as 51 (94.4%) are associated with strong asymmetries in transcript levels,  
386 with high expression of the dominant *SCR* allele and low expression of the recessive *SCR*  
387 allele (Figure 2). Hence, our expression data were largely consistent with the hypothesis of  
388 transcriptional control of the dominance hierarchy in pollen genotypic combinations. *SCR*  
389 transcripts of the most recessive allele (S1) were only detected in an S1S1 homozygote  
390 genotype, but not in any other genotypic combination. Climbing up the dominance  
391 hierarchy from most recessive to most dominant, expression of *SCR* was detected in an  
392 increasing number of heterozygous combinations, in strong agreement with phenotypic  
393 dominance (Figure 2). At the top of the dominance hierarchy, the two most dominant  
394 alleles, *SCR13* and *SCR20*, were expressed in all heterozygous contexts, including when  
395 they formed a heterozygote combination with one another (S13S20), also as expected

396 given the codominance observed between them at the phenotypic level (Durand *et al.*,  
397 2014). This general rule had a few exceptions however (indicated by arrows on Figure 2).  
398 Specifically, we observed low expression for both *SCR01* and *SCR12* when in  
399 heterozygote combination (*S01S12* genotypes) and for both *SCR10* and *SCR12* in  
400 heterozygote combination (*S10S12* genotype), which is not consistent with the  
401 documented phenotypic dominance of these alleles in pollen (*S12* > *S01* and *S12* > *S10*; see  
402 Table S3). We also detected expression of both *SCR02* and *SCR29* when placed in  
403 heterozygote combination, which might explain the unusual phenotypic data indicating  
404 robust rejection of pollen from this heterozygote genotype on the [S02] tester line, but only  
405 partial compatibility on the [S29] tester line (Table S3). Hence, the dominance interaction  
406 between these two alleles may be partial, both at the transcriptional and phenotypic levels.  
407 Interestingly, these two alleles belong to class III, which in *A. lyrata* tend to show  
408 inconsistent (or leaky) SI responses (Kusaba *et al.* 2001).

409 Overall, in spite of these three exceptions, we observed a striking contrast in transcript  
410 levels for a given allele according to its relative phenotypic dominance status in the  
411 genotype ( $F$ -value = 19.538;  $p$ -value < 2.2e-16, Table S5c), suggesting complete silencing  
412 of recessive alleles. Specifically, we observed an average 145-fold decrease in transcript  
413 abundance in genotypes where a given focal allele was phenotypically recessive as  
414 compared to genotypes in which the same focal allele was dominant. We note that the  
415 silencing was so strong that the  $C_t$  values associated with recessive *SCR* transcripts were  
416 comparable with those of the negative controls (Figure S3) and close to the detection limits  
417 of our method as determined by the break of linearity of the dilution experiment, such that  
418 the magnitude of the calculated fold-change value is probably under-estimated (Figure S1).

419 In strong contrast, we found no significant effect of dominance in pistils on *SRK*  
420 expression (F-value: 6.8884 p-value: 0.068244; Figure 3, Table S5h), confirming the  
421 absence of transcriptional control of dominance for *SRK*.

#### 422 Target features and silencing effect

423 Levels of *SCR* expression of any given focal allele varied sharply with the alignment score  
424 of the “best” target available for the repertoire of canonical sRNAs produced by the other  
425 allele present in the genotype (Figure 4a). Specifically, we observed on average high levels  
426 of *SCR* transcripts when the score of their best predicted target was low, but consistently  
427 low levels of *SCR* transcripts when the score of the best target was high (Figure 4a, Table  
428 S5d). Strikingly, the transition between high expression and low expression was abrupt  
429 (around an alignment score of 18), suggesting a sharp threshold effect rather than a  
430 quantitative model for transcriptional silencing.

431 In two cases, the presence of a target with a high score within the *SCR* gene of the  
432 dominant allele was associated with high relative *SCR* expression (in agreement with the  
433 dominant phenotype established by controlled crosses), confirming the absence of  
434 silencing (target of Ah04mir4239 on *SCR20*, score=20; and target of Ah10mir4239 on  
435 *SCR20*, score =21; Figure 5a) and suggesting that these interactions are not functional.  
436 Examining in detail these two exceptions did not reveal mismatches at the 10-11<sup>th</sup>  
437 nucleotide position, suggesting that mismatches at other positions have rendered these  
438 sRNA-target interactions inactive (Figure 5a). We note that the target of Ah10mir4239  
439 with the highest score is predicted for a sRNA with non-canonical size (25nt), but this  
440 precursor also produces a canonical 24nt isomir with a score above the threshold (score =  
441 20, Table S6). These two sRNAs (Ah04mir4239 and Ah10mir4239) have a 5' nucleotide

442 different from the expected “A” for 24nt sRNAs, possibly suggesting that loading into an  
443 improper AGO protein may have rendered these predicted interactions inactive. Another  
444 exception concerns the observed low score (15.5) for the best match between a sRNA from  
445 the dominant allele Ah04mirS4 and its best putative target at the recessive *SCR03* (Figure  
446 5b). Whether *SCR04* silences *SCR03* through this unusual target or through another elusive  
447 mechanism remains to be discovered.

448 In spite of the generally very low expression of all recessive alleles, we found marginal  
449 evidence that the strength of silencing experienced by a given *SCR* allele varies across  
450 genotypic combinations for a given allele (F-value=2.221,  $p$ -value = 0.0756, Table S5i).  
451 However, there was no evidence that the position of the target site on the measured allele  
452 (promoter; intron; intron-exon boundary; upstream vs. downstream) could explain this  
453 variation (F-value=1.7061,  $p$ -value = 0.1928, TableS5e). We also found no effect of the  
454 inferred age of the miRNA on the mean alignment score (mean= 20.41 and 20.22 for  
455 recent or ancient miRNAs, respectively; F-value: 0.0362;  $p$ -value = 0.8504, Table S5j).  
456 Finally, we compared the alignment scores observed here in Arabidopsis with those in  
457 Brassica for *Smi* & *Smi2* on their *SCR* target sequences. A clear threshold was also  
458 observed, but in Brassica the alignment score threshold distinguishing dominant from  
459 recessive interactions was 16.5 instead of 18 (Table S6), suggesting distinct base-pairing  
460 requirements for effective silencing in these two systems.

461

462

## Discussion

463 Determining the base-pairing requirement for sRNA silencing in plants has remained  
464 challenging because the “rules” used for target prediction have typically been deduced  
465 from observations that conflate distinct microRNA genes and their distinct mRNA targets  
466 over different genes. Moreover, detailed evaluations of the functional consequences of  
467 mismatches have relied on heterologous reporter systems (typically GFP in transient  
468 tobacco assays), hence limiting the scope of the phenotypic consequences that can be  
469 studied. Here, we build upon the inter-allelic regulatory system controlling transcriptional  
470 activity of alleles of the SI system in Arabidopsis revealed in Durand *et al.* (2014), where  
471 multiple sRNAs regulate target sites on alleles of a single gene (*SCR*), and in which we are  
472 able to make a direct link between the sRNA-target interactions, the level of *SCR* transcript  
473 and the encoded phenotype (dominance/recessivity interaction). The first step was to  
474 clarify several aspects of the expression pattern of the genes controlling SI in *A. halleri*,  
475 which was necessary to confirm that the dominance interactions in Arabidopsis involve  
476 transcriptional regulation.

### 477 Expression profile

478 Earlier accounts had suggested that alleles of the allelic series may differ from one another  
479 in their expression profile (Kusaba *et al.*, 2002). In line with Kakizaki *et al.*, (2003),  
480 Suzuki *et al.*, (1999); Schopfer *et al.*, (1999); Takayama *et al.*, (2000) and Shiba *et al.*,  
481 (2002), we found maximal expression of *SCR* in early buds but low or no expression at the  
482 open flower stage. This expression pattern is consistent with in situ hybridization  
483 experiments showing that *SCR* transcripts are localized in the tapetum, a specialized layer  
484 of cells involved in pollen grains coating (Iwano *et al.*, 2003) which undergoes apoptosis

485 and is quickly degraded as the development of pollen grains inside the anther progresses  
486 (Murphy & Ross, 1998; Takayama *et al.*, 2000). We confirmed that differences exist in the  
487 temporal dynamics of expression among alleles, as suggested by Kusaba *et al.* (2002) in *A.*  
488 *lyrata*, possibly as the result of strong sequence divergence of the promotor sequences of  
489 the different *SCR* alleles. Finally, we confirmed that *SCR* and *SRK* have sharply distinct  
490 expression dynamics throughout flower development. Indeed, transcript levels of *SRK*  
491 increased steadily along development and were very low in early buds, consistent with the  
492 observation that SI can be experimentally overcome to obtain selfed progenies by “bud-  
493 pollination” (Llaurens *et al.* 2009).

#### 494 **Generality of the transcriptional control of dominance in Arabidopsis**

495 Based on this clarified transcriptional dynamics, we confirmed the generality of the  
496 transcriptional control of dominance for *SCR*, with as much as 96.3% of the documented  
497 dominance interactions associated with mono-allelic expression of the dominant *SCR* allele  
498 and complete silencing of the recessive *SCR* allele in heterozygote genotypes. Even in the  
499 single heterozygote genotype where in our previous study (Durand *et al.*, 2014) no sRNA  
500 produced by the phenotypically dominant allele was predicted to target the sequence of the  
501 phenotypically recessive *SCR* allele (e.g. S04>S03), transcripts from the recessive *SCR03*  
502 allele were undetected. This suggests either that some functional sRNAs or targets have  
503 remained undetected by previous sequencing and/or by our *in silico* prediction procedures,  
504 or that mechanisms other than sRNAs may cause transcriptional silencing for some S-allele  
505 combinations. Regardless of the underlying cause, the generality of the transcriptional  
506 control of dominance suggests that the simple comparison of transcript levels between the  
507 two alleles in a heterozygote genotype could be used as a first approximation to determine



508 their relative dominance levels. In contrast, we confirmed the absence of transcriptional  
509 control for *SRK*, for which both alleles were consistently expressed at similar levels in all  
510 heterozygote genotypes examined, irrespective of the (pistil) dominance phenotype. For  
511 *SRK*, other dominance mechanisms must therefore be acting, which are yet to be  
512 discovered (*e.g.* Naithani *et al.*, 2007).

### 513 **Variation in the strength of silencing**

514 An important feature of the silencing phenomenon is that the decrease of transcript levels  
515 for recessive *SCR* alleles was very strong in heterozygous genotypes, bringing down  
516 transcript levels below the limits of detection in most cases. This is in line with the  
517 intensity of transcriptional silencing by heterochromatic siRNAs (typically very strong for  
518 transposable element sequences, see Marí-Ordóñez *et al.*, 2013), while post-transcriptional  
519 gene silencing by microRNAs can be more quantitative (Liu *et al.*, 2014). As a result of  
520 this strong decrease of transcript levels, the strength of silencing appeared independent  
521 from the position of the sRNA target along the *SCR* gene (promoter *vs.* intron), although  
522 we note that our power to distinguish among levels of transcripts of recessive alleles,  
523 which were all extremely low, is itself fairly low. It remains to be discovered whether the  
524 different positions of the sRNA targets (Durand *et al.*, 2014) do indeed imply different  
525 transcriptional silencing mechanisms.

### 526 **A simple threshold model for sRNA-based silencing**

527 Based on the many allelic combinations where we could compare the agnostic prediction  
528 of putative target sites with the level of transcriptional silencing, we find that a simple  
529 threshold model for base-pairing between sRNAs and their target sites captures most of the  
530 variation in *SCR* expression in heterozygotes. This result provides a direct experimental

531 validation of the *ad-hoc* criteria used in Durand *et al.*, (2014). However, our results also  
532 indicate that this quantitative threshold is not entirely sufficient to capture the complexity  
533 of targeting interactions. Indeed, in three of the 54 cases tested this simple threshold model  
534 would inappropriately predict targeting of a dominant *SCR* allele by a sRNAs from a more  
535 recessive allele, yet the dominant *SCR* allele was expressed at normal levels with no sign  
536 of silencing in these heterozygote genotypes (Figure 5a). The targeting interaction may be  
537 abolished either by defects in the sRNA itself (e.g. for Ah04mir4239 the 5' nucleotide is a  
538 G, while the majority of functional 24 nt small RNA molecules end with a 5'A, which may  
539 interfere with loading in the appropriate AGO protein). Alternatively, the targeting  
540 interactions may be abolished by the position of the mismatches (at position 14 and 22 of  
541 the Ah10mir4239 and at position 13 and 21 of the Ah04mir4239, both on *SCR20*).  
542 Similarly, a single mismatch at position 10 in the *Smi* interaction in Brassica (Tarutani *et*  
543 *al.*, 2010) and in other microRNA-targets interactions (Franco-Zorrilla *et al.*, 2007) was  
544 shown to result in loss of function of the interaction (Table S6). Interestingly, quantitative  
545 differences may exist between Arabidopsis and Brassica, as the experimentally validated  
546 targets in Brassica (Tarutani *et al.*, 2010; Yasuda *et al.*, 2016) correspond to base-pairing  
547 threshold below the one that we find in Arabidopsis (*i.e.* a target score of 16.5 seems  
548 sufficient for silencing in Brassica *vs.* 18 in Arabidopsis). For Brassica, both class I and  
549 class II alleles have *Smi*, but a mismatch at the 10<sup>th</sup> position was proposed to explain why  
550 the class II *Smi* is not functional. Here, we show that this mismatch drives the alignment  
551 score below the 16.5 threshold and could be sufficient to explain the loss of function,  
552 regardless of its position. Overall, although these small RNAs achieve their function in a  
553 way that may be sharply different from classical microRNAs (DNA methylation *vs.*

554 mRNA cleavage), our results suggest that the sRNA-target complementarity rules for  
555 silencing in both cases are qualitatively consistent (Liu *et al.*, 2014). Better understanding  
556 the molecular pathway by which these sRNAs epigenetically silence their target gene  
557 (*SCR*) will now be key to determine whether this threshold model can be generalized to  
558 more classical siRNAs found across the genome, as evidence is still missing for such  
559 classes of sRNAs.

### 560 **Implications for the evolution of the dominance hierarchy**

561 The existence of a threshold model has important implications for how the dominance  
562 hierarchy can evolve. In fact, our model suggests that a single SNP can be sufficient to turn  
563 a codominance interaction into a dominance interaction (and vice-versa), making this a  
564 relatively trivial molecular event. This is actually what Yasuda *et al.*, (2016) observed in *B.*  
565 *rapa*, where the combination of single SNPs at the sRNA *Smi2* and its *SCR* target  
566 sequences resulted in a linear dominance hierarchy among the four class II S-alleles found  
567 in that species. Strikingly, in some cases, we observed base pairing at sRNA-target  
568 interactions with very high alignment scores (up to 22), *i.e.* above the threshold at which  
569 transcriptional silencing was already complete (score =18). Under our simple threshold  
570 model, such interactions are not expected since complete silencing is already achieved at  
571 the threshold, and no further fitness gain is therefore to be expected by acquiring a more  
572 perfect target. A first possibility is that these interactions reflect the recent emergence of  
573 these silencing interactions. In fact, one of the models for the emergence of new  
574 microRNAs in plant genomes involves a partial duplication of the target gene, hence  
575 entailing perfect complementarity at the time of origin that becomes degraded over time by  
576 the accumulation of mutations (Allen *et al.*, 2004). Under this scenario, the higher-than-

577 expected levels of sRNA-target complementarity could reflect the recent origin of these  
578 sRNAs but we found no evidence of a difference in alignment score for young *vs.* old  
579 sRNA precursors. A second possibility is that selection for developmental robustness is  
580 acting to prevent the phenotypic switch from mono- to bi-allelic expression of *SCR*  
581 (especially during stress events, Boukhibar & Barkoulas, 2016) that could be devastating  
582 for the plant reproductive fitness (Llaurens *et al.* 2009). Indeed, we observed strong  
583 variation in overall *SCR* expression when the sRNA target score of the companion allele is  
584 below the threshold in the benign greenhouse conditions under which we grew our plants,  
585 and it is possible that under stress conditions the epigenetic machinery may be less  
586 efficient, hence requiring stronger base-pairing to achieve proper silencing. Finally, a third  
587 possibility is that sRNA-target complementarity above the threshold reflects the pleiotropic  
588 constraint of having a given sRNA from a dominant allele control silencing of the  
589 complete set of target sequences from the multiple recessive alleles segregating, and  
590 reciprocally of having a given *SCR* target in a recessive allele maintaining molecular match  
591 with a given sRNA distributed among a variety of dominant alleles. Comparing the  
592 complementarity score of sRNA/target interactions among sRNAs or targets that contribute  
593 to high versus low numbers of dominance/recessive interactions will now require a more  
594 complete depiction of the sRNA-target regulatory network among the larger set of S-alleles  
595 segregating in natural populations.

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597

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607

## Author Contribution

608 NB, SS, SB, ACH performed the molecular biology experiments. CP and ES obtained and  
609 took care of the plants. SS, IFL and XV provided advice on the experimental strategy and  
610 interpretations. NB performed the statistical analyses. VC supervised the work. NB and  
611 VC wrote the manuscript.

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753

## 754 **Figure legends**

755 **Figure 1:** Expression dynamics of **a. SCR** and **b. SRK** during flower development, from  
756 early buds (<0.5mm) to open flowers. For *SCR*, only genotypes in which a given allele was  
757 either dominant or co-dominant were included (recessive *SCR* alleles were strongly  
758 silenced at all stages and were therefore not informative here). For each allele,  $2^{-\Delta Ct}$  values  
759 were normalized relative to the developmental stage with the highest expression. For each  
760 stage, the thick horizontal line represents the median, the box represents the 1<sup>st</sup> and 3<sup>rd</sup>  
761 quartiles. The upper whisker extends from the hinge to the largest value no further than 1.5  
762 \* Inter Quartile Range from the hinge (or distance between the first and third quartiles).

763 The lower whisker extends from the hinge to the smallest value at most  $1.5 * IQR$  of the  
764 hinge and the black dots represents outlier values.

765 **Figure 2:** Expression of individual *SCR* alleles in different genotypic contexts. Pollen  
766 dominance status of the S-allele whose expression is measured relative to the other allele in  
767 the genotype as determined by controlled crosses are represented by different letters (**D**:  
768 dominant; **C**: codominant; **R**: recessive; **U**: unknown; **H**: Homozygote, Table S3). In a few  
769 instances, relative dominance status of the two alleles had not been resolved  
770 phenotypically and were inferred from the phylogeny (marked by asterisks). Thick  
771 horizontal bars represent the median of  $2^{-\Delta Ct}$  values, 1<sup>st</sup> and 3<sup>rd</sup> quartile are indicated by the  
772 upper and lower limits of the boxes. The upper whisker extends from the hinge to the  
773 largest value no further than  $1.5 * Inter\ Quartile\ Range$  from the hinge (or distance  
774 between the first and third quartiles). The lower whisker extends from the hinge to the  
775 smallest value at most  $1.5 * IQR$  of the hinge and the black dots represents outlier values.  
776 We normalized values relative to the highest median across heterozygous combinations  
777 within each panel. Alleles are ordered from left to right and from top to bottom according  
778 to their position along the dominance hierarchy, with SCR01 the most recessive and  
779 SCR13 and SCR20 the most dominant alleles. Under a model of transcriptional control of  
780 dominance, high expression is expected when a given allele is either dominant or co-  
781 dominant and low expression when it is recessive. Exceptions to this model are marked by  
782 black vertical arrows and discussed in the text. “Na” marks homozygote or heterozygote  
783 genotypes that were not available.

784 **Figure 3:** Expression of individual *SRK* alleles in different genotypic contexts. Putative  
785 pistil dominance status of the S-allele whose expression is measured relative to the other

786 allele in the genotype is represented by different letters (**D**: dominant; **R**: recessive; **U**:  
787 unknown; **H**: Homozygote). Note that the pistil dominance hierarchy of the S-allele have  
788 been less precisely determined than the pollen hierarchy, and so many of the pairwise  
789 dominance interactions were indirectly inferred from the phylogenetic relationships (and  
790 marked by an asterisk) rather than directly measured phenotypically. Thick horizontal bars  
791 represent the median of  $2^{-\Delta C_t}$  values, 1<sup>st</sup> and 3<sup>rd</sup> quartile are indicated by the upper and  
792 lower limits of the boxes. The upper whisker extends from the hinge to the largest value no  
793 further than  $1.5 * \text{Inter Quartile Range}$  from the hinge (or distance between the first and  
794 third quartiles). The lower whisker extends from the hinge to the smallest value at most  $1.5$   
795  $* \text{IQR}$  of the hinge and the black dots represents outlier values.. We normalized the values  
796 for each allele relative to the higher median across heterozygous combination. We  
797 normalized values relative to the highest median across heterozygous combinations within  
798 each panel. Alleles are ordered from left to right and from top to bottom according to their  
799 position in the pistil dominance hierarchy, with SRK01 the most recessive and SRK04 the  
800 most dominant allele in our sample, based on the phenotypic determination in Llaurens *et*  
801 *al.* (2008).

802 **Figure 4:** Base-pairing requirements for the transcriptional control of *SCR* alleles by  
803 sRNAs suggest a threshold model. **a.** Relative expression of *SCR* alleles as a function of  
804 the alignment score of the “best” interaction between the focal allele (including 2kb of  
805 sequence upstream and downstream of *SCR*) and the population of sRNAs produced by  
806 sRNA precursors of the other allele in the genotype. For each allele, expression was  
807 normalized relative to the genotype in which the  $2^{-\Delta C_t}$  value was highest. Dots are coloured  
808 according to the dominance status of the focal *SCR* allele in each genotypic context (black:

809 dominant; white: recessive; grey: undetermined). The black line corresponds to a local  
810 regression obtained by a smooth function (loess function, span=0.5) in the ggplot2 package  
811 (Wickham, 2009) and the grey area covers the 95% confidence interval. Vertical arrows  
812 point to observations that do not fit the threshold model of transcriptional control and are  
813 represented individually on Figure 5. **b.** Barplots of the Akaike Information Criteria (AIC)  
814 quantifying the fit of the generalized linear model for different target alignment scores  
815 used to define functional targets. Lower AIC values indicate a better fit.

816 **Figure 5:** Predicted sRNA/target interactions that do not fit with the documented  
817 dominance phenotype or the measured expression. For each alignment, the sequence on top  
818 is the sRNA and the bottom sequence is the best predicted target site on the *SCR* gene  
819 sequence (including 2kb of sequence upstream and downstream of *SCR*). **a.** sRNA targets  
820 with a score above 18, while the S-allele producing the sRNA is phenotypically recessive  
821 over the S-allele containing the *SCR* sequence. **b.** sRNA target with a score below 18,  
822 while the S-allele producing the sRNA (S04) is phenotypically dominant over the S-allele  
823 containing the *SCR* sequence and transcript levels of the *SCR03* allele is accordingly very  
824 low. This is the best target we could identify on SCR03 for sRNAs produced by S04.

825

## 826 **Supplementary figures**

827 **Figure S1.** Position of the *SCR* qPCR primers. Each primer is represented by a black  
828 arrow, according to its relative position to the gene. Positions indicated correspond to base  
829 pairs from the start codon on the cDNA.

830 **Figure S2.** Position of the *SRK* qPCR primers. Each primer is represented by a black  
831 arrow, according to its relative position to the gene. Positions indicated correspond to base  
832 pairs from the start codon on the cDNA.

833 **Figure S3.** Validation of the *SCR* qPCR primers. “Positive control”: amplification assay  
834 with primers for *SCR* alleles that are expressed in the cDNA used. “Cross Amplification”:  
835 amplification assay with primers for *SCR* alleles that are different from the ones in the  
836 cDNA used. “Water” : amplification assay with primers but water instead of cDNA.  
837 “Recessive”: amplification with primers for the phenotypically recessive *SCR* allele in the  
838 cDNA (mean *Ct* values of the biological and technical replicates). “Dominant”:  
839 amplification with primers for the phenotypically dominant *SCR* allele in the cDNA (mean  
840 *Ct* values of the biological and technical replicates). Thick horizontal bars represent the  
841 median of  $2^{-\Delta Ct}$  values, 1<sup>st</sup> and 3<sup>rd</sup> quartile are indicated by the upper and lower limits of  
842 the. The upper whisker extends from the hinge to the largest value no further than  $1.5 * IQR$   
843 Inter Quartile Range from the hinge (or distance between the first and third quartiles). The  
844 lower whisker extends from the hinge to the smallest value at most  $1.5 * IQR$  of the hinge  
845 and the black dots represents outlier values.

846 **Figure S4:** qPCR amplification (non-transformed *Ct* values) in serial dilutions for each  
847 *SCR* (a) and *SRK* (b) allele. Solid lines are the linear regressions over all *Ct* values. Dashed  
848 lines are linear regressions excluding the highest dilution level.

849 **Figure S5.** Expression of individual *SCR* alleles in different genotypic contexts,  
850 representing each biological and clone replicate separately. Symbols on top of the boxes  
851 indicate measures from identical clone replicates. See legend of Figure 2 for a full  
852 description.



853 **Figure S6.** Generalized linear mixed model used to test the effect of developmental stage  
854 and dominance status on the expression of *SCR* alleles ( $C_t$  values). The distribution shows  
855 that the residues of the full model are approximately normally distributed when taking  
856 allele identity, developmental stage and dominance status into account and using a  
857 logarithmic transformation of the  $C_{tSCR} / C_{tactin}$  ratios.

858 **Table S1.** *SCR* samples analysed for each S-locus genotype, showing the number of  
859 biological and clone replicates over the four developmental stages sampled. “Allele 1”  
860 refers to the first allele noted in the genotype (for example in the S1S2 genotype, “allele 1”  
861 is S1 and “allele 2” is S2).

862 **Table S2:** *SRK* samples analysed for each S-locus genotype, showing the number of  
863 biological and clone replicates over the four developmental stages sampled. The alleles are  
864 named accordingly to the Table S1.

865 **Table S3:** Dominance relationships between alleles from the different genotypes included  
866 in this study as determined by controlled crosses.

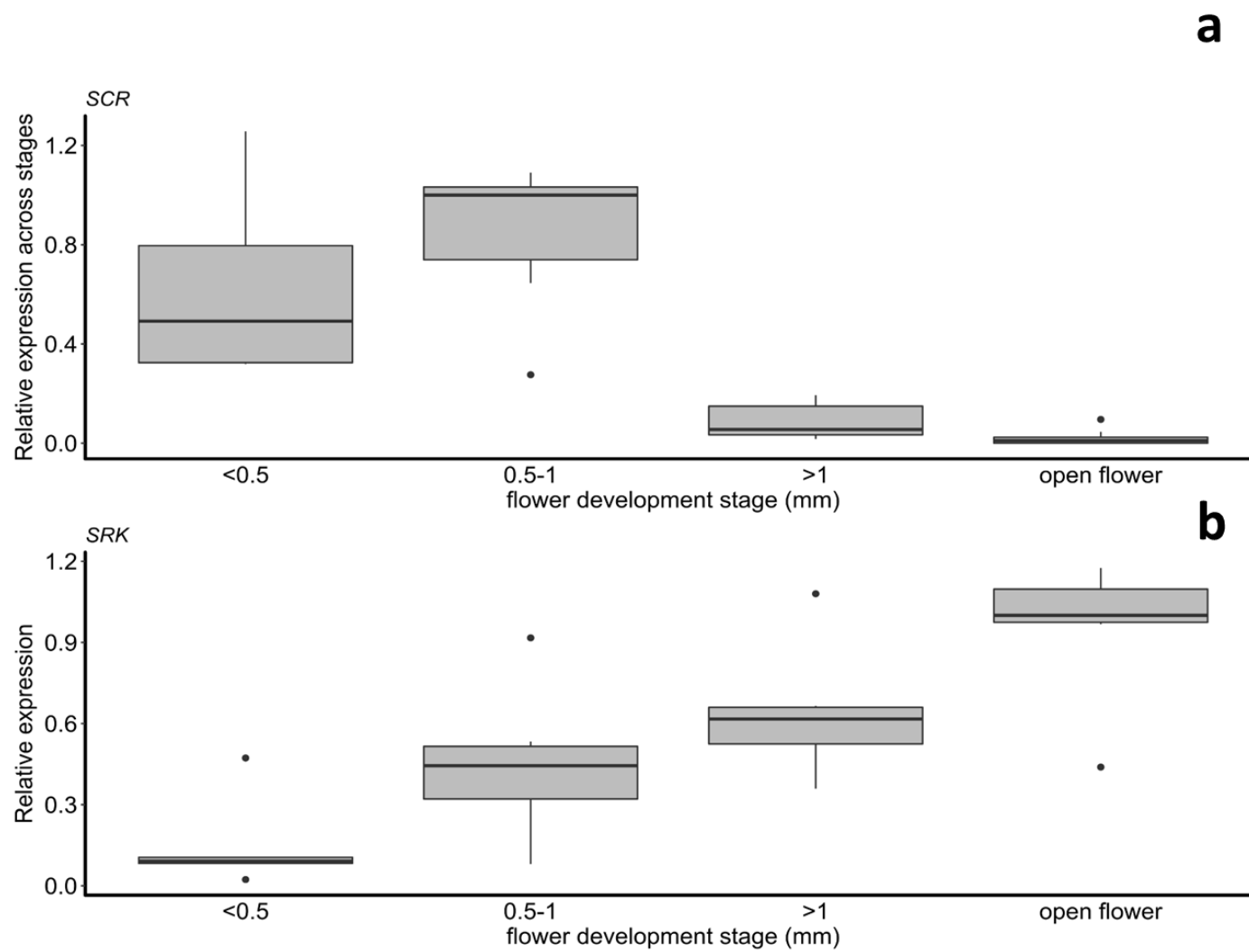
867 **Table S4:** qPCR primer sequences for each *SCR* and *SRK* alleles studied.

868 **Table S5:** Detailed results from the generalized linear mixed models. **a.** Decomposition of  
869 the sources of variance across allele identity and the hierarchical levels biological, clones  
870 and technical replicates for *SCR*. **b.** Test of the variation of expression dynamic across *SCR*  
871 alleles. **c.** Test of the dominance and stage effects on *SCR* transcript levels, showing a  
872 significant interaction. **d.** Comparison of the fit of the model under different base-pairing  
873 score thresholds. **e.** Test of the effect of the position of the target on the strength of  
874 silencing. **f.** Decomposition of the source of variance across the technical replicates and the

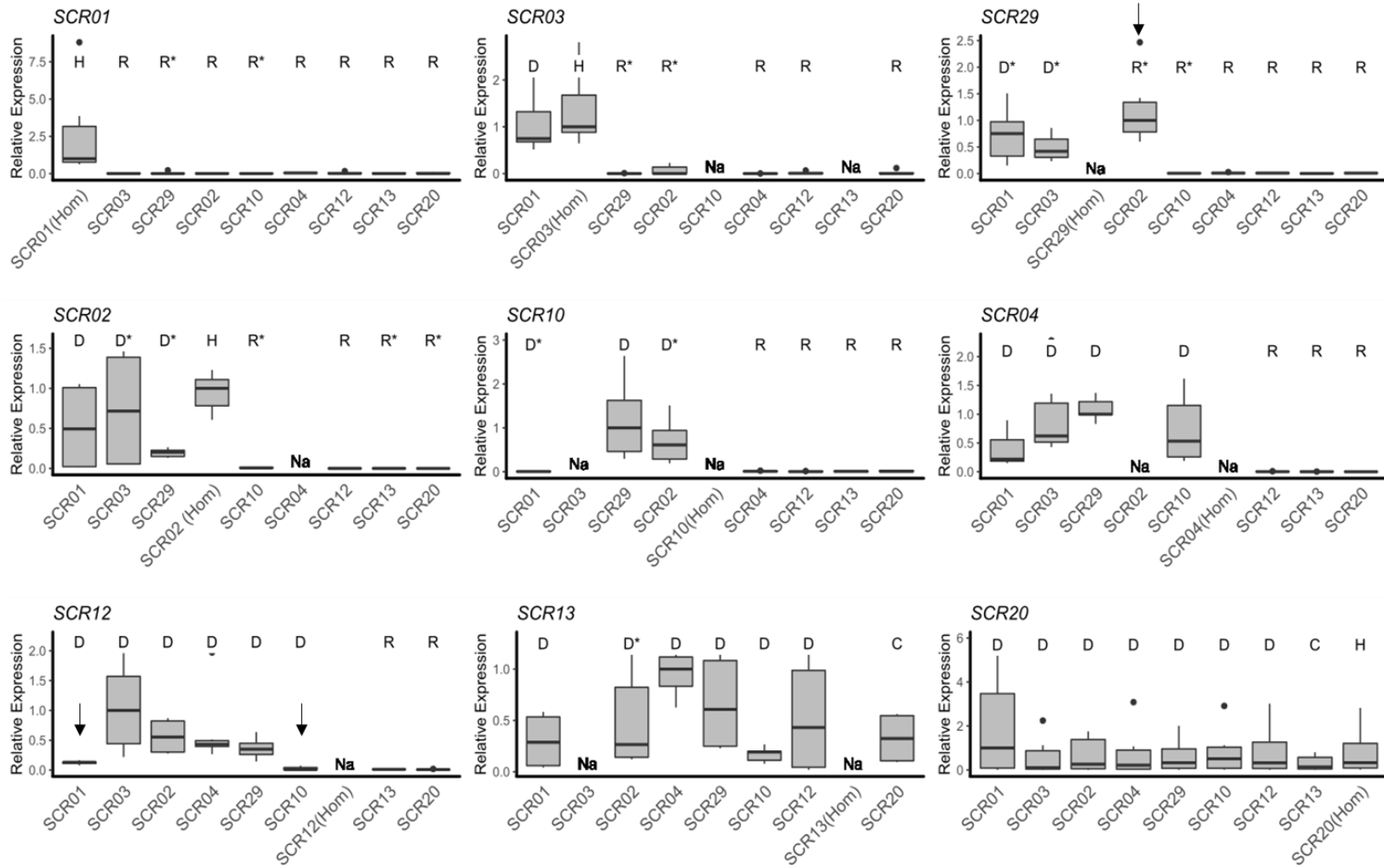
875 allele identity for *SRK*. **g.** Test of the variation of expression dynamic across *SRK* alleles.  
876 **h.** Test of the effect of stage and dominance on *SRK* transcript levels. **i.** Test of the effect  
877 of the identity of the companion allele on *SCR* transcript levels. **j:** Test of the effect of age  
878 on alignment score above the threshold of 18.

879 **Table S6:** sRNA and *SCR* target identified as the best match for every pair of S-alleles.  
880  $Ct_{SCR}/Ct_{actin}$  ratios are given for the target S-allele in the interaction and is calculated as the  
881 mean value across the two earliest developmental stages (see Figure 1). The positions of  
882 the targets are given relative to the beginning of the nearest exon of *SCR* for targets  
883 upstream from the gene or in the intron, and relative to the stop codon for downstream  
884 targets. R: Recessive; D: dominant as phenotypically determined by controlled crosses; H:  
885 homozygote; R\* or D\*: dominance as indirectly inferred from the phylogeny of S-alleles.  
886 The size of the canonical (21 or 24nt-long) isomiR with the highest targeting score is given  
887 in parentheses. The targeting score of the best canonical isomiR is given in parentheses).

888

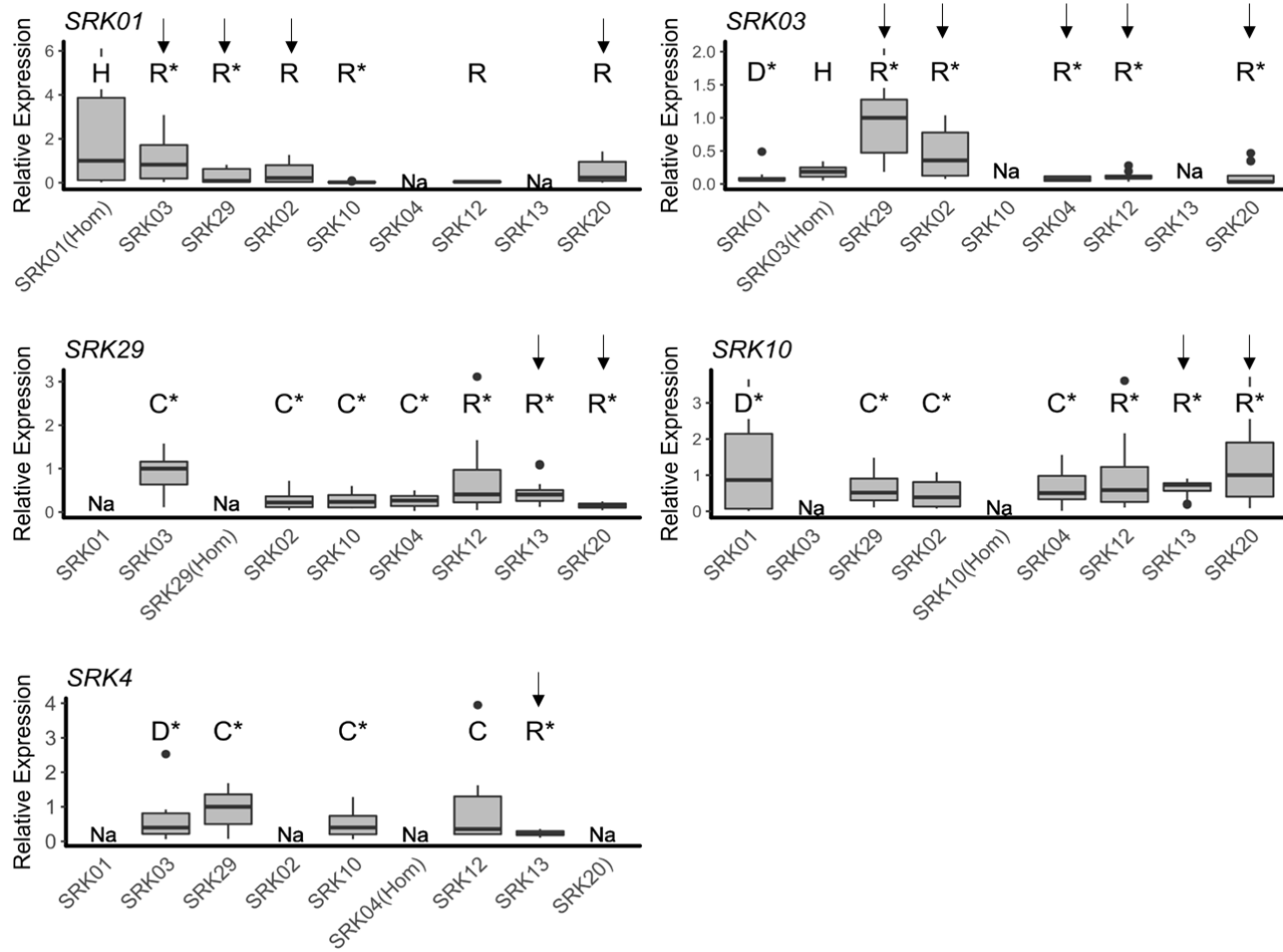


891 Figure 2



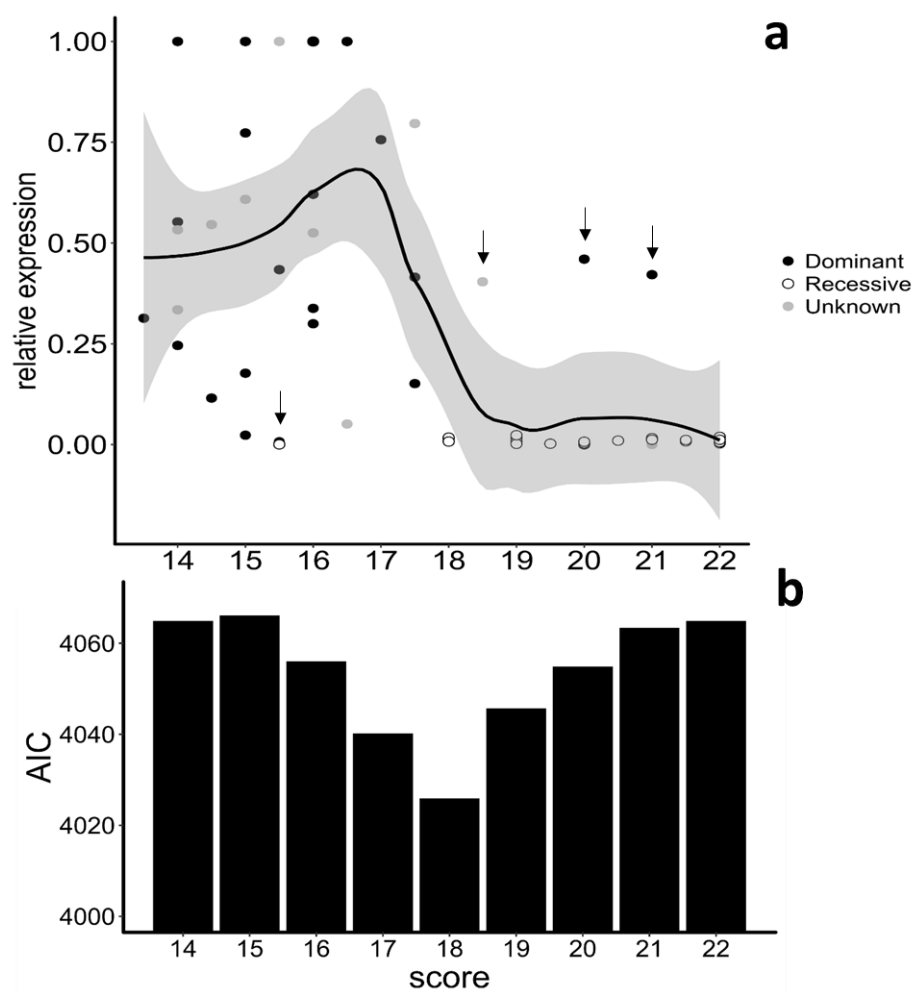
892

893 Figure 3



894

895 Figure4



896

897 Figure5

**a**

```
                20    15    10    5    1
S04 Mir4239  3' CCUCGUACACCUUUUAUUGCCUUUG 5'
              |||x|||||||x|||||||
S20 target   5' GGAACATGTGGCAATAACGGAAAC 3'
Score = 20
```

```
                25    20    15    10    5    1
S10 Mir4239  3' CCUCGUACACCUUUUAUUGCCUUUGU 5'
              |||x|||||||x|||||||
S20 target   5' GGAACATGTGGCAATAACGGAAACA 3'
Score = 21
```

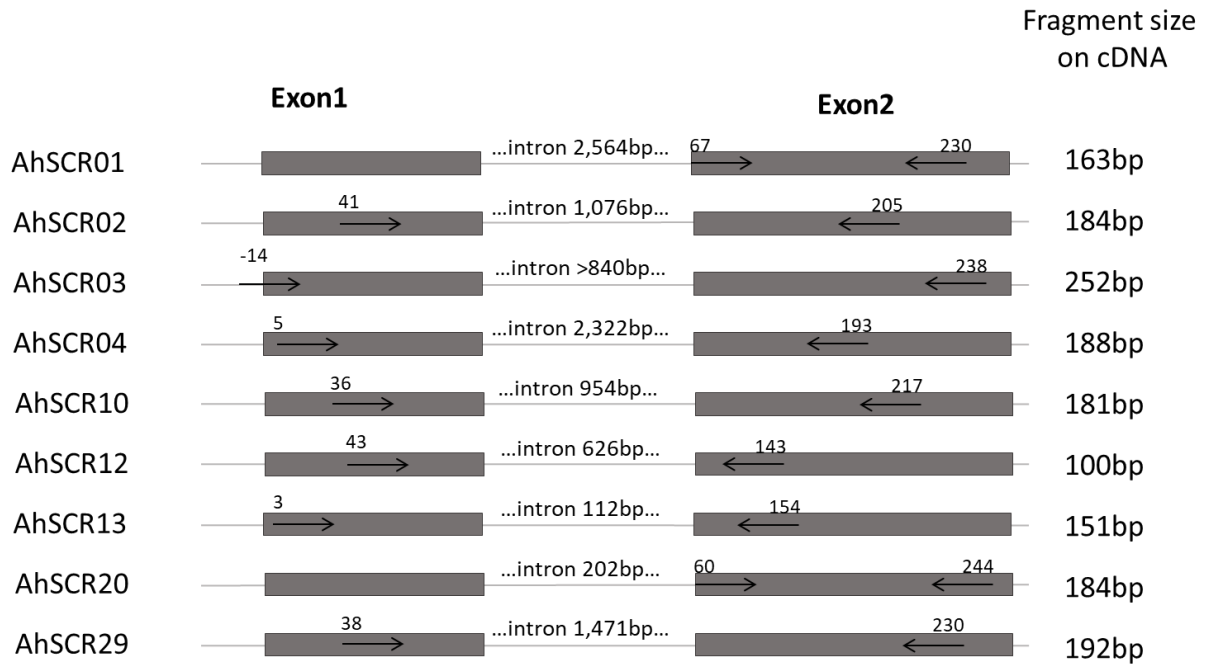
**b**

```
                20    15    10    5    1
S04 MirS4    3' GUAUGAUUCUUGUUAGAUUCA 5'
              ~~~|||||||o|||||||~
S03 target   5' ACTACTAAGAATAATCTAAGA 3'
Score = 15,5
```

898

899

**Figure S1**

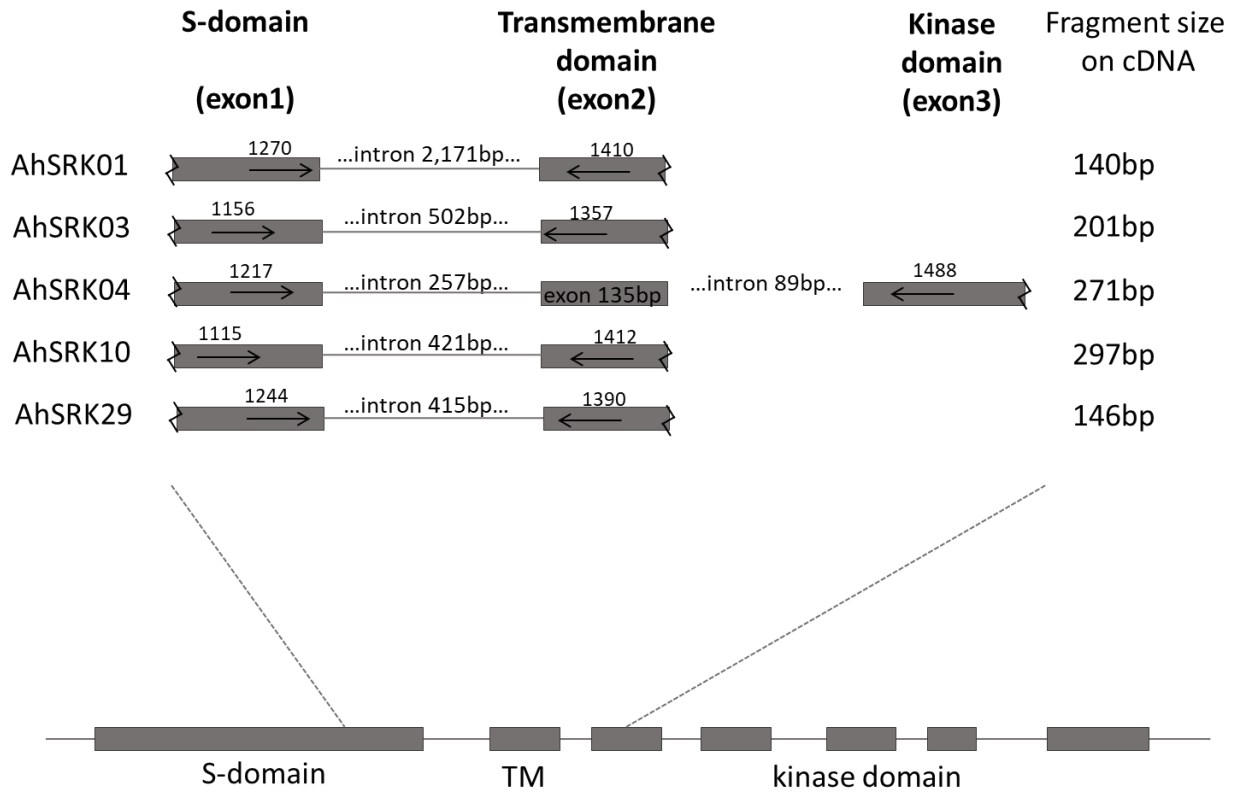


Positions indicated correspond to base pairs from the start codon on the cDNA

900  
901

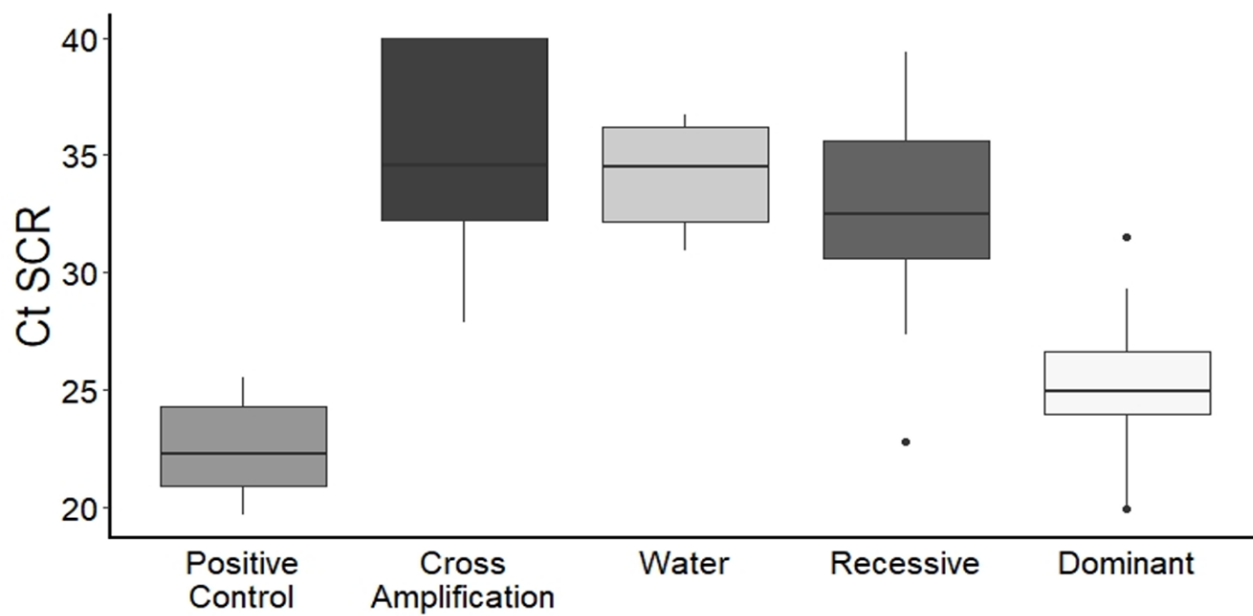


**Figure S2**



902  
903

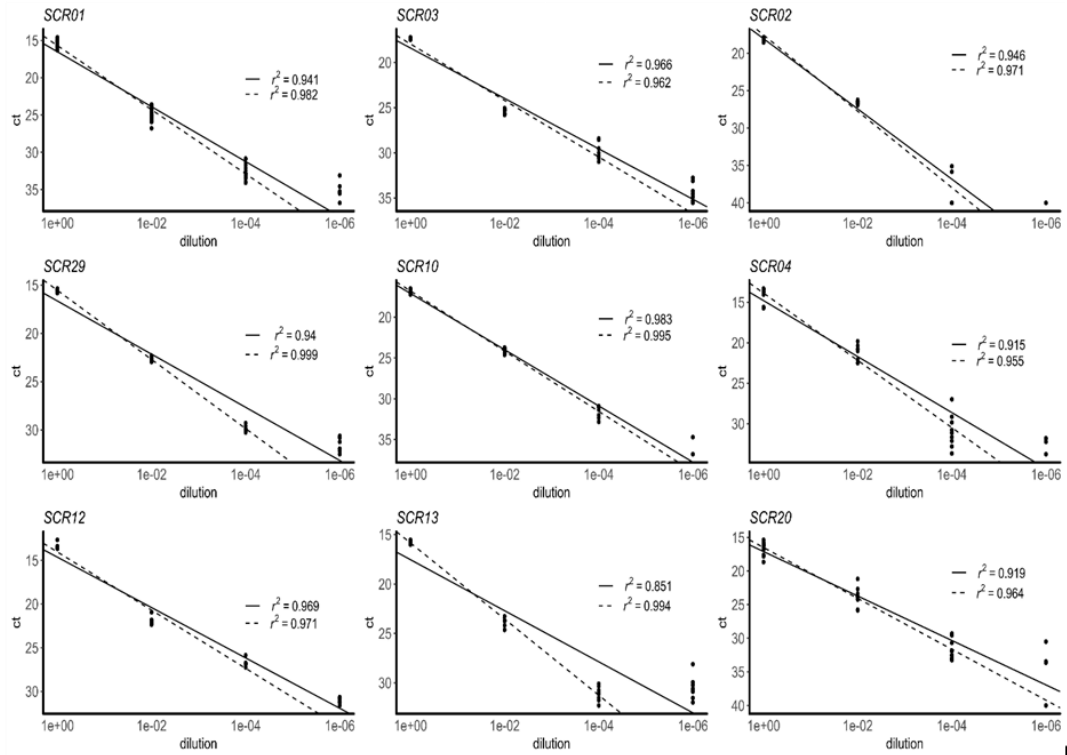
Figure S3



904  
905

Figure S4

a



b

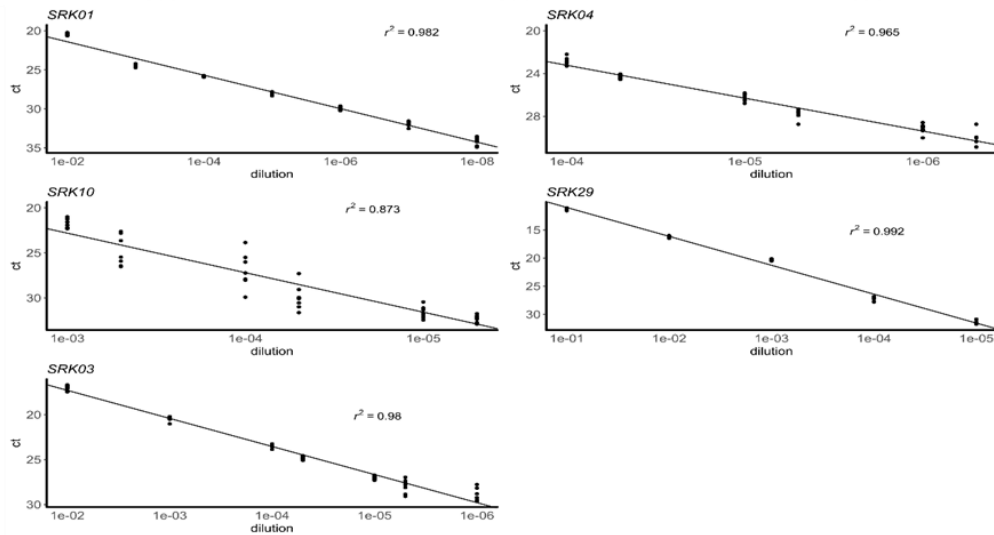
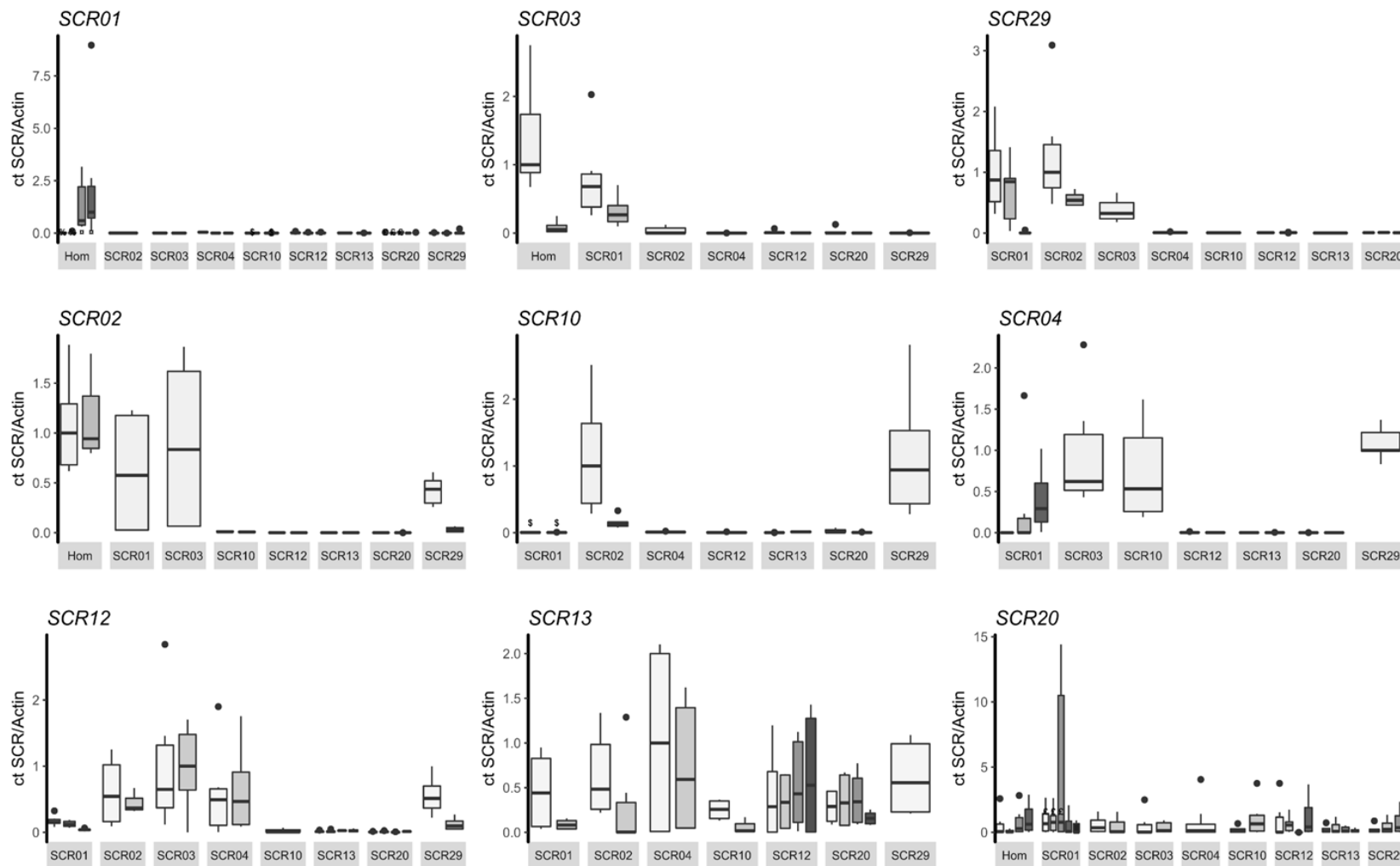
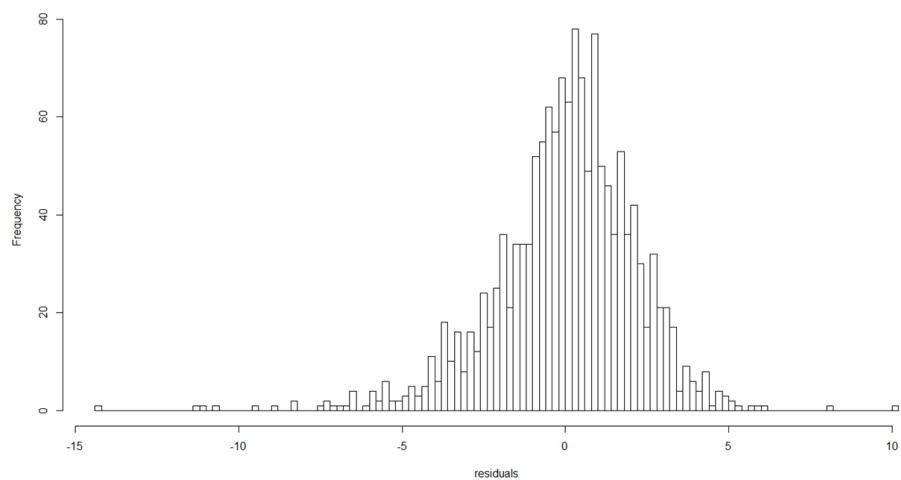


Figure S5



**Figure S6**

```
lmer(log(s1$Ct_SCR.actine) ~  
(1|allele_measured:stage)+stage*dom_phenotype+  
(1|replicatBiol_genotype/replicat_Techclone), na.action=na.omit)
```



908