Title: *Cis*-regulatory evolution spotlights species differences in the adaptive potential of gene expression plasticity

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Abstract

Phenotypic plasticity is the variation in phenotype that a single genotype can produce in different environments and, as such, is an important component of individual fitness. However, whether the effect of new mutations, and hence evolution, depends on the direction of plasticity remains controversial. Here, we identify the *cis*-acting modifications that have reshaped gene expression in response to dehydration stress in three Arabidopsis species. Our study shows that the direction of effects of most *cis*-regulatory variants differentiating the response between *A. thaliana* and the sister species *A. lyrata* and *A. halleri* depends on the direction of pre-existing plasticity in gene expression. A comparison of the rate of *cis*-acting variant accumulation in each lineage indicates that the selective forces driving adaptive evolution in gene expression favors regulatory changes that magnify the stress response in *A. lyrata.* The evolutionary constraints measured on the amino-acid sequence of these genes support this interpretation. In contrast, regulatory changes that mitigate the plastic response to stress evolved more frequently in *A. halleri*. Our results demonstrate that pre-existing plasticity can be a stepping stone for adaptation, but its selective remodeling differs between lineages.

Introduction

Phenotypic plasticity provides most lineages with the ability to alter their development or their physiology in response to the environment [1,2]. The evolution of plastic traits has been proposed to follow one of two major models, depending on whether plasticity is adaptive or maladaptive in novel environments[3–8]. If the phenotypic state reached after activation of a plastic response (plastic state) is more adaptive than the basal phenotypic state, plasticity will promote survival. In this case, directional selection will favor mutations that move the mean phenotype towards the plastic state, via changes in the basal state or in the magnitude of the plastic response[4,9,10]. This process has been called the "Baldwin effect" [4,9,10]. If the plastic state is optimal, novel alleles will be selected that prevent the phenotype from returning to its basal state. This particular "Baldwin effect" has been described as "genetic assimilation". It is predicted to help canalize the phenotype around its new optimal state, and was experimentally documented by Waddington[6–8,11]. Prominent examples have since confirmed that adaptive plasticity can provide a stepping stone for the emergence of novel genetic adaptations[12–15].

If instead the phenotype determined by a plastic reaction decreases fitness, mutations will be selected that prevent or even oppose its activation[3,16,17]. By comparing the direction of genetic change with the direction of the plastic reaction over a large number of transcripts, transcriptome studies have revealed that evolution in a novel environment often opposes the pre-existing ancestral plastic reaction[18–24]. As a result, the process of correcting maladaptive plasticity has been proposed to occur more frequently than the enhancement of plastic phenotypes via the above-mentioned "Baldwin effect" [5,18–24]. Yet, relying on expression levels alone to identify the general rules that govern how pre-existing plasticity influences the evolution of plastic reactions in a novel environment can be misleading. A single master regulator, for example, can change the expression of many genes in the same direction, even in the absence of a selective force [25]. In addition, new mutations can erode pre-existing plastic reactions, if selection is relaxed[29–33], and mutations can have functional effects whose size or direction is not uniformly distributed[26–28]. To unravel the general rules governing the evolution of transcriptional plasticity and determine whether plasticity itself directs adaptive evolution, rates of evolution must be compared among lineages to distinguish the action of natural selection from the random accumulation of new regulatory mutations.

62

63 To this aim, we investigate how *cis*-acting modifications have shaped the divergence in gene
64 expression of the sister species *A. lyrata* and *A. halleri* in reaction to stress triggered by acute
65 dehydration. The ecology of these species predicts that the reaction to dehydration stress
66 has the most relevance in *A. lyrata*, which has the highest tolerance to dehydration[34]. In
67 contrast, the reaction of *A. halleri* to stress is expected to be less relevant, because this
68 species grows in highly competitive environments, and prioritizes its growth over its stress
69 response[34]. We identify a total of 6360 and 6780 *cis*-acting regulatory variants in *A. lyrata*
70 and *A. halleri,* respectively, that differentiate the stress response of these species from that
71 of their common outgroup species, *A. thaliana*. We compared the direction of effects of
72 these variants with the estimated direction of pre-existing plasticity and quantified their
73 relative rate of evolution in each lineage. We conclude that natural selection promoted
74 increased expression plasticity in response to dehydration stress in *A. lyrata*, whereas
75 mutations decreasing stress plasticity were more frequent in *A. halleri*.

76

77 **Results and Discussion**

78 To determine how gene expression plasticity has changed between species, we
79 monitored alterations in gene expression in *A. lyrata*, *A. halleri,* the outgroup *A. thaliana*
80 and their F1 hybrids in response to dehydration stress. This involved sequencing the
81 transcriptome at six time points between 0 and 24 hours, replicated four times (Fig. S1).
82 Compared to *A. halleri* and *A. thaliana*, we confirmed that the drought-adapted species
83 *A. lyrata* had a higher survival rate to acute stress (Fig. S2). In this species, we therefore
84 expect that plastic reactions in gene expression will have been selected to restore
85 homeostasis, whereas maladaptive responses that manifest as physiological and cellular
86 stress will have been disfavored [35,36]. A principal component analysis showed that
87 samples grouped by species and by the duration of exposure to dehydration (Fig. S3).
88 We detected marked differences in the stress reactions of each species, both in initial
89 expression levels and in the slope of the response (Fig. S4). Among the genes whose
90 expression was increased or decreased in response to dehydration (plastic genes), we
91 found that, depending on the pair of species compared, between 39.5-57.9%) (Table S1)
92 showed a significant difference in initial expression, i.e. before the onset of dehydration
93 stress. In addition, between 22.7 and 37.4% (depending on the pair of species

compared) of the plastic genes showed a significant change in the response slope to dehydration (false discovery rate – FDR- <0.05, Table S1).

Basal expression evolution depends on the direction of ancestral plasticity

In the vigorously growing unstressed plants sampled just before initiation of the dehydration treatment, we assume that gene expression reflects the basal expression level. The pattern of up- or down-regulation of gene expression in response to dehydration stress was strongly correlated between species pairs. After 6h of dehydration, for example, 90% of genes that changed expression in *A. thaliana* had also changed expression in the same direction in the other two species (Fig. S4, S5, Fig. 1). We thus assume that the direction of gene expression plasticity in *A. thaliana* in response to dehydration is a proxy for the direction of plasticity that existed in the common ancestor of the three species. If the direction in which basal expression levels evolved is independent of such ancestral plasticity, we expect that the direction of interspecific differences in expression and the direction of plasticity will be independent. To test this, we determined the genes in *A. lyrata* and *A. halleri*, whose basal expression level differed significantly from that of *A. thaliana* (there were 3262 and 4634 of these, respectively, FDR<0.05, Table S2). Among these, we identified those genes whose basal expression differed from *A thaliana* in the same direction as the ancestral plastic reaction to dehydration and called such genetic changes in "orthoplastic" (see Fig. S1, Fig. 1A). As discussed by Crispo (2007), this term is consistent with the definition proposed by Baldwin (1896). Genetic changes in the opposite direction to the ancestral plasticity we called "paraplastic" (see Fig. 1A). In the absence of any direct observation of the common ancestor of the species, we note that orthoplastic basal regulatory changes are defined for a given lineage, because they are *de facto* paraplastic in the species it is compared to and *vice-versa*. This terminology thus helps describe the evolutionary change in a given species. We observed that a majority of genes in *A. lyrata* (1975/3263, 60.5%, Fig. 1C) had a basal expression level that was different from *A. thaliana* in a direction orthoplastic to the direction of ancestral plasticity (Fig. 1D, chi-squared test, p = 2.0e-33). When we compared basal expression levels in *A. halleri* and *A. thaliana*, we observed a similar pattern, with 64.4% (2986/4634- Fig1B, Table S2) showing orthoplastic gene expression changes (Figure 1D, chi-squared test, p = 5.2e-86). The predominantly orthoplastic regulatory evolution we

126 observe when comparing *A. lyrata* or *A. halleri* to *A. thaliana* was significant at all time
127 points (Table S2-S6), but was strongest 6h into the stress treatment (Fig. S5AB, chi-squared
128 tests, minimum p= 2.0e-8). The direction of the stress response was largely conserved across
129 species, and the results were essentially unchanged if we used the reaction of *A. lyrata* or *A.*
130 *halleri* as alternative proxy for ancestral plasticity (Fig. S6A-F). We conclude that, for genes
131 responding to dehydration stress, the evolution of basal expression in the Arabidopsis genus
132 is not independent of the direction of ancestral plasticity.
133
134 This pattern could plausibly result from a single genetic change, if e.g. species differed for
135 only one major regulator[25]. To investigate whether this pattern resulted from more than a
136 few trans-regulatory changes, we determined how many of these changes were due to *cis*-
137 acting regulatory modifications. *Cis*-acting variation in gene expression can be directly
138 inferred from allele-specific expression differences observed in RNA collected from F1
139 hybrids between *A. thaliana* and either *A. lyrata* or *A. halleri* (Fig. 2A, S7). The relative
140 expression levels of the alleles within hybrids correlated positively with parental differences
141 in expression (Table S7, Fig. S7). Of the 3262 genes with an altered basal expression in *A.*
142 *lyrata vs. A. thaliana*, 1243 presented a significant *cis*-acting difference between alleles in
143 the hybrid (FDR 0.05). A majority of these *cis*-acting modifications (776, 62.4%) were
144 orthoplastic (Odds ratio 1.65, *p*=5.8E-10). In *A. thaliana-A. halleri* F1 hybrids, the total
145 number of basal *cis*-acting variants distinguishing *A. halleri* from *A. thaliana* was in the same
146 order of magnitude (1112 *cis*-acting variants at FDR 0.05), 65.6% (730) of which contributed
147 to orthoplastic changes in basal expression (Odds ratio 1.91, *p*=9.6E-14). Therefore, *cis*-
148 acting regulatory variants were predominantly orthoplastic. Because the allele-specific
149 expression of individual transcripts reveals individual *cis*-acting variants, we conclude that
150 the orthoplastic evolution of basal gene expression has a polygenic basis, both in *A. lyrata*
151 and in *A. halleri*. The association we observed between the direction of plasticity and the
152 evolution of basal expression levels is the result of many independent regulatory mutations
153 (Fig. 3B, S2, S8A).
154
155
156 **The response is attenuated in *A. lyrata* and *A. halleri*, compared to *A. thaliana***

157 In both *A. lyrata* and *A. halleri,* we further determined the genes whose plastic response to
158 stress had a significantly steeper slope than the response measured in their outgroup
159 species *A. thaliana* (Fig. 1F-H). A comparison of the expression fold-change in *A. thaliana* to
160 the fold-changes in the other two species revealed that for most genes, the plastic responses
161 were attenuated in both *A. halleri* and *A. lyrata* (*A. halleri*: Fig. 1F,H; and *A. lyrata*: Fig. 1G,H).
162 The reaction to stress observed in *A. thaliana* thus tended to be predominantly mitigated in
163 both *A. halleri* and *A. lyrata* (Table S2). This pattern was also observed at all other time
164 points in both species (chi-squared test, minimum p < 1.8E-7, Fig. S5EF, Table S3-S6),
165 confirming that the effect did not result from a delayed stress reaction in one of the
166 species[37]. The predominant evolution of a mitigated response to stress in *A. halleri* was also
167 observed if we used the reaction measured in *A. lyrata* as a proxy for the magnitude of
168 ancestral plasticity (Fig. S6G-I). Interestingly, the pattern was less pronounced in *A. lyrata*
169 (Fig. S5F): we observed 2.5 fold more genes in *A. lyrata* than *A. halleri* with a reaction to
170 stress that was greater than in the outgroup *A. thaliana* after 3-6h of stress (Fig. 1H, Fig. S4F,
171 Fig, S6J-L). Furthermore, for 578 of the 910 genes whose expression was down-regulated
172 after 6h of stress in *A. halleri*, the slope of the stress reaction was steeper in *A. lyrata* (Fig.
173 S6H).
174
175 As mentioned earlier, interspecific differences in the slope of the reaction to stress could
176 plausibly result from a single change in a major regulator of the stress response[25]. We
177 therefore determined the *cis*-acting basis of changes in the slope of the plastic response to
178 stress in *A. lyrata* and in *A. halleri*, compared to *A. thaliana*. If the allelic ratio measured
179 within the hybrid altered significantly after initiation of the stress, we concluded that a *cis*-
180 acting variant had modified the response of gene expression to stress (Fig. S7). Changes in
181 allelic ratios within the hybrid were positively correlated with parental differences in gene
182 expression plasticity (Fig S7, Table S7). After 6h of dehydration, a significant fold-change
183 difference was apparent between parental species for 2588 genes, 51.4% of which were also
184 observed in hybrids. Of these *cis*-acting modifications of plasticity in *A. lyrata*, a large
185 majority (72.9%) contributed to mitigating the gene expression response after 6h (Odds ratio
186 2.68, p<2.2E-16). In *A. halleri*, the contribution of *cis*-acting modifications was also strongly
187 skewed towards mitigation (88.1% of 1382, Fig. 2B, Odds ratio 7.37, p<2.2E-16). Because the
188 mitigated stress response observed in *A. lyrata* and *A. halleri,* compared to *A. thaliana,* was

189 clearly supported by a large number of *cis*-acting modifications, we conclude that it is
190 polygenic.
191
192 **Derived *cis*-acting variants reveal interspecific differences in the rate of plasticity**
193 **evolution**
194 A combination of basal expression and plasticity to stress underlies the expression
195 profile of stress responsive genes and thus their molecular effect. Unchanged,
196 orthoplastic or paraplastic basal expression that combine with either unchanged,
197 magnified or mitigated plasticity define nine possible modes of plasticity evolution (Fig.
198 3A). A chi-squared test showed that genes were not distributed uniformly across these
199 modes (Fig. 3A, χ^2=217.4, df = 4, p = 6.93e-46, and χ^2= 363.7, df = 4, p = 1.89e-77, in *A.*
200 *halleri* and *A. lyrata*, respectively, Table S2). However, to understand how the stress
201 response evolves, we must determine how often changes in each of these modes
202 evolve. Our experimental design allows us to compare the rate at which changes in basal
203 expression and slope have evolved in the two sister lineages *A. lyrata* and *A. halleri*. To
204 distinguish derived regulatory changes in each lineage, we assumed parsimony in the
205 evolution of *cis*-regulation: *cis*-acting variants detected in only one of the two hybrid
206 types are more likely to have occurred after the split between *A. lyrata* and *A. halleri*
207 (Fig. 3B, S9). Those detected in both hybrids were called "undetermined" as it is not
208 possible to infer whether they predated the split of the two species or evolved in the
209 outgroup lineage. We note that it is not possible to assign a clear direction of effect for
210 *cis*-regulatory mutations of undetermined origin. The direction of effect of a derived *cis*-
211 regulatory variant, instead, can be deduced by comparison with the expression level of
212 the *A. thaliana* allele in the hybrid. We observed that in the sister species, the majority
213 of derived *cis*-regulatory changes contributed to orthoplastic basal changes in
214 expression and/or to the mitigation of the stress response observed in *A. thaliana* (Fig.
215 3C, Fig. S8). *Cis*-regulatory variants, however, tended to remodel plasticity in different
216 ways in *A. halleri* and *A. lyrata*. Grouping genes into the 9 modes of plasticity evolution
217 defined previously, we observed that the proportion of derived *cis*-acting variants in
218 each of the two lineages was not the same across these gene groups (Fig. 3D, χ^2=189.3,
219 df=7 p=2.02E-37). The contrast was strongest after 3h and 6h of stress (Fig. S5D,H, Fig.
220 S10). *Cis*-acting variants in *A. halleri* were proportionately more frequent than in *A.*

221 *lyrata* among genes combining an orthoplastic basal expression change with a decrease
222 in the slope of the response to stress (Fig. 3D, ortho-mit genes, p<1.42E-06 after 6h).
223 This combination corresponds to the genetic assimilation of the plastic response
224 observed by Waddington in his seminal experiment [4,8,11]. We also observed that *A.*
225 *halleri* genes displaying a paraplastic basal expression change combined with a mitigated
226 response had an excess of derived *cis*-acting variants (p=3.6E-08). This excess indicates
227 that regulatory modifications opposing the plastic reaction to stress were more favored
228 in *A. halleri* than in *A. lyrata*. Because mutations that reduce plasticity are over-
229 represented in the *A. halleri* lineage, we conclude that a greater fraction of ancestral
230 gene expression plasticity was not adaptive in this lineage.
231
232 In contrast, derived *cis*-acting variants were enriched in *A. lyrata* among genes
233 displaying a magnification of the stress response, and this was true regardless of
234 whether or not the magnification was combined with a change in basal expression (Fig.
235 3D, partial test χ^2 tests, p=0.003, p=1.4E-19, p=3.9E-08 for ortho-mag, mag-only and
236 para-mag, respectively). Although a magnified response to stress was not the most
237 frequent mode of plasticity evolution, the comparatively faster rate at which *cis*-acting
238 variants accumulated among these genes indicates that mutations leading to this mode
239 of plasticity evolution are more favored in the *A. lyrata* than in the *A. halleri* lineage.
240 Interestingly, the functions enriched among them indicate that the regulation of leaf
241 development could be one of the targets of selection for increased plasticity in *A. lyrata*.
242 Gene Ontology categories such as GO:0010207 ”Photosystem II Assembly” (p=1.6E-06),
243 GO:009965 ”Leaf Morphogenesis” (p=8.4E-05), GO:0010103 “Stomatal Complex
244 Morphogenesis” (p= 4.5E-06) or GO: 0034968 “Histone Lysine Methylation” (p=4.5E-03)
245 were enriched (Table S8, S9).
246
247 **Genes with magnified plastic responses in *A. lyrata* evolve under increased**
248 **evolutionary constraints**
249 The accumulation of derived *cis*-acting modifications indicates that the expression of
250 genes displaying magnified plasticity after 6h of stress evolves under positive selection
251 in *A. lyrata*. If such genes with magnified plasticity are subject to stronger selection
252 overall in this lineage, we also might expect that the genes will be exposed to stronger

253 evolutionary constraints than other plastic genes. We grouped *A. lyrata* genes according
254 to their mode of plasticity and assessed the ratio of the divergence at non-synonymous
255 and synonymous sites (*Ka/Ks*) between *A. lyrata* and *A. thaliana*. We included genes
256 that did not change their expression as a control group and used bootstrapping to
257 determine confidence intervals and identify significant differences between groups. For
258 three of the eight modes of regulatory evolution, the number of genes was small,
259 resulting in high bootstrap variance (Fig. 3A, Fig. S11); these modes were therefore
260 excluded from the analysis. We observed that genes of all remaining modes of plasticity
261 evolution showed a significantly lower *Ka/Ks* than the control group (Fig. 4F, Fig. S11J).
262 Genes with modified plasticity therefore all experienced stronger evolutionary
263 constraints. Interestingly, however, we observed that the *Ks* of control genes was
264 significantly lower than the *Ks* of all gene groups, with the exception of the genes that
265 evolved a magnified response to stress (Fig. 4AB). To gain further insight into the
266 evolutionary forces operating on these genes in *A. lyrata,* we examined nucleotide
267 polymorphism within a local *A. lyrata* population[39,40]. The average number of pairwise
268 differences did not differ significantly across groups of genes, and was similar to that of
269 the control genes (Fig. 4G). We also used the Tajima's *D* statistic to quantify the
270 contribution of low-frequency variants to nucleotide diversity within each gene group.
271 Mean *D* for non-synonymous sites differed significantly among modes of plasticity
272 evolution (Fig. 4C), but mean *D* did not differ significantly for synonymous sites (Fig. 4D).
273 *A. lyrata* genes that evolved a magnified plastic reaction, but no basal change in
274 expression, displayed the most negative Tajima's *D* of all modes of plasticity evolution at
275 non-synonymous sites (Fig 4C-D). We performed the same analysis on the
276 polymorphism for orthologous gene sets within *A. thaliana.* Compared to *A. lyrata*,
277 Tajima's *D* in *A. thaliana* was generally lower, presumably due to its history of
278 population expansion[40]. Yet, the distinctive Tajima's *D* of *A. lyrata* genes with a
279 magnified plasticity was specific to *A. lyrata,* because the Tajima's *D* of its orthologous *A.*
280 *thaliana* genes was not lower than that of orthologous genes in the other plasticity
281 groups (Fig. 4E,F). This finding indicates that amino-acid variants in *A. lyrata* tend to
282 segregate at lower frequencies, if they affect genes with a magnified plasticity (Fig. 4E).
283 To confirm that the amino-acid sequence of genes with a magnified plasticity are subject
284 to stronger evolutionary constraints, we estimated the distribution of fitness effects

285 (DFE) of new non synonymous mutations for each gene group, using the software
286 fitδaδi[41,42]. Parameters of a demographic model for the *A. lyrata* population have been
287 previously determined[38]. Population data, simulated under this model by fitδaδi, fitted
288 well with the site frequency spectrum of synonymous variation observed in the
289 population (Fig. S12). The DFE was then subsequently estimated by fitting a gamma
290 distribution of fitness effects to the frequency spectrum of non-synonymous variants[41]
291 (Fig. 4H). This analysis showed that, of all modes of plasticity evolution, the genes with a
292 magnified plastic reaction to stress showed the lowest proportion of variants with weak
293 fitness effects. Bootstrapping confirmed that the predicted proportion of nearly neutral
294 deleterious variants was significantly lower among these genes (Fig. S11). This pattern is
295 in agreement with the higher proportion of low-frequency variants indicated by the
296 lower Tajima's *D* statistic of this group of genes, compared to other plastic genes (Fig
297 4H, Fig. S11). In conclusion, *A. lyrata* genes that evolved a magnified response after 6h
298 of stress not only accumulated an excess of derived *cis*-acting variants, they also appear
299 to be subject to increased selective constraints at the amino-acid level.
300
301 Waddington proposed that stabilizing selection around the new optimum (canalization)
302 would favor the genetic assimilation of plastic responses[11]. In *A. lyrata*, the constraints
303 detectable on genes with an assimilated stress response that combine the two
304 predominant types of basal and plastic regulatory changes have neither increased nor
305 decreased (Fig. 4A-D). The fitness effects of new mutations in these genes do not differ
306 significantly from those in genes whose regulation remained unchanged (Fig. 4A-D, Fig.
307 S11). This result indicates that the genetic assimilation of the ancestral plastic response
308 is not associated with increased canalization at the amino-acid level in *A. lyrata*. Future
309 studies will have to examine whether the excess of *cis*-acting changes leading to a
310 decrease in plasticity in *A. halleri* reflects adaptation or whether it is a result of relaxed
311 constraints on the regulation of the stress response.
312
313 **Conclusion:**
314 Because plants are sessile, they evolved rapid and complex plastic responses that allow them
315 to face ever-changing environmental stressors[43]. By documenting the *cis*-regulatory
316 divergence between *A. halleri* or *A. lyrata* and their common outgroup *A. thaliana,* we show

317 that the divergence of plastic stress responses in the *Arabidopsis* genus has a broad
318 polygenic basis. By comparing the direction of *cis*-acting changes with the direction of the
319 plastic reaction, we demonstrate the profound impact of plasticity on the accumulation of
320 regulatory mutations in each lineage, lending the strongest support to date for the idea that
321 plasticity can be stepping stone for the evolution of transcription[7]. Previous studies
322 concluded that most transcript changes in reaction to stress were maladaptive because they
323 were predominantly lost during adaptation[18,20–22]. Here, we challenge these approaches by
324 using two sources of information to infer the adaptive relevance of regulatory changes in the
325 plastic response to stress. First, by comparing allele-specific expression across the two
326 interspecific hybrids, we estimated lineage-specific rates of accumulation of derived *cis*-
327 acting variants, an approach that has proven successful in revealing the polygenic response
328 to selection on complex traits [32,42]. Second, we used the site frequency spectrum of
329 synonymous and non-synonymous mutations in *A. lyrata* to test whether the fitness effects
330 of new mutations in plastic genes depends on the mode of plasticity evolution [44]. In *A.*
331 *lyrata*, the lineage that best survived the severe dehydration imposed in this experiment
332 (Fig. S2) and displays the strongest drought tolerance [34,45], both approaches point to the
333 sustained action of polygenic selection for an increase in expression plasticity of many genes,
334 rather than its loss. In *A. halleri*, instead, we did observe an excess of *cis*-acting variants
335 leading to a decreased transcriptional plasticity to stress. We conclude that plasticity can
336 potentiate the adaptation of gene expression, but its selective remodeling depends on the
337 ecological context of each lineage.
338
339 **Material and Methods**
340 Methods are described in detail in supplementary information.
341
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345

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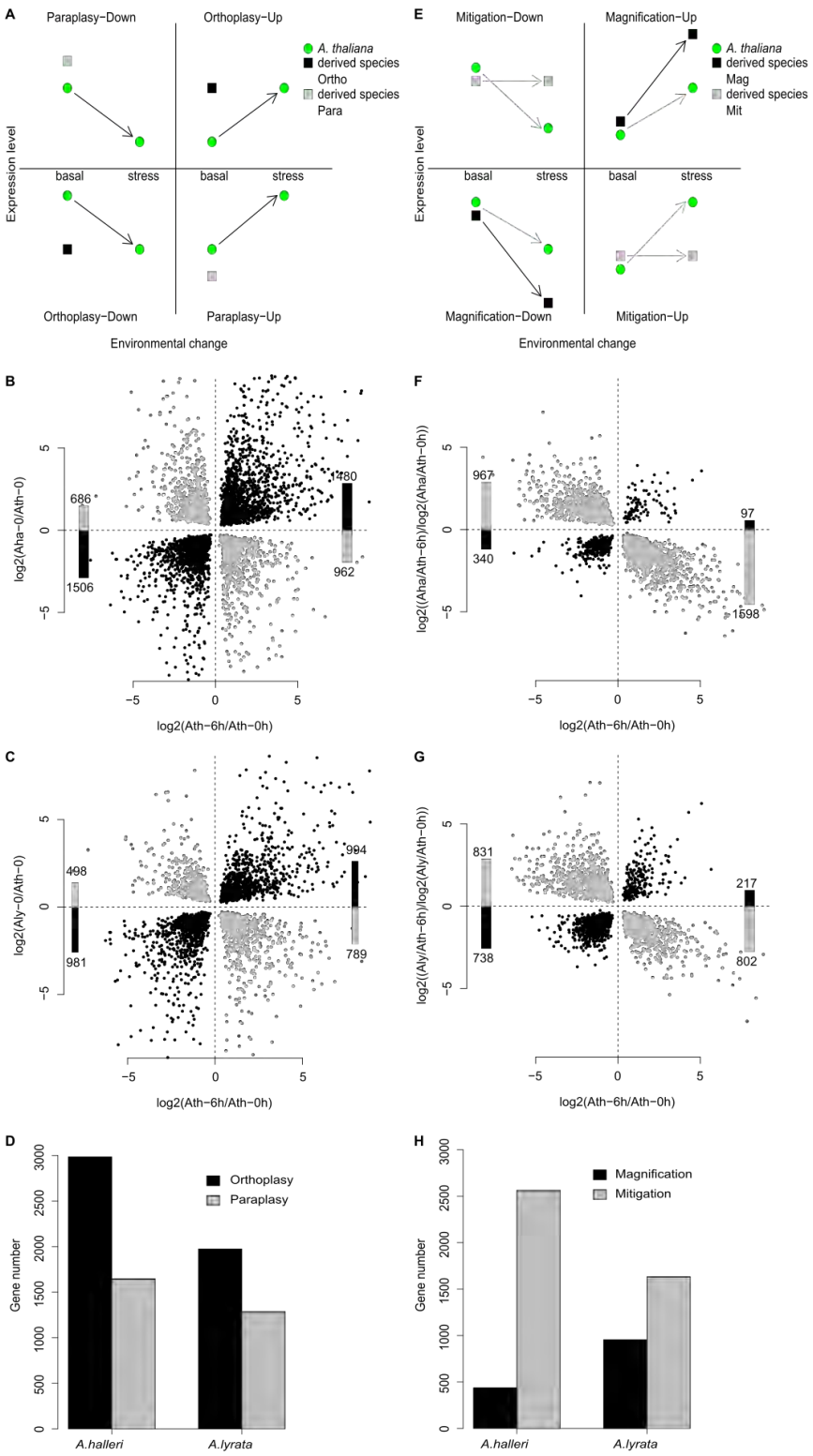

Figure 1: **Gene expression differences between species depend on the direction of gene expression plasticity.** A. Sketch illustrating what ortho- or para-plastic changes in the derived species (*A. halleri* or *A. lyrata*) correspond to for up- and down-regulated genes, using *A. thaliana* as a proxy ancestral plasticity and ancestral gene expression level. Black dot: ortho-plastic change, grey dot: para-plastic change. Ortho- and para-plastic changes in *A. halleri* (B) and *A. lyrata* (C). x-axis: ratio of gene expression after 6 hours in *A. thaliana,* y-axis: ratio of basal expression in each species, compared to expression in *A. thaliana* after 6h. Bars represent the number in each quadrant of the plot. D) Number of ortho- (black) and para-plastic (grey) changes in *A. halleri* and *A. thaliana*. This terminology helps describe the evolutionary change in any given species, but we note that orthoplastic basal regulatory changes in one species are de facto paraplastic in the outgroup species and *vice-versa*.
E. Sketch illustrating what magnified or mitigated response to stress in the derived species (*A. halleri* or *A.* lyrata) correspond to for up- or down-regulated genes, using *A. thaliana* as proxy for ancestral plasticity. Black arrow: magnified response, grey arrow: mitigated response. Magnified and mitigated response in *A. halleri* (F) and *A. lyrata* (G). y-axis: ratio of the response ratios, x-axis: ratio of gene expression after 6 hours in *A. thaliana*. Bars represent the number in each quadrant of the plot. H). Number of genes with a magnified (black) and mitigated (grey) response in *A. halleri* and *A. lyrata,* compared to *A. thaliana*.

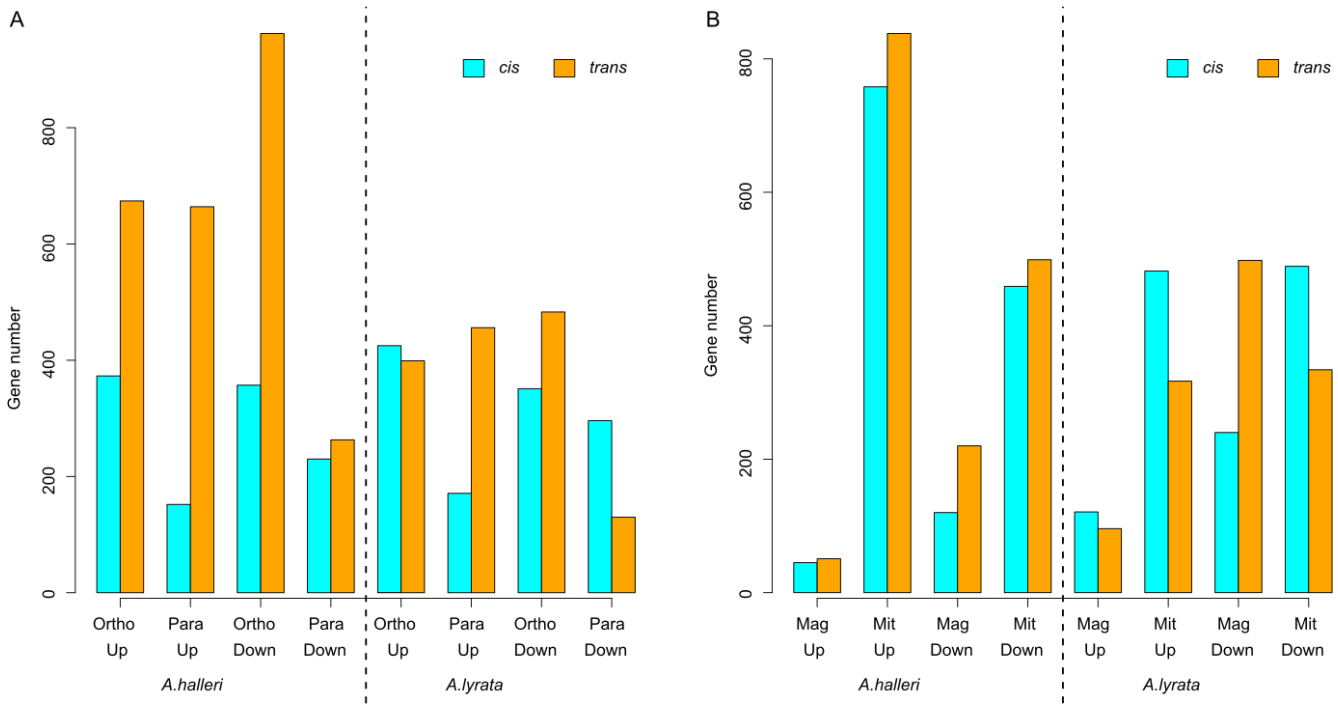

Fig 2. ***Cis*-regulatory changes predominantly contribute to orthoplastic basal gene expressio changes, or a mitigation of the response to stress, both in *A. halleri* and *A. lyrata,* compared to *A. thaliana*.** (A) The number of genes for which a basal *cis*-regulatory change was detected in hybrids (significant log2ratio of *A. halleri*/*A. thaliana* or *A. lyrata*/*A. thaliana* among alleles in F1 hybrid and between the corresponding parental species) or no *cis*-regulatory change (parental difference are not associated with a bias in allele specific expression, assumed to be controlled in *trans*). (B) The number of genes that have a plastic *cis*-regulatory change (the ratio of the parental response is explained by the log2 ratios at xh vs. 0h for *A. halleri*/*A. thaliana* or *A. lyrata*/*A. thaliana* in the F1 hybrid) or no *cis*-regulatory change (the ratio of the parental response is not associated with a bias in the allele specific expression, assumed to be controlled in *trans*).

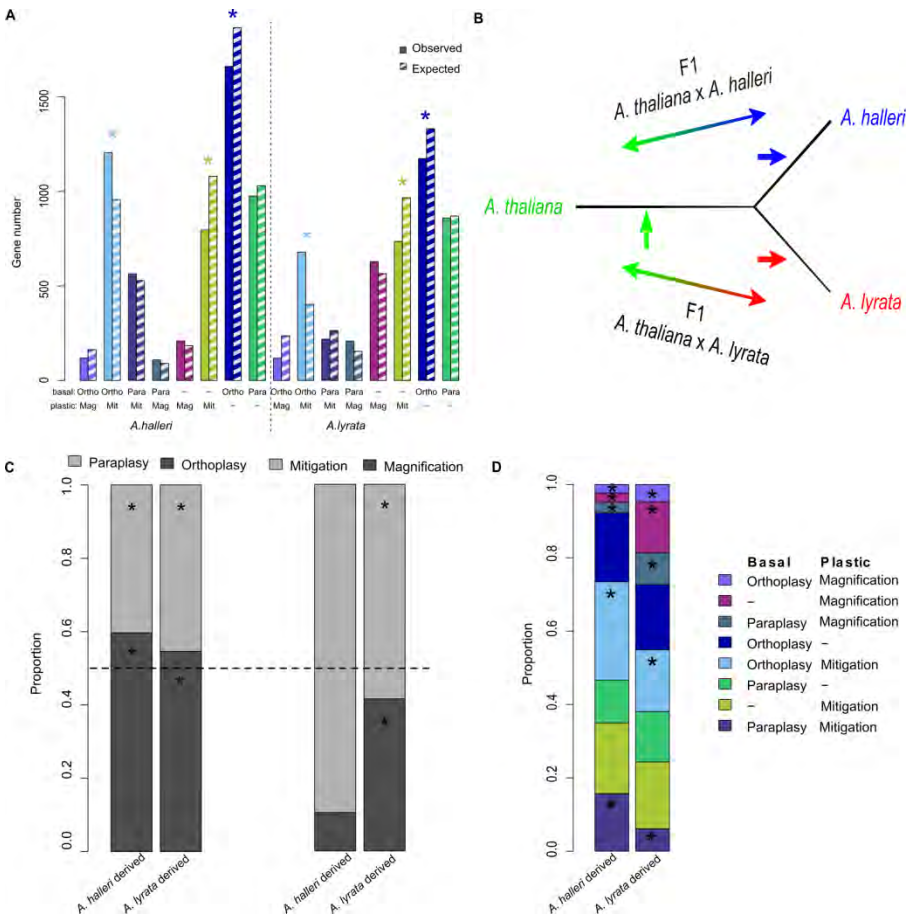

Fig. 3. **Distribution of derived cis-acting8 genes sets defined by their basal and/or plastic expression changes in *A. halleri* or *A. lyrata* compared to *A. thaliana*.** Ortho-Mag: orthoplasy and magnification, Ortho-Mit: orthoplasy and mitigation, Para-mit: paraplasy and mitigation, Para-Mag: paraplasy and magnification, Only-Mag: magnification only, Only-mit: Mitigation only, Only-Ortho: Orthoplasy only, Only-Para: Paraplasy only.
(A). The number of observed (solid bars) and expected (hatched bars) genes in each of the 8 sets. Left: *A. halleri;* right: *A. lyrata*. "*": Significant excess or depletion, inferred by a partial Chi square test with one degree of freedom.
(B) Phylogenetic relationship between the three species allowed differentiating undetermined *cis*-acting changes (green arrow), which are shared between the two F1 hybrids, from the derived *cis*-acting changes, which are specific to one hybrid (blue and red arrow).
(C) Proportion of derived and *cis*-acting modifications within all genes with a basal change (left) or a plastic response (right). Dashed line shows 50%, i.e. the expectation in the absence of a mutational bias. The total number of genes in these groups are shown in Figure S8.
(D) Proportion of derived and *cis*-acting modifications within each gene set. "*": Significantly increased or decreased number of derived changes in *A. halleri* compared to *A. lyrata*. The association of basal and plastic changes in gene expression is not random (Chi-sq =47.7 df=7 p=4.13e-08 and Chi-sq =302.5 df=7 p=1.84e-61).

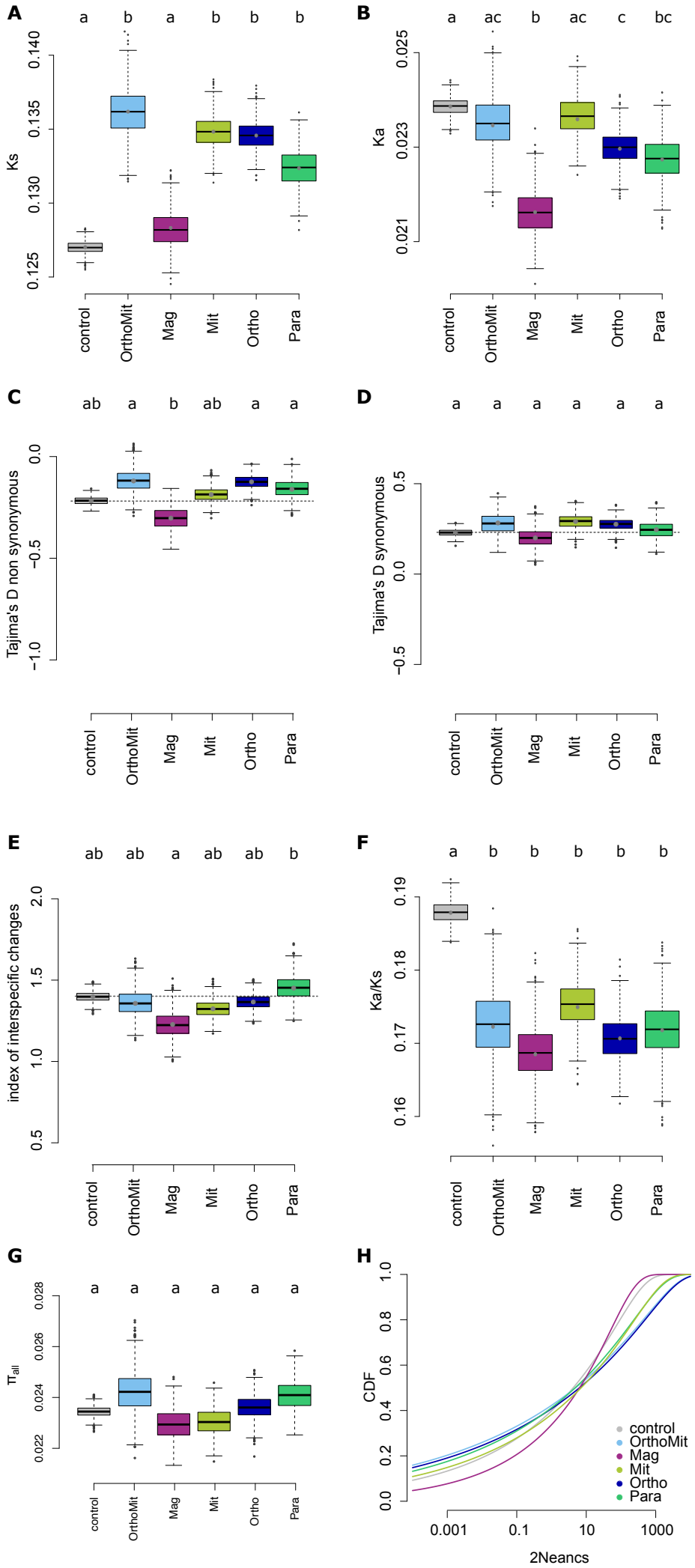

Fig. 4. **Tajima's D at synonymous and non-synonymous sites differ between 5 modes of plastictiy evolution.** Ortho-Mit: orthoplasy and mitigation, Only-Mag: magnification only, Only-mit: Mitigation only, Only-Ortho: Orthoplasy only, Only-Para: Paraplasy only. The plasticity categories Ortho-Mag, Para-Mit and Para-Mag were excluded due to having too few genes (see Fig. 3A) and therefore too high variance. (A) Ks between *A. lyrata* and *A. thaliana*, (B) Ka between *A. lyrata* and *A. thaliana,* (C) Tajima's D for synonymous sites and (D) nonsynonymous sites in *A. lyrata.* Significance between the groups was estimated based on pairwise comparison of bootstrap replicates. No shared letters mean that the groups are different with a significance cutoff at $p < 0.05$. (E) Relative change in mean Tajima's D for non-synonymous sites between *A. lyrata* and *A. thaliana.* ((*A. lyrata* +2) / (*A. thaliana* +2)), (F) Ka/Ks ratio, (G) average **π** per gene and (H) Cumulative distribution function of the gamma distribution for the control and the 5 classes of plasticity. OrthoMag, ParaMit and ParaMag were removed due to the low number of genes in these plasticity classes.

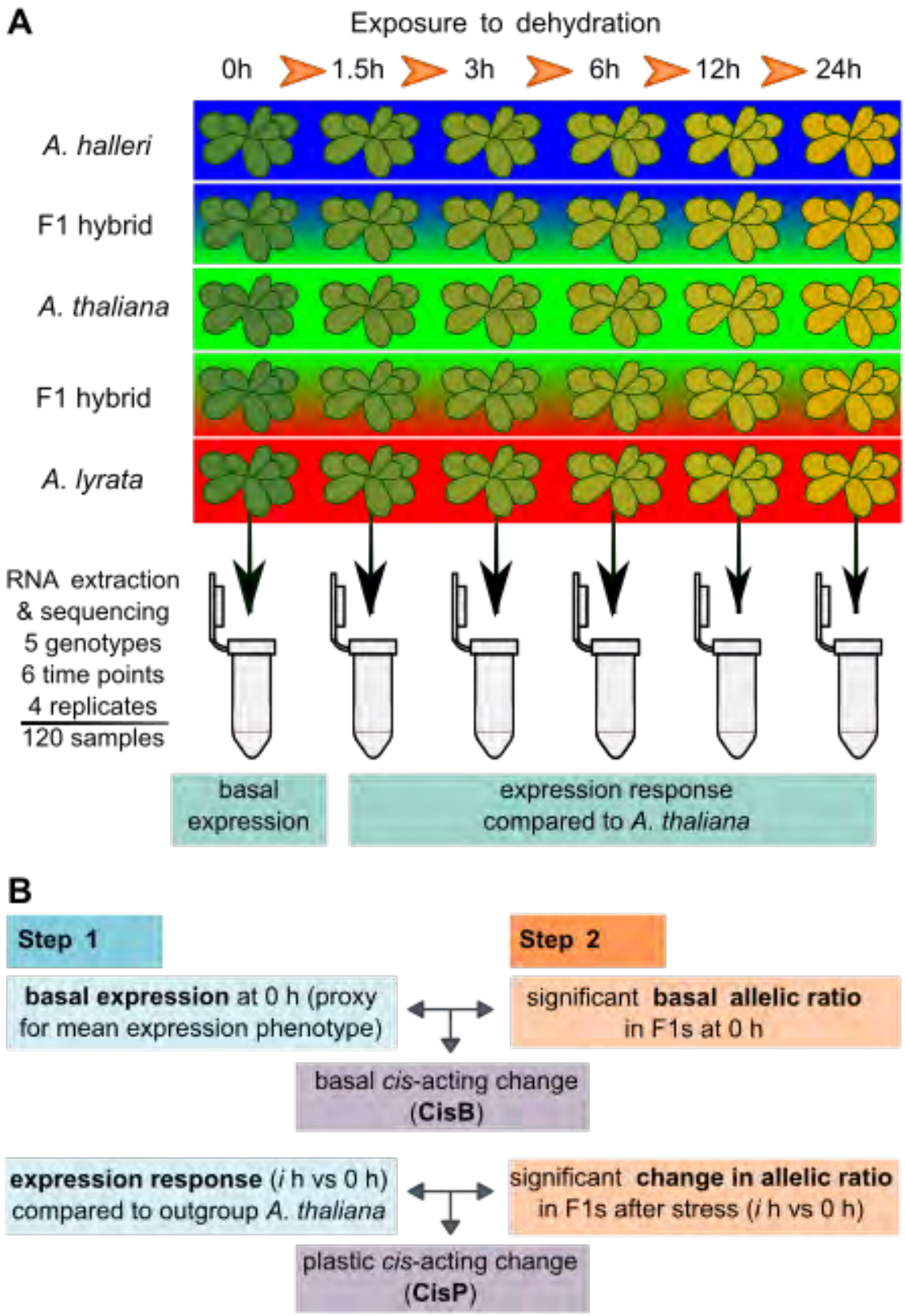

Figure S1. (A) Overview of the experimental setup for the desiccation stress for the three *Arabidopsis* species and two F1 hybrids *A. thaliana* x *A. lyrata* and *A. thaliana* x *A. halleri*. In four independent trials, aerial parts of the plants were cut and left to dry out on absorbent paper for 0, 1.5, 3, 6, 12 and 24 hours under growth conditions. Samples for transcriptome sequencing were flash frozen after the treatment and used for RNA extraction. (B) Definition of basal and plastic *cis*-regulatory changes. If basal expression differences between the parents (step 1) can be explained by a significant basal allelic ratio (step2) in the F1, a basal *cis*-acting change is contributing to the change in gene expression. If the ratio of the expression response between the parents (step 1) due to the stress can be explained by the significant change in the allelic ratio in the F1s (step 2), a plastic *cis*-acting change is contributing to the change in slope. Details for the analytical pipeline in Figure S7.

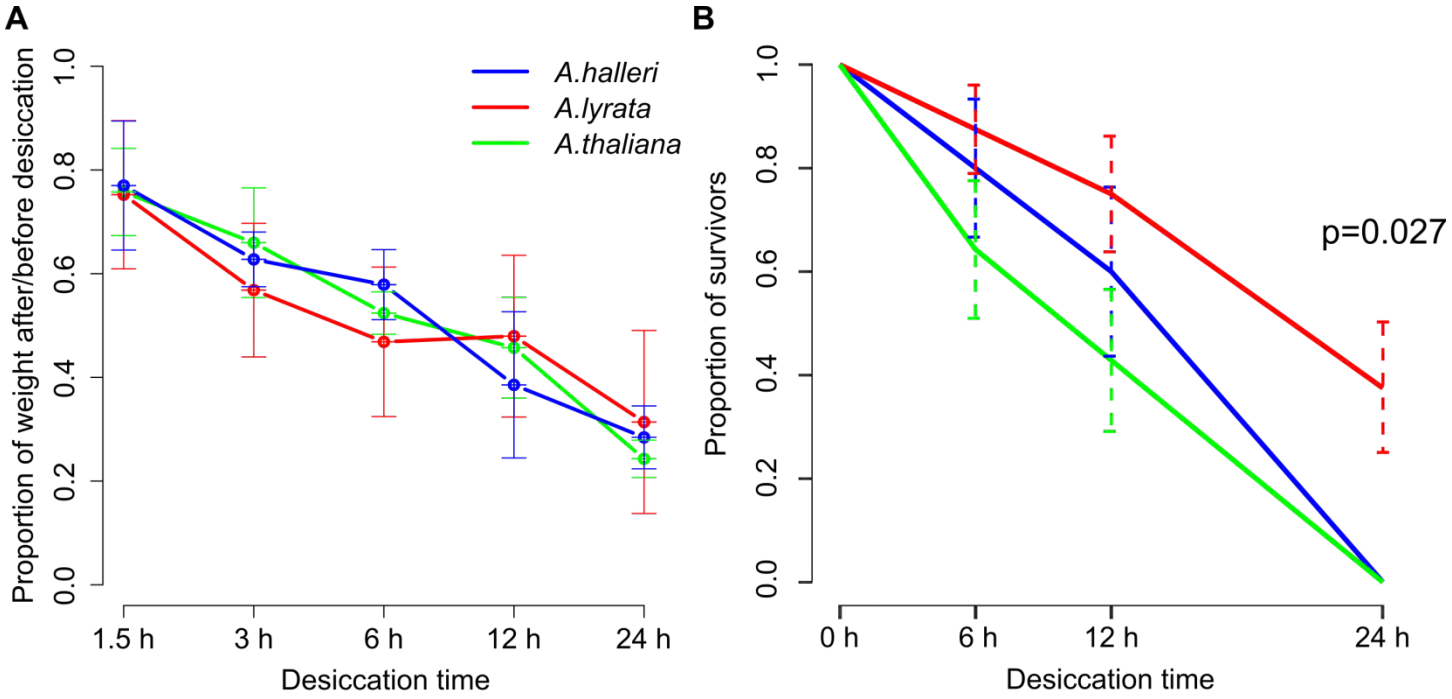

Fig. S2: (A) **The rate of water loss after 1.5 to 24h of desiccation is similar between the species.** The rate of water loss was inferred by the ratio of plant weight after / before the desiccation stress. Samples were weighted before the initiation of stress and immediately prior to RNA sampling. Species did not differ significantly in their rate of water loss (interaction of species:duration of dehydration term in glm model, F=0.562, *p*=0.803).
(B) ***A. lyrata* is the most resistant to acute dehydration.** We performed the same dehydration experiment as for the transcriptome analysis, but instead of sampling material, we repotted the plants after 0h, 6h, 12h and 24h of dehydration and measured survival after 18 days. The survival rate after stress among species was significantly different and after 24h of dehydration, only *A. lyrata* survived (interaction term species:duration of dehydration in glm model, $F_{6,111}$= 8.71, *p*= 8.841e-08). This experiment included a representative set of 10, 17 and 14 genotypes of *A. halleri, A. lyrata* and *A. thaliana*, respectively (Table S13).

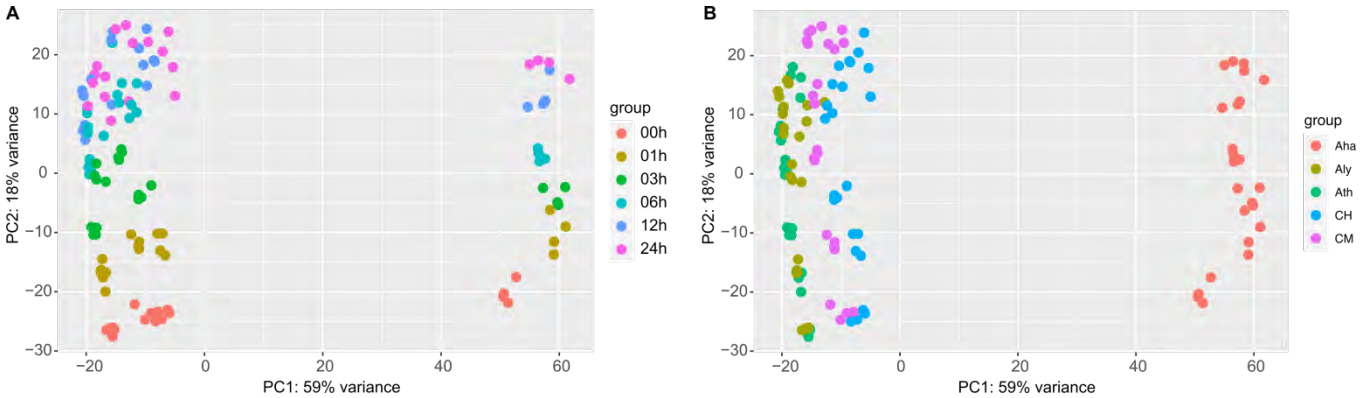

Figure S3. **Differences between species and time points have a clear genetic basis.** Principal component analysis (PCA) of the 120 transcriptome samples collected over a desiccation time course for three species and two of their hybrids colored by A) time points and B) species (Aha – *A. halleri*, Aly – *A. lyrata*, Ath – *A. thaliana*, CH – F1 *A. thaliana* x *A. halleri*, CM – F1 *A. thaliana* x *A. lyrata*). The PCA separates the samples by species (PC1 59% variance) and by time-points (PC2 18% variance).

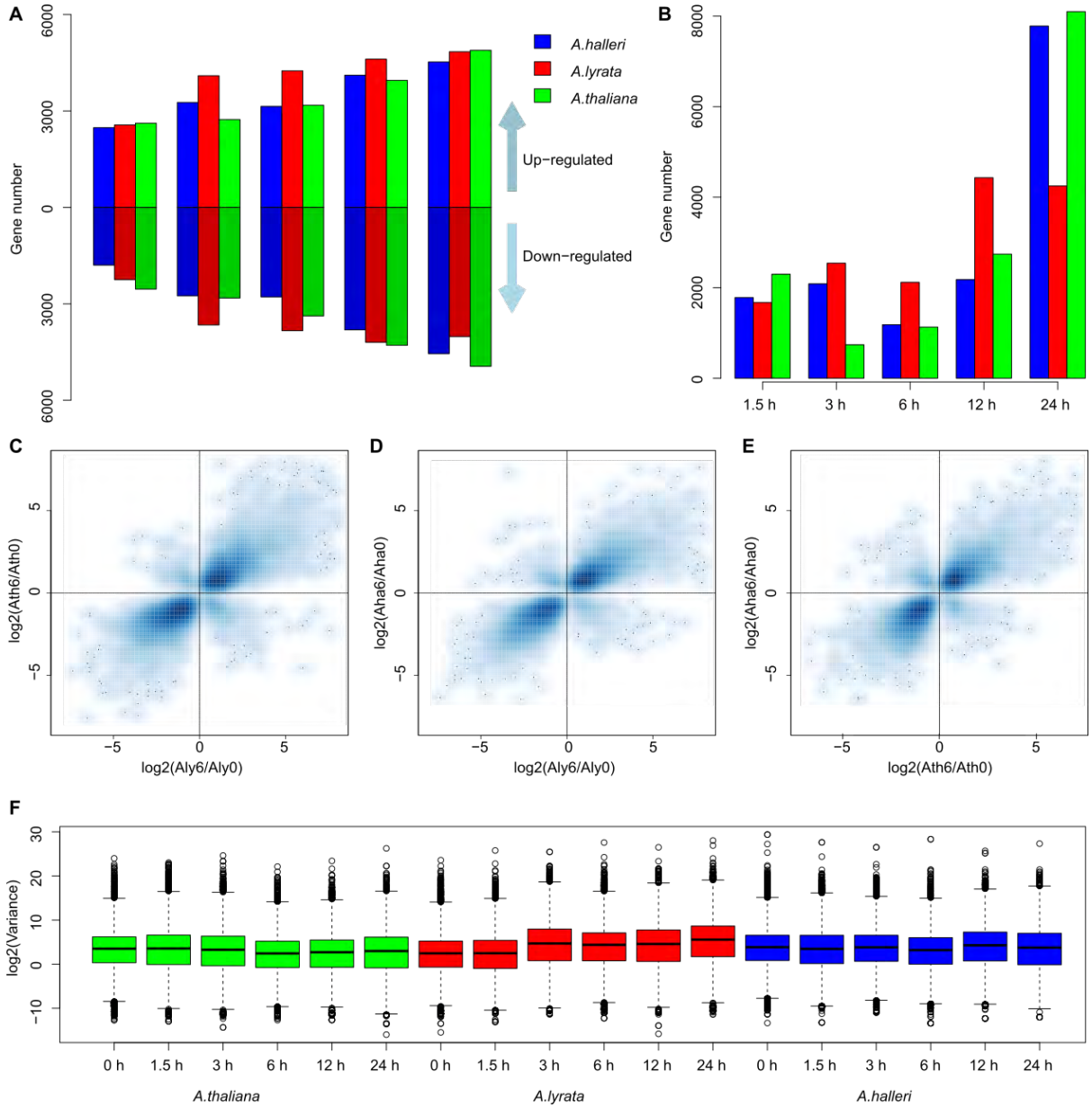

Fig. S4. Supplementary information regarding the number of genes responding to stress response, the time point of maximal gene expression change, and the variance of gene expression among biological replicates. A) Number of genes showing significant up- and down-regulation at each time point after initiation of the stress in each of the three species. *A. lyrata* has a higher number of up- and down-regulated genes at intermediate time points. B) Number of genes reaching their maximum log-fold change at each time point in each of the three species confirms the stronger response in *A. lyrata* at intermediate points C-E) Strong pairwise correlation in gene expression fold change between 0 and 6 hours into the desiccation stress for each species pairs : C) *A. thaliana* vs *A. lyrata* (R=0.84, p < 1e-321), D. *A. halleri* vs *A. lyrata* (R=0.85, p < 1e-321) and E) *A. halleri* vs *A. thaliana* (R=0.83, p < 1e-321). This indicates that the overall response to the stress is similar between the species and that the direction of transcriptional plasticity predates species divergence. F) The variance of the expression level in the samples does not increase with the duration of exposure to the stress. The variance of each gene, in each species and at each time point, was calculated based on the four independent biological replicates of the experiment.

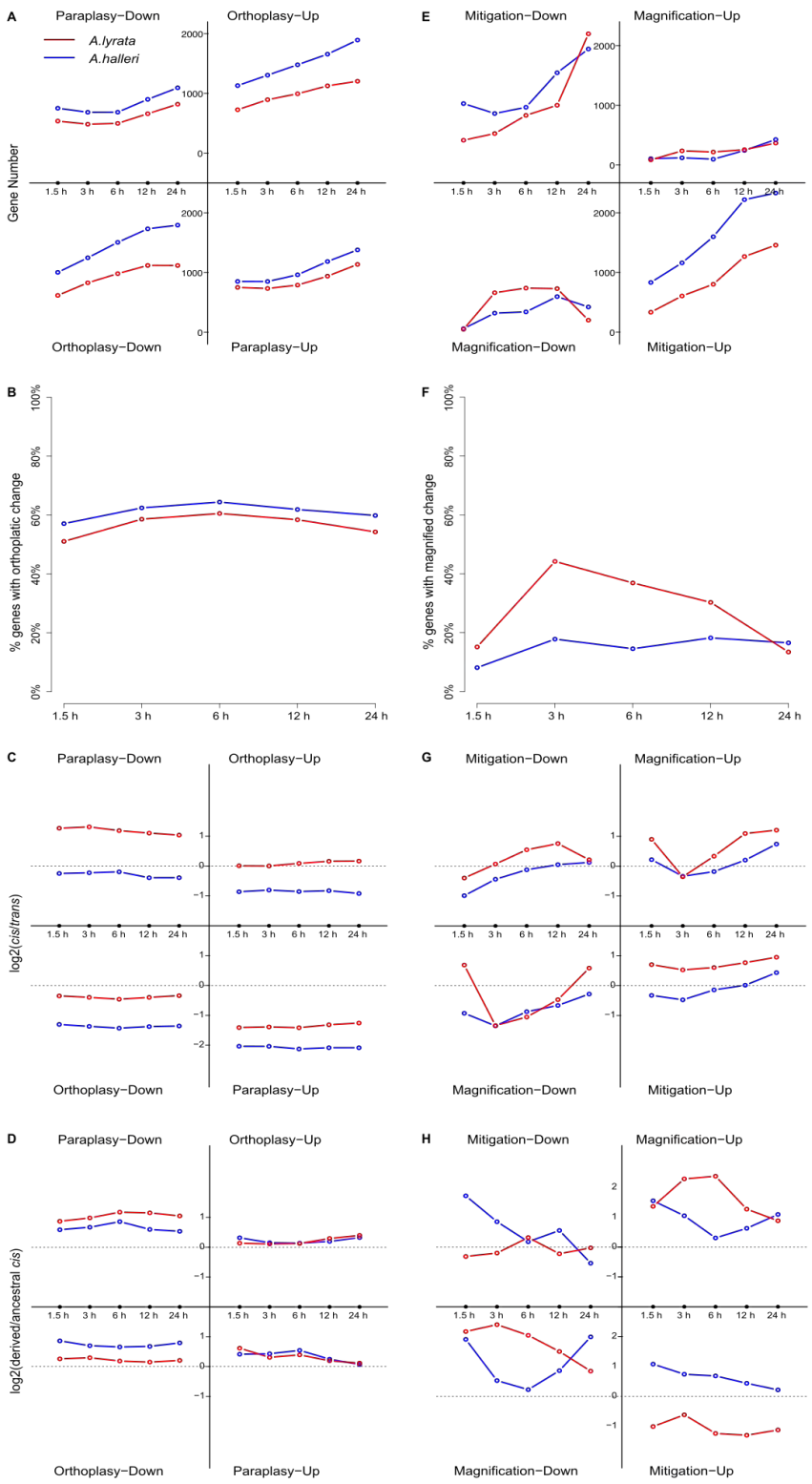

Fig S5. **Extension of Fig. 1 to all time points of the experiment.** Basal changes (A – D) and plastic responses to the stress (E – H) in *A. lyrata or A. halleri*, compared *to A. thaliana*.
(A) The number of genes with a basal change in the direction (ortho-) or opposite (para-) to the direction of the stress reaction in *A. thaliana* increases over the time course. (B) The proportion of genes with an orthoplastic change is not dependent on the time point at which plasticity is determined. (C) The proportion of basal changes explained by *cis*-acting differences is not dependent on the time point at which plasticity is determined. (D) The proportion of basal changes explained by a derived *cis* is not dependent on the time point at which plasticity is observed.
E) The number of genes with a mitigated response increase during the time course. The number of genes with a magnified response do not increase to the same extent. (F) In *A. halleri*, the proportion of genes with a magnified response is not dependent on the time point, whereas in *A. halleri* the proportion of magnified genes is increased between 6 and 12 hours. (G) The proportion of responses to the stress explained by plastic *cis*-acting differences is dependent on the time point in *A. lyrata*. H) The proportion of responses to the stress explained by derived *cis*-acting changes is dependent on the time point.

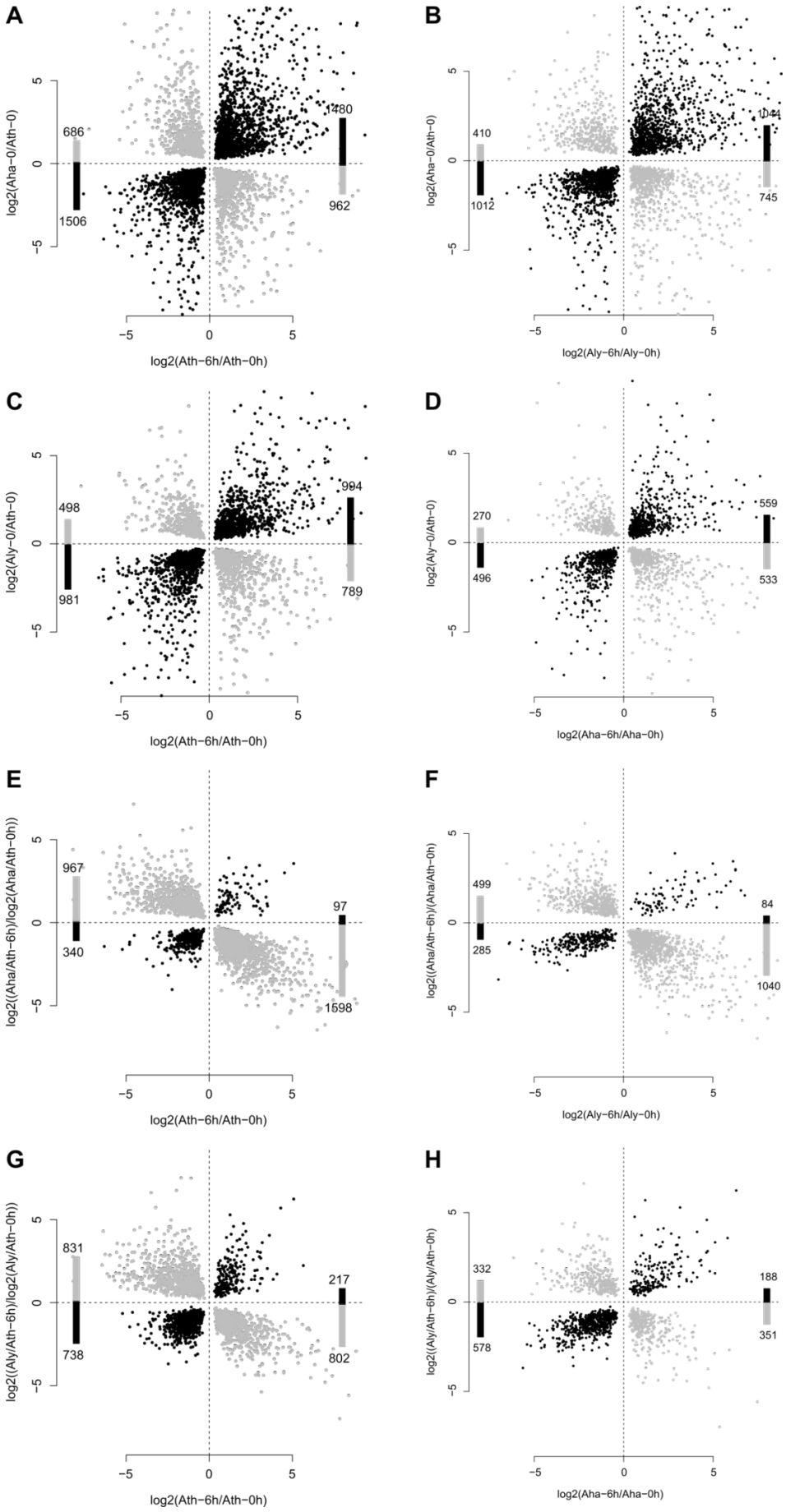

Figure S6. Orthoplastic evolution of basal (A – D) gene expression in *A. lyrata* or *A. halleri* is robust to the species used as a proxy for ancestral expression plasticity. A mitigated response to stress in *A. halleri* is robust to the proxy used for plastic changes (E – F) . This is not the case in *A. lyrata* (G-H). A-B: Basal changes in *A. halleri* vs *A. thaliana* using *A. thaliana* (A) and *A. lyrata* (B) as a proxy for ancestral expression change.
C-D: Basal changes in *A. lyrata* vs *A. thaliana* using *A. thaliana* (C) and *A. halleri* (D) as a proxy for ancestral expression change.
E-F: Plastic changes in *A. halleri* using *A. thaliana* (E) and *A. lyrata* (F) as a proxy for ancestral expression change.
G-H: Plastic changes in *A. lyrata* using *A. thaliana* (E) and *A. halleri* (F) as a proxy for ancestral expression change.
A-D: orthoplastic: black, paraplastic: grey
E-H: magnification: black, mitigation: grey

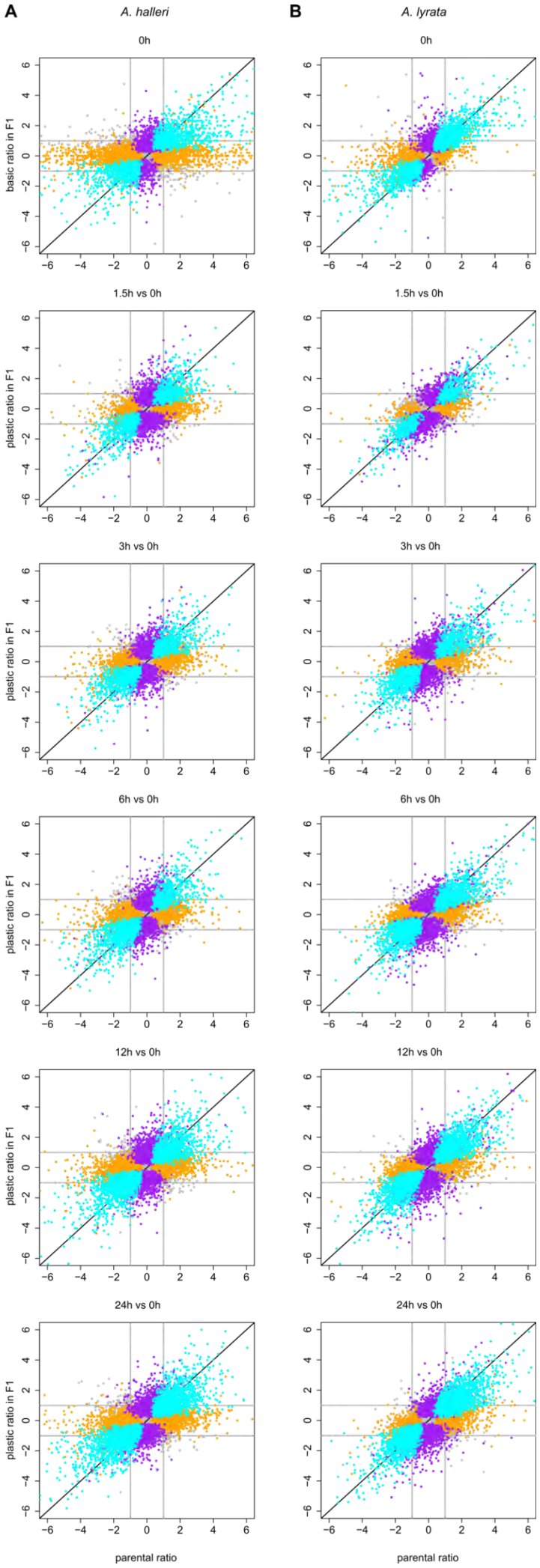

Fig. S7A. ***Cis* regulatory changes explain a large part of the interspecific differences between the species.** A) *A. halleri*. B) *A. lyrata*. Cyan: *cis* change explains most of the parental difference; Orange parental difference are not associated with a bias in allele specific expression and thus assumed to be controlled in trans- (no significant *cis*-acting change); purple: Significant cis-acting changes are detected but the difference between parents is not significant. The allele-specific expression ratio of purple genes is however strongly correlated with the parental expression ratio, indicating that this class of genes reflect lower power for detecting parental differences (shown in Table S2).

At 0h, the genetic basis of regulatory differences in basal expression is shown by plotting log2ratio of *A. halleri*/*A. thaliana* or *A. lyrata*/*A. thaliana* of alleles in the hybrid (y-axis) against the ratio of parental expression (x-axis). From 1.5h to 24h, the genetic basis of regulatory changes is determined by comparing ratio of log2 ratios at xh vs. 0h for *A. halleri*/*A. thaliana* or *A. lyrata*/*A. thaliana* alleles in hybrid (y-axis) with parents (x-axis) at each time point.

Pearson correlations for *cis* (cyan), non-*cis* (trans; orange) and purple (compensatory; *cis*-change but no significant parental difference) are shown in Table S2.

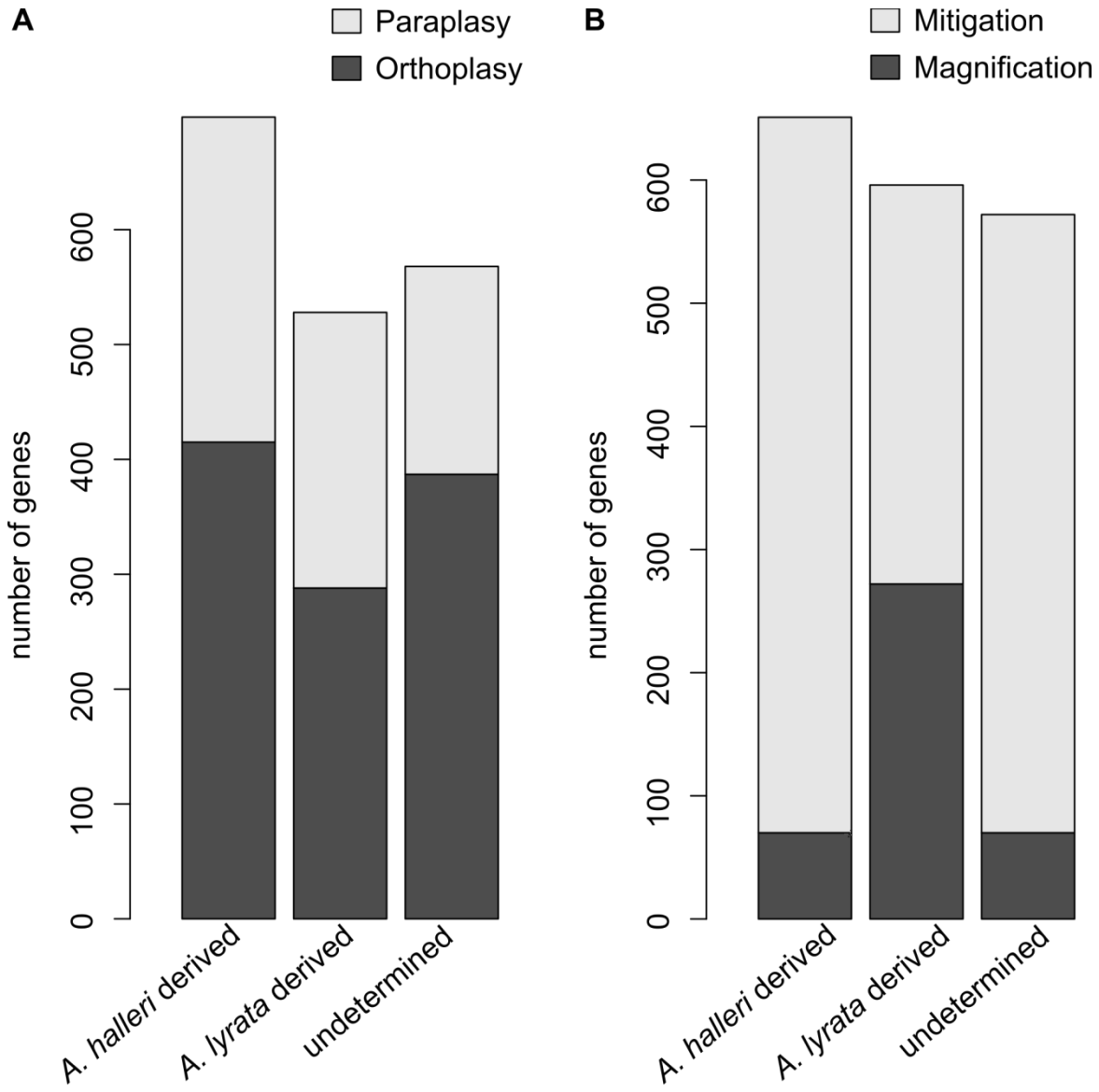

Figure S8. **The total number of genes with derived and undetermined *cis*-regulatory changes are similar.** The number of genes that have (A) a basal change and either an undetermined or derived *cis*-regulatory change or a (B) plastic response and either an undetermined or derived *cis*-regulatory change. Genes are partitioned by the direction of the cis-acting effect detected in basal expression (A) or in the response slope (B). The proportions from Fig3B are based on these numbers. Because the direction of effect of cis-regulatory changes of undetermined origin cannot be ascertained, the number of genes with these changes are not partitioned functionally.

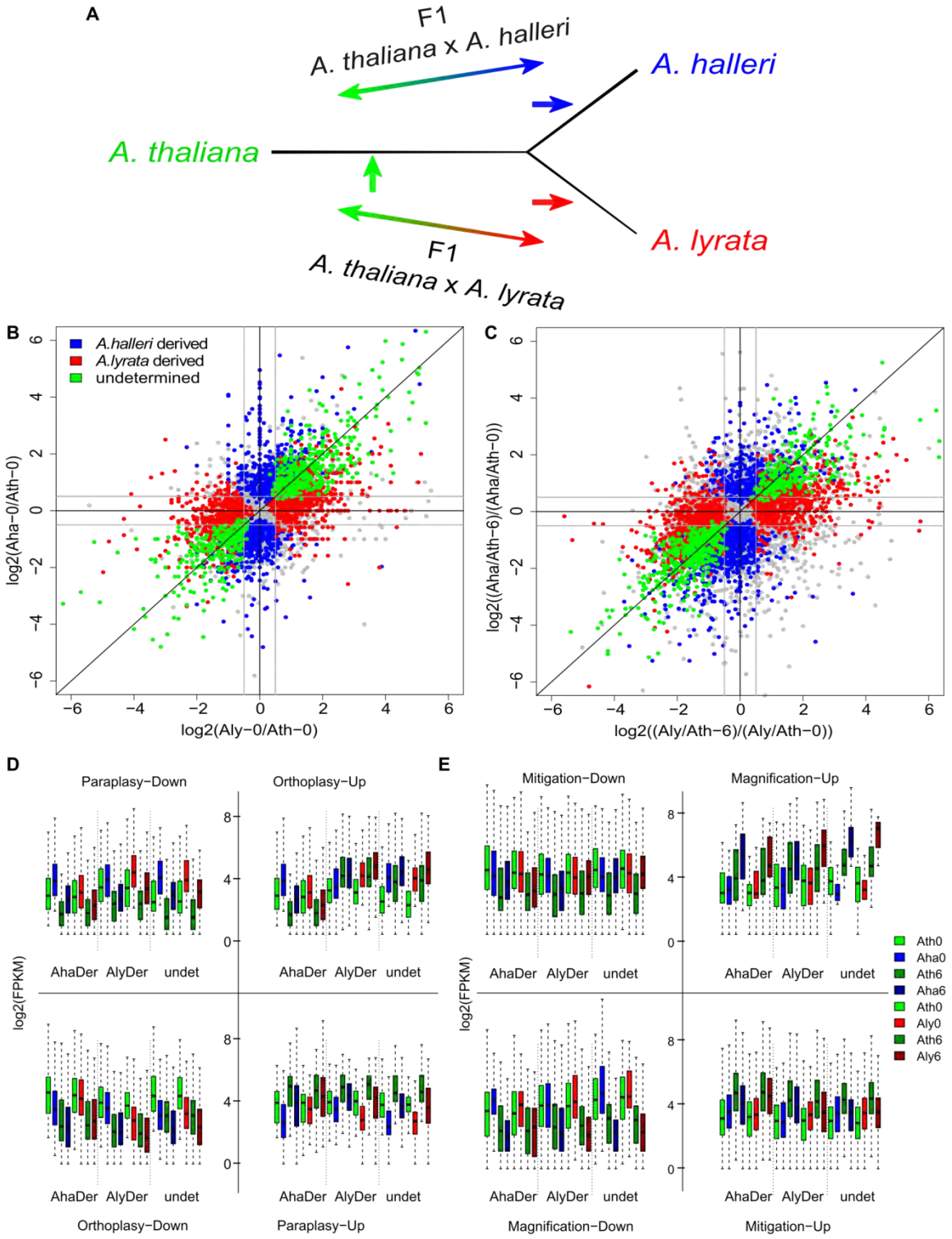

Fig. S9. **The phylogenetic origin of cis-acting changes was determined based on the comparison of allele-specific expression ratios in the two F1 hybrids A. thaliana-A. lyrata and A. thaliana-A. halleri.** (A) Phylogenetic relationship between the three species allowed distinguishing cis-acting changes of undetermined origin (green arrow), which are shared in the two F1 hybrids, from the derived cis-acting changes, which are specific (blue and red arrow). Also shown in Fig. 3C. (B) basal and (C) plastic cis-acting changes. Log2 allelic ratios in AthxAha hybrids plotted against log2 allelic ratios in AthxAly hybrids in basal cis mutations (B), genes with an undetermined *cis*-regulatory change are on the diagonal (green), genes with a derived *cis*-regulatory change in *Arabidopsis lyrata* are on the horizontal central lines (red) and genes with a derived *cis*-regulatory change in *A. halleri* are on the vertical line (blue). Log2 of allelic ratio changes between 6h and 0h in AthxAha hybrids plotted against log2 allelic ratio changes in AthxAly hybrids in plastic cis mutations (C).

The inferred phylogenetic inference of derived cis-acting changes is independent of read count levels. D). The total expression level before stress for the orthoplastic and paraplastic genes in the F1 hybrids. Dark colors are for time point 6h, light colors for time point 0 hours. *A. thaliana*: green, *A. halleri*: blue and *A. lyrata*: red. E). The total expression level before and after stress for the magnified and mitigated genes in the F1 hybrids. Dark colors are for time point 6h, light colors for time point 0 hours. *A. thaliana*: green, *A. halleri*: blue and *A. lyrata*: red.

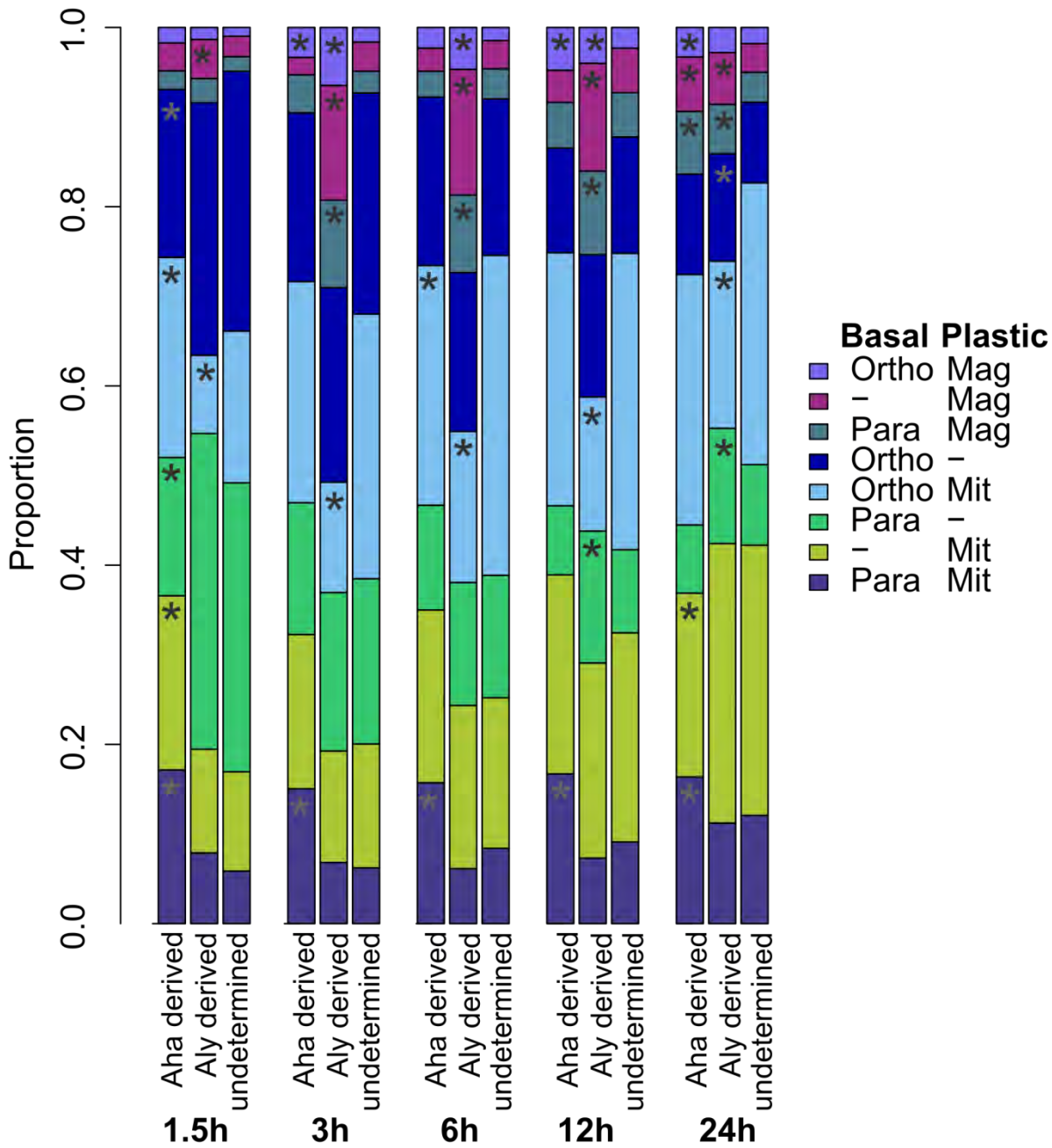

Figure S10. **The pattern reported in fig. 3C is also apparent in samples collected after 3h or 12h.**
Ortho-Mag: orthoplasy and magnification, Ortho-Mit: orthoplasy and mitigation, Para-mit: paraplasy and mitigation, Para-Mag: paraplasy and magnification, Only-Mag: magnification only, Only-mit: Mitigation only, Only-Ortho: Orthoplasy only, Only-Para: Paraplasy only.
The proportion of derived and undetermined *cis*-regulatory modifications observed after 6h within each class of genes combining basal and/or plastic expression changes is similar after 3h or 12h of desiccation stress, at the time points where the largest numbers of genes are observed that show a magnified response to stress. Aha - *A. halleri,* Aly– *A. lyrata*.
"*": Significantly increased or decreased number of derived compared to undetermined changes in *A. halleri* or *A. lyrata* at each time point.

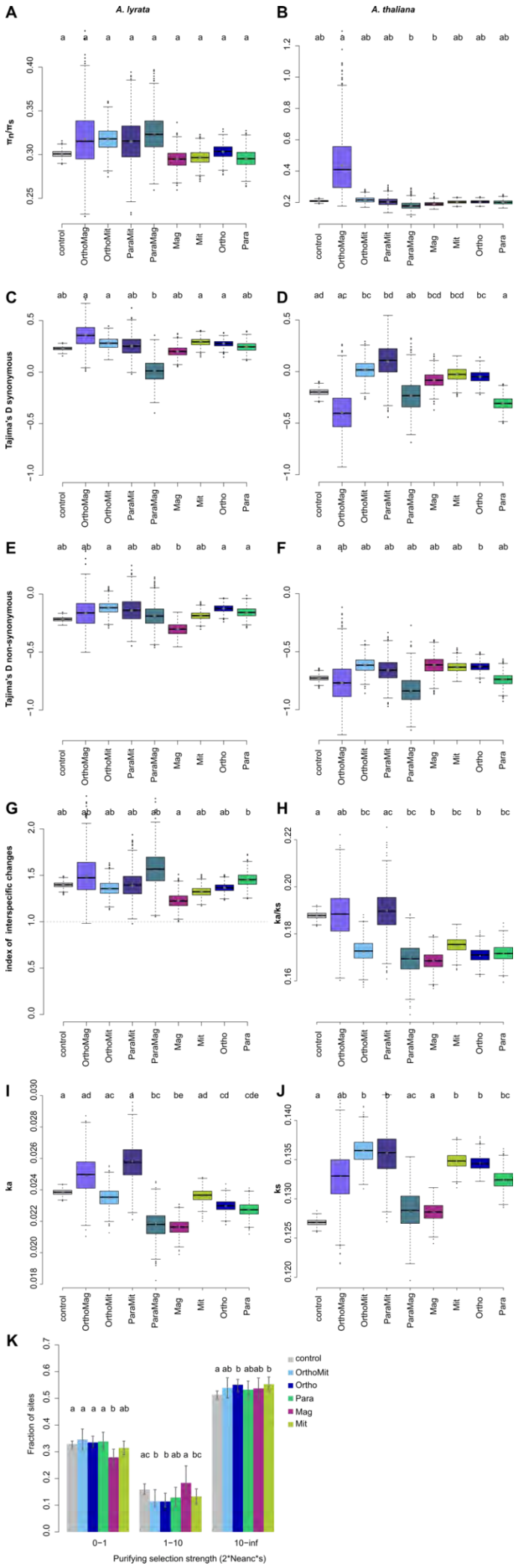

Fig. S11: Summary statistics for the 8 plasticity groups and the control for *A. lyrata* (a,c,e) and *A. thaliana* (b,d,f). Boxplots show 1000 bootstrap replicates of the data for each group.
a) and b) pi_n/pi_s, c and d) Tajima's D for synonymous sites, e and f) Tajima's D for non-synonymous sites. g) index of interspecific change for nonsynonymous sites ((A. lyrata +2) / ((A. thaliana +2)), h) ka/ks between *A. thaliana* and *A. lyrata,* i) ka and j) ks.
The plasticity classes OrthoMag, ParaMit and ParaMag were removed from analysis due to the low number of genes and the high variance between the bootstrap in these categories.
k) binned DFE for the control and 5 of the plasticity classes. 95% conficance intervals based on 200 bootstrap replicates. P-values can be found in Table S14.
No shared letters between plasticity groups mean that there is a significance difference between the groups.

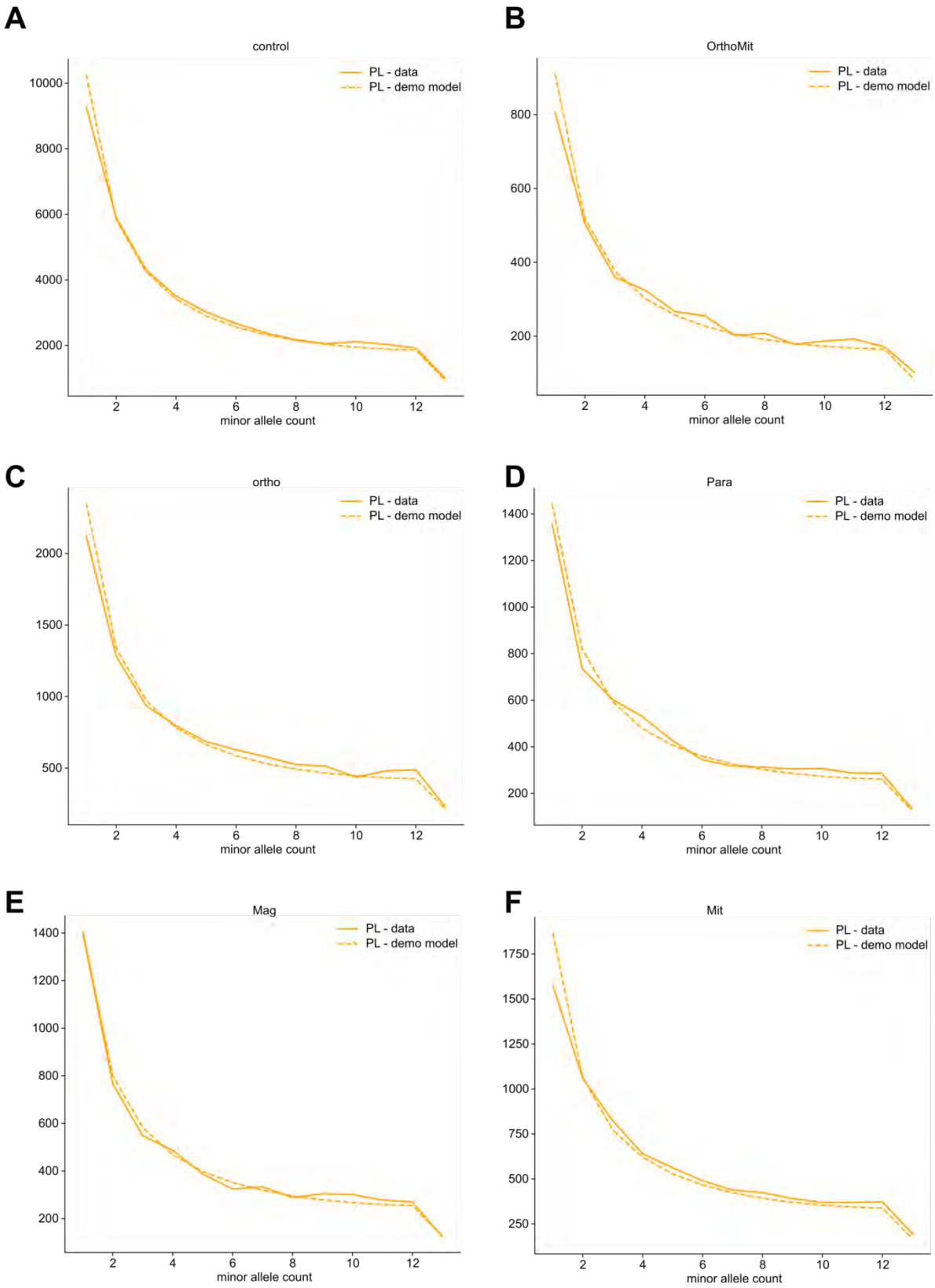

Figure S12. Folded site frequency spectra for synonymous sites based on the data (solid line) and based on the expected number of sites of the demographic model (dashed line) for 5 sufficiently large gene sets, that grouped genes by their mode of plasticity evolution, as well as for the group of control genes, which had the same expression levels at all time points in all three species.

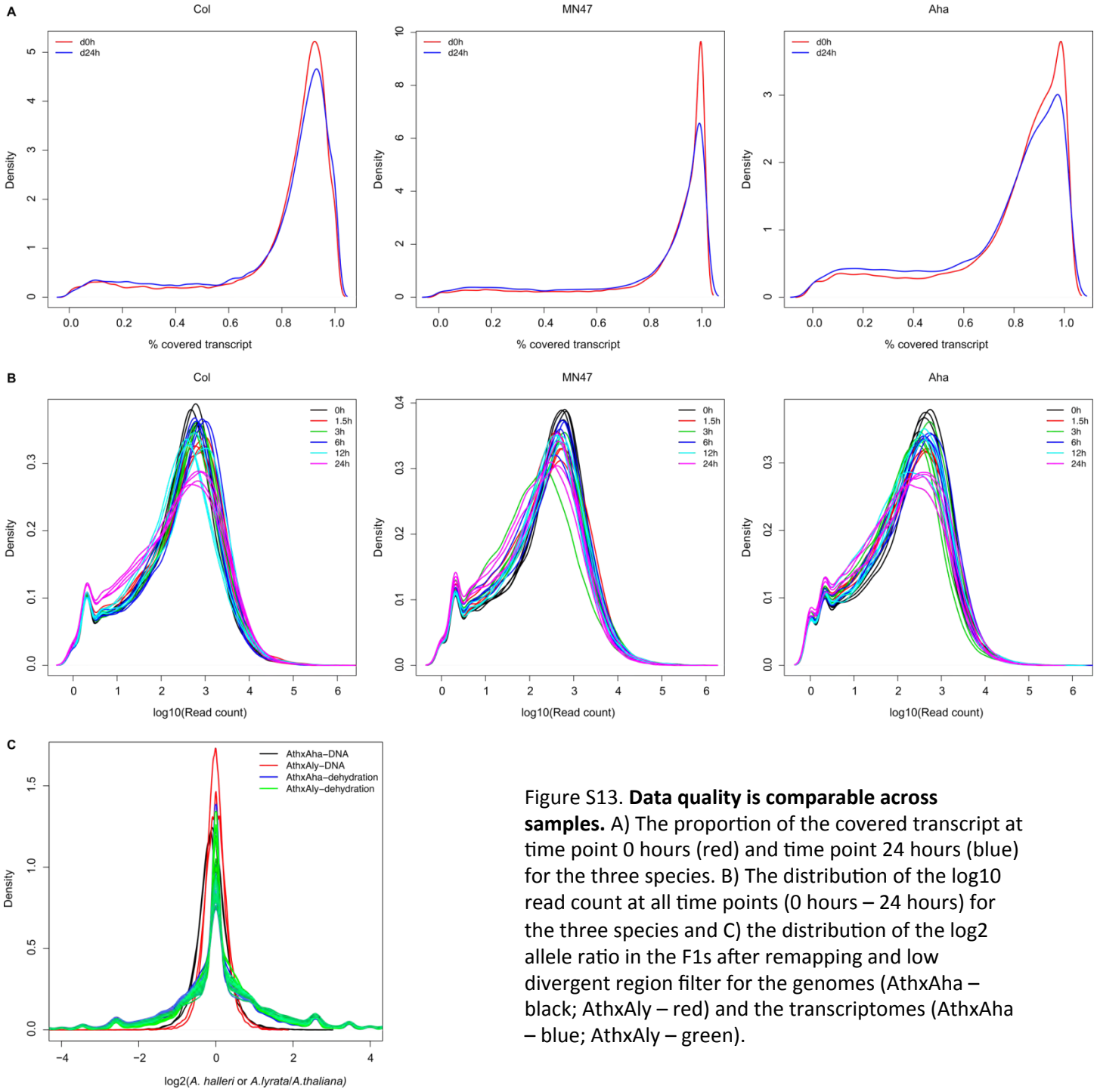

Figure S13. **Data quality is comparable across samples.** A) The proportion of the covered transcript at time point 0 hours (red) and time point 24 hours (blue) for the three species. B) The distribution of the log10 read count at all time points (0 hours – 24 hours) for the three species and C) the distribution of the log2 allele ratio in the F1s after remapping and low divergent region filter for the genomes (AthxAha – black; AthxAly – red) and the transcriptomes (AthxAha – blue; AthxAly – green).

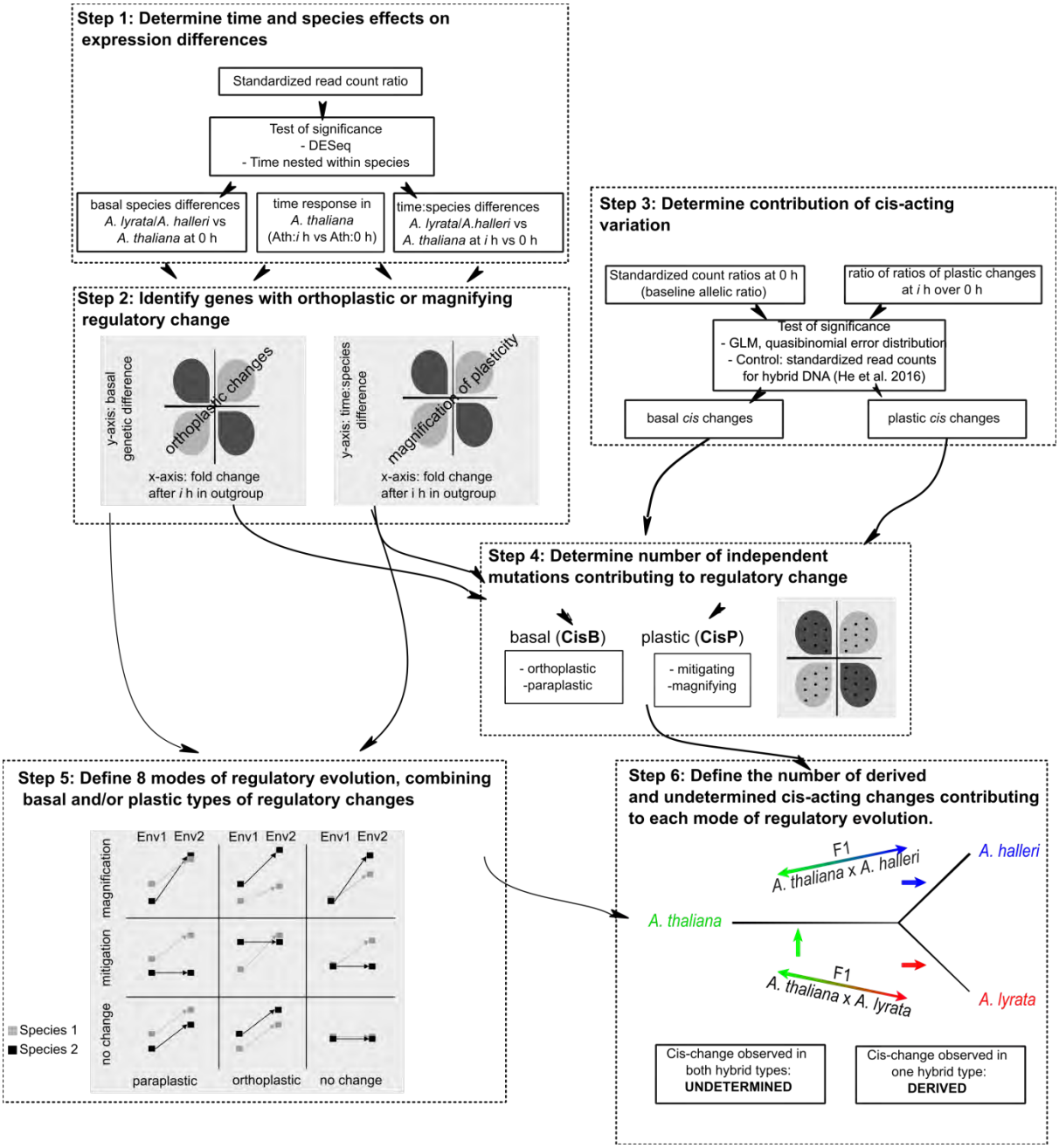

Figure S14. Overview of the analytical pipeline to determine basal and plastic expression changes (step 1, step 2) and infer the number of independent *cis*-changes (Step 3, Step 4). Step 5: integration of analyses describing basal and plastic changes in gene regulation in *A. lyrata* and *A. halleri* to define 8 gene sets. Step 6: inferring the derived state of the *cis*-changes observed.

Supplementary information on materials and methods

Plant materials and stress treatments

Seeds of *A. thaliana* accession Col-0 and *Arabidopsis lyrata* ssp. *lyrata* genotype MN47 were obtained from the Arabidopsis Biological Resource Center (ABRC, USA). Seeds of *A. halleri* h2-2 (Gorno, Italy) were obtained from Pierre Saumitou-Laprade (University of Lille, France). F1 crosses were generated by pollinating emasculated *A. thaliana* flowers with pollen of *A. lyrata* (AthxAly) or *A. halleri* (AthxAhal), as described in de Meaux et al. (2006)[1]. Crosses with *A. thaliana* as the pollen parent were unsuccessful and thus no reciprocal F1s were included. Col-0, MN47, AthxAly and AthxAha F1 hybrids were germinated and grown on germination medium containing Murashige and Skoog salts, 1% sucrose, and 0.8% agar. The plants were stratified for 3 days at 4°C, and then transferred to soil. Plants were grown for 4 weeks. *A. halleri* plants were multiplied by clonal amplification and grown for three weeks. Plants were all randomly placed and grown in a chamber at 20°C under 14 h light/ 16°C 10 h dark under dim light (100 mmol sec±1 m±2). Dehydration treatment was applied in four independent trials performed at one-week intervals and followed a published protocol [2]. Germination or cuttings were staggered over four weeks to ensure that plant size at harvest was comparable across the four trials. For the dehydration treatment, the aerial part of the plants was cut at the base of the roots to mimic a rupture of the water column, as occurs when the plant wilts, and deposited on absorbent paper for 0, 1.5, 3, 6, 12 and 24 hours in the growth chamber under the same growth conditions. Plants were weighed before and after the stress prior to flash freezing for RNA extraction to verify that the rate of water loss was similar for all three species (Fig. S2). The ecological relevance of this treatment was confirmed in a second experiment by determining the different survival rates of the plants in response to the stress (Fig. S2). *A. lyrata* was the only species to survive 24h of acute dehydration, a result in line with its known ability to withstand several days of wilting [3]. Additionally, there was a significant overlap of stress-responsive genes in this experiment to the stress-responsive genes in Bouzid et al. [3] (hypergeometric test, Table S10), where the authors performed a more progressive dry-down experiment conducted in potted plants.

RNA isolation, preparation of cDNA libraries and sequencing

The whole aerial part of parental or hybrid individuals was flash frozen in Eppendorf tubes using liquid nitrogen and homogenized to fine powder in a Homogenizer of Precellys

33 Evolution (Bertin Technologies). Total RNA was extracted in 1 ml of Invitrogen TRIzol Plus
34 RNA Purification System (Thermo Fisher Scientific) and decontaminated with the DNA-free
35 kit (Thermo Fisher Scientific). RNA quality and quantity were examined with the Bioanalyzer
36 2100 (Agilent) and Qubit® 2.0 Fluorometer (Thermo Fisher Scientific). Two microgram of
37 Total RNA was used for library preparation. Library preparation followed the TruSeq®
38 Illumina RNA Sample Preparation v2 Guide. Sequencing was performed on Illumina
39 HiSeq2000 following the manufacturer's protocols, and paired-end 100 bp long reads were
40 obtained. 15.6-21.5 million paired reads for each sample of the parents and 33.4-41.1 million
41 reads for each sample of the hybrids were produced. The sequencing data has been
42 submitted to GENBANK under the study PRJNA640858.
43
44 **Read processing**
45 After FastQC quality check (www.bioinformatics.babraham.ac.uk/projects/fastqc/), the
46 FastX-toolkit was used for sequence trimming and filtering. Low-quality nucleotides were
47 removed from the 3' ends of the sequences (fastq_quality_trimmer -Q 33 -t20 -l 50).
48 Sequences were reverse-complemented by fastx_reverse_complement, and 5' ends were
49 cut following the parameter described above, before being reverse-complemented back to
50 the original direction of reads. Reads with less than 90% bases above quality threshold
51 (fastq_quality_filter -Q 33 -q 20 -p 90), and paired-end reads with a single valid end were
52 removed by a custom R script. Trimmed and filtered reads were saved for further analysis.
53 For *A. halleri,* we generated a pseudo-genome using the *A. lyrata* genome and an assembly
54 of the *A. halleri* transcriptome. For this, the *A. halleri* transcriptome was first assembled as
55 described below using the bulk of 24 *A. halleri* transcriptomes yielding a total of 456 million
56 paired-end reads generated in this study. After verifying assembly quality (91% transcripts
57 are single-copy orthologues), we used the assembly to generate a pseudo-reference genome
58 for *A. halleri*.
59
60 The *A. halleri* pseudo-reference genome was generated by aligning the *A. halleri*
61 transcriptome to the *A. lyrata* genome with BLAT[4], calling the SNP variants after the
62 alignment and substituting them on the *A. lyrata* genome with a custom script. The
63 proportion of mapped reads for samples of *A. halleri* and its hybrid using *A. halleri* pseudo
64 genome was similar to the proportion of mapped reads for samples of *A. lyrata* and its

65 hybrid obtained using *A. lyrata* genome as a reference (see Table S11, Fig. S13). When *A.*
66 *lyrata* is used as a reference for *A. halleri* transcriptome, ~70% of the total reads map to the
67 reference. However, 90% of the *A. halleri* reads can be uniquely mapped on the *A. halleri*
68 pseudogenome, a percentage of mapped reads comparable to the percentage obtained for
69 *A. lyrata* reads mapped to the *A. lyrata* reference (~90%). For the hybrids, using a hybrid
70 pseudo-reference genome based on the two parental species increased read mapping from
71 ~80 to ~90% for both the *A. thaliana x A. halleri* and *A. thaliana x A. lyrata* transcriptome,
72 decreased mapping bias and improved the quantification of allele-specific expression (Fig.
73 S13C and below).
74
75 ***A. halleri* transcriptome assembly**
76 An assembly of the transcriptome of *A. halleri* was generated using a total of 456 million
77 paired-end reads generated from all A. halleri transcriptome samples during the desiccation
78 time-course. For this, transcriptome reads were first concatenated into two groups (*_left.fq
79 > all_left_reads.fq; *_right.fq > all_right_reads.fq). These reads were then mapped to the
80 rDNA/TEs/REs plant databases SILVA (https://www.arb-silva.de) and MIPS PlantsDB[5] using
81 STAR version 2.5.0c [6]. All reads mapping to rRNA and TEs were removed using SAMtools
82 version 1.8 (samtools fastq -f 4, Li 2011 Bioinformatics) and read errors were corrected using
83 CORAL version 1.4.1[7]. We further removed low quality reads and unpaired reads using
84 TRIMMOMATIC version 0.36[8].For the remaining reads, we normalized read counts using the
85 trinity-in-silico-normalize function and then preformed a de novo assembly using TRINITY
86 version 2.2.0 [9].The assembly was then filtered based on IsoPct and length (the longest
87 isoform with the highest IsoPct) using RSEM version 1.3.0[10] and duplicated contigs were
88 removed using CAP3 [11]. Using BUSCO version 1.2 [12], we confirmed the high quality of the
89 assembly we obtained (90% single-copy).
90
91 **Parental and hybrid gene expression**
92 The complete analytical pipeline is summarized in Fig. S1 and S14. For *A. thaliana, A. lyrata*
93 and *A. halleri* samples, all the reads were mapped to their own genomes using STAR with
94 default parameters [6]. Read count numbers were calculated by HTSeq-count [13] and
95 differential gene expression was analyzed using DESeq2 [14] with a nested model (Expression ~
96 Species/time). Read counts were computed as number of fragments per kilobase per million

97 reads (FPKM). Variance in standardized read counts did not increase with the duration of
98 stress, thus indicating that the reaction to acute stress remained tightly controlled
99 throughout the stress treatment (Fig. S4F). A Spearman correlation coefficient was
100 computed for each gene, based on gene rank for read count, to quantify the among sample
101 correlation in expression level reported in Fig. S4C-E. The significance of gene expression
102 variation over time (plasticity), gene expression variation across species (genetic variation)
103 and gene expression variation as a result of time nested within species (genetic variation in
104 plasticity) was tested with a generalized linear model implemented in DESeq with read count
105 as dependent variable and time nested within species as independent variable (note that
106 DESeq normalizes read counts across samples). Gene expression differences at 0h (e.g. just
107 before initiation of the stress) were assumed to be constitutive and reflect basal expression
108 level (Table S2-S6). Gene expression levels measured 1.5 to 24h after initiation of the stress
109 were standardized to gene expression differences at 0h to quantify variation in gene
110 expression plasticity. The significance of the plasticity was tested with the CONTRAST feature
111 of the RESULTS function of DESeq2 [14]. Differences in plasticity between species were further
112 computed as the ratio of gene expression plasticity (e.g. ratio of ratio of allele count at 6h
113 after stress over 0h) measured in each species (Table S2-S6). In *A. lyrata* and *A. halleri*, genes
114 with species-specific differences in plasticity overlapped significantly with plasticity
115 differences reported after exposing plants to progressive soil dehydration [3].
116
117 To define the genetic basis of gene expression change, we determined allele-specific
118 expression levels within the F1 hybrids obtained from crossing each of the two sister species
119 *A. lyrata* and *A. halleri* with their outgroup relative *A. thaliana* as described in *de Meaux et*
120 *al., 2006*[1] (Fig. S1, S14). Allele specific expression was quantified as previously described [15]
121 with the exception that the pseudo-reference genome of *A. halleri* was used to map *A.*
122 *halleri* parental reads of the *A. hallerixA.thaliana* F1 hybrids. Hybrid DNA was used to design
123 a pipeline minimizing mapping differences between alleles, and subsequently infer allele-
124 specific read counts [15]. P-values were adjusted to control the false discovery rate (FDR
125 correction[16]). An FDR threshold of 5% was fixed to call significant ASE genes.
126
127 **Inferring *cis*-acting contribution to parental differences**

128 We analyzed separately constitutive expression difference (i.e. allele or parental expression
129 changes measured at time point 0, before initiation of the stress), and differences in
130 expression plasticity, to account for interspecific differences in the timing and magnitude of
131 gene activation or repression (Fig. 1A, E, Fig. S1, Fig. S14). Low expression genes in either
132 parent or hybrid (mean count <10) were removed from the analysis.
133
134 Comparing parental and allele-specific expression differences, we defined three categories
135 of gene expression changes, akin to the categories defined by Wittkopp et al. (2004)[17]. We
136 first defined basal expression differences and their genetic architecture (Fig. S14, Fig. S7). If
137 parental genotypes differed significantly in expression (FDR<0.05) while no significant
138 difference in allele-specific expression (ASE) was detected in their hybrid (FDR>0.05), the
139 genetic basis of the expression change was defined as *trans*-only change. If significant
140 differences were detected both between parental genotypes (FDR>0.05) and between
141 alleles in the hybrid (FDR>0.05), a *cis*-acting variant was inferred to contribute to the
142 expression change. We did not consider genes where *ASE* was detected in the absence of a
143 significant parental difference (Fig. S7, purple). Indeed, since alleles are expressed in the
144 same environment in F1s, we have greater power to detect allele specific differences than
145 differences between parents. We also did not consider the (rare) genes where the preferred
146 expressed alleles in the hybrid did not belong to the parent expressing the gene at the
147 highest level (Fig. S7).
148 We then determined genes with a plastic *cis*-acting variant as the genes for which a change
149 in allele-specific expression contributed to change in the plastic response after initiation of
150 the stress (Fig. S7, S14, Table S7). For the analysis of variation in plasticity of gene expression
151 during stress, we used the ratio of plastic expression after 1.5-24h of stress over gene
152 expression at 0h. Significant difference was determined in a single GLM model with quasi-
153 binomial distribution nesting the duration of stress within hybrid type or parental difference.
154 For the hybrids, we used the ratio of the ASE ratio at 1.5-24h of stress vs the ratio of ASE at
155 time point 0h (tp 0, FDR < 0.1). Genetic variants of expression plasticity in response to stress
156 were called significant at FDR<0.1 (Fig. S7 – xh vs 0h).
157
158 **Testing for the impact of plasticity on the direction of gene expression evolution**

159 *A. thaliana* genes with a significantly higher or lower expression after 1.5h-24h compared to
160 0h (FDR<0.05) were defined as plastic and partitioned into up-regulated or down-regulated
161 genes. To test for the impact of the direction of plasticity on the direction of evolutionary
162 change in basal expression level, we used the plastic response of *A. thaliana* as a proxy for
163 the ancestral direction of plastic stress response, since this species forms an outgroup to the
164 sister species *A. halleri* and *A. lyrata.* We then determined whether changes in basal
165 expression in each species were orthoplastic (i.e. in the direction of ancestral plasticity) or
166 paraplastic (i.e. in the direction opposite to ancestral plasticity).
167 For each gene, we plotted the ratio of expression level of *A. lyrata or A. halleri* over the fold
168 change in expression observed in *A. thaliana* (e.g. interspecific evolutionary change in
169 expression significant at FDR 0.05) as a function of the plastic response in *A. thaliana*
170 (significant stress response at FDR = 0.05) (Fig 1A). We then counted the number of genes
171 whose interspecific difference in basal expression (at 0h, before initiation of the stress) was
172 in the direction of the stress response in *A. thaliana* (orthoplastic) or opposite (paraplastic).
173 We subsequently used a chi-squared test with 1 degree of freedom to test whether gene
174 counts were randomly distributed in the 4 quadrants of the plots, thereby estimating the
175 proportion of genes evolving by orthoplasy, given their probability to be up- or down-
176 regulated by stress (Fig 1 A-C). We subsequently used a chi-squared test with 1 degree of
177 freedom to test whether the proportion of genes with a detectable *cis*-acting variant were
178 randomly distributed among these genes. Finally, we compared the distribution in each
179 quadrant in *A. lyrata* with the distribution expected based on the distribution observed in *A.*
180 *halleri,* and used a chi-squared test with 4 degrees of freedom to determine significant
181 differences in the contribution of cis- acting variants to the modification of basal expression
182 levels.
183 A similar approach was used to test for the impact of the direction of plasticity on the
184 direction of evolutionary change in plasticity*, i.e*. the ratio of gene expression at 1.5-24h vs.
185 0h in *A. lyrata* or *A. halleri*, divided by the ratio of gene expression at 1.5-24h vs. 0h in *A.*
186 *thaliana* (Significant plasticity changes were taken at FDR 0.1, Fig. 1E-G). In this way, we
187 computed the proportion of genes evolving by a magnified vs. mitigated plastic response as
188 well as the contribution of *cis*-acting variants to these evolutionary outcomes (Fig. S1H).
189

190 **Comparative analysis of the distribution of fitness effects in *A. lyrata* and *A. thaliana***

191 For analyzing the strength of selection, whole genome data for 16 *A. lyrata* individuals
192 collected in Plech (Germany) were taken from Takou et al. 2019[18], and 40 *A. thaliana*
193 individuals from Spain were taken from 1001 genomes [19]. Individuals were randomly down-
194 sampled to have the same number of observed alleles for all sites. Vcf files were filtered to
195 remove Indels, sites with more than two alleles, sites with coverage < 10, genotype quality <
196 20 or quality < 30 as well as positions with more than 80% of the individuals missing data, as
197 described in Takou et al. 2019[18]. We focused on orthologous genes in this dataset that had
198 polymorphism information in both species. Synonymous and nonsynonymous positions were
199 retained and folded site frequency spectra were generated for non-overlapping groups of
200 genes defined for each mode of plasticity evolution using R (Fig. S12). As an additional
201 control, we included a group of plastic genes, whose plastic reaction did not change
202 between *A. lyrata* and *A. thaliana.* Additionally, summary statistics (π_n/π_s, Tajima's D, π)
203 were calculated using custom R scripts for each of the categories. Ka and Ks were calculated
204 using an alignment of *A. lyrata* and *A. thaliana* as described in He et al. [15], with the "kaks"
205 function of R package seqinr. For each summary statistic 1000 bootstrap replicates were
206 generated by resampling the genes with replacement, and recalculating the statistics over all
207 genes. An index of interspecific changes was calculated by the ratio between the species
208 ((D_{Aly}+2)/ (D_{Ath}+2)) for the bootstrap replicates. Differences between plasticity groups were
209 assessed by calculating if the difference between the groups significantly differed from 0 for
210 each bootstrap. Note that for three mode of plasticity evolution (paraplasy-mitigation,
211 orthoplasy-magnification, paraplasy-magnification), the number of genes was small and
212 bootstraps revealed high variance. Those mode of plasticity evolution were therefore not
213 further considered in the analysis.

214 The program fitδaδi [20] was used to estimate the distribution of fitness effects. This extension
215 to the δaδi program [21], which infers demographic history and infers selection based on
216 genomic data, allows us to specify the demographic model when inferring selection. In short,
217 we are using the simplified demographic model for the *A. lyrata* Plech population based on
218 the demographic model in Takou et al. 2019[18]. The synonymous population scaled mutation
219 rate was estimated using the δaδi function Inference.optimal_sfs_scaling and multiplied by
220 2.76 to get the nonsynonymous mutation rate. The nonsynonymous SFS was then used to
221 estimate the selection parameters which are given by the shape and scale parameter of the

222 gamma distribution. 200 bootstrap replicates of the resampled SFS were used to get 95%
223 confidence intervals for the gamma distribution parameters.
224
225 **Inferring the phylogenetic origin of *cis*-acting variants and their lineage-specific rate of**
226 **accumulation**
227 To infer the phylogenetic origin of *cis*-acting modifications, we defined *cis* acting changes as
228 ancestral if they were shared between the two types of F1 hybrids, which shared *A. thaliana*
229 as an outgroup. If the *cis*-acting variant was specific to one of the hybrids, this change was
230 considered to be derived in the corresponding parent, i.e *A. lyrata*- or *A. halleri*-derived.
231 Genes that displayed a change in plasticity contributed by at least one *cis*-acting
232 modification (e.g. basal *cis*-change, plastic *cis*-change or both) were considered as
233 independent variants (although they could have been controlled by more than one
234 mutation) and were assigned to one of the eight combinations of basal change (invariable /
235 Orthoplasy / Paraplasy) and plastic change (invariable /Magnification / Mitigation): Paraplasy
236 Magnification, Paraplasy Mitigation, Paraplasy only, Orthoplasy Magnification, Orthoplasy
237 Mitigation, Orthoplasy only, Magnification only, and Migation only (Table S12). Genes with
238 *cis*-acting variants that were shared in both comparisons of *A. halleri* or *A. lyrata* to *A.*
239 *thaliana* were inferred as ancestral and differentiated from the lineage-specific derived *cis*-
240 acting variants. A chi-squared test with seven degrees of freedom was performed to test
241 whether the proportion of derived *cis*-acting variants in one of the lineages was different
242 from either the distribution expected based on the other lineage or on the distribution of
243 ancestral *cis* acting changes.
244
245
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