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

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Short title: The effect of plasticity on cis-regulatory evolution

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 reaction promotes survival in a new environment, then plasticity is adaptive under these conditions. 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]. This may include the selection of novel alleles that prevent the phenotype resulting from the plastic reaction 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[26–33], and mutations can have functional effects whose size or direction is not uniformly distributed[31–28]. The study of allele-specific expression in F1 hybrids, instead, offers a powerful avenue to identify myriads of cis-regulatory variants that evolved independently at each transcribed locus[27,29,34-38]. With such a population of variants, it becomes possible to compare rates of evolution among lineages and distinguish the action of natural selection from the random accumulation of new

62 regulatory mutations. The study of cis-regulatory divergence can therefore help explore the
63 general rules governing the evolution of transcriptional plasticity.
64
65 To this aim, we investigate how cis-acting modifications have shaped the divergence in gene
66 expression of the sister species *A. lyrata* and *A. halleri* in reaction to stress triggered by acute
67 dehydration. The ecology of these species predicts that the reaction to dehydration stress
68 has the most relevance in *A. lyrata*, which has the highest tolerance to dehydration[34]. In
69 contrast, the reaction of *A. halleri* to stress is expected to be less relevant, because this
70 species grows in highly competitive environments, and prioritizes its growth over its stress
71 response[39]. Here, we describe how a total of 6360 and 6780 cis-acting regulatory variants
72 identified in *A. lyrata* and *A. halleri,* respectively, contribute to reshape the transcriptome
73 reaction to stress. Results indicate that mutations increasing expression plasticity to
74 dehydration stress were favored in *A. lyrata*, whereas mutations decreasing stress plasticity
75 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. 1A).
82 Plants were grown under controlled conditions for four weeks and then exposed to
83 dehydration by cutting the aerial part of the plant at the base of the root (see
84 Supplementary Methods for details). This treatment mimicked a rupture of the water
85 column. Our experimental design allowed us to investigate different aspects of
86 expression changes in regards to dehydration stress. In detail, this includes the
87 significance of gene expression variation over time (plasticity), gene expression variation
88 across species (genetic variation) and gene expression variation as a result of time
89 nested within species (genetic variation in plasticity). The inclusion of F1 hybrids in the
90 experiment allowed us to simultaneously determine the contribution of cis-regulatory
91 changes (Fig. 1B)
92 Compared to *A. halleri* and *A. thaliana*, we confirmed that the drought-adapted species
93 *A. lyrata* had a higher survival rate, despite being exposed to the same level of

94 dehydration (Fig. S1, Supplementary Data 1, 2), and that gene expression changes
95 overlapped with those observed in a previous study where *A. halleri* and *A. lyrata* were
96 sampled at the onset of wilting after exposure to more progressive drought stress (Table
97 S1). In this species, we therefore expect that plastic reactions in gene expression will
98 have been selected to restore homeostasis, whereas maladaptive responses that
99 manifest as physiological and cellular stress will have been disfavored[40,41]. A principal
100 component analysis showed that samples grouped by species and by the duration of
101 exposure to dehydration (Fig. S2). We detected marked differences in the stress
102 reactions of each species, both in initial expression levels and in the slope of the
103 response (Fig. 1 C,D), although gene expression fold changes were strongly correlated
104 between the species pairs (Fig. 1 E-G). Among the genes whose expression was
105 increased or decreased in response to dehydration (plastic genes), we found that,
106 depending on the pair of species compared, between 39.5-57.9% (Table S2) showed a
107 significant difference in initial expression, i.e. before the onset of dehydration stress. In
108 addition, between 22.7 and 37.4% (depending on the pair of species compared) of the
109 plastic genes showed a significant change in plasticity itself, that is in the response slope
110 to dehydration (false discovery rate – FDR- <0.05, Table S2).
111
112 **Basal expression evolution depends on the direction of plasticity**
113 In the vigorously growing unstressed plants sampled just before initiation of the dehydration
114 treatment, we assume that gene expression reflects the basal expression level. The pattern
115 of up- or down-regulation of gene expression in response to dehydration stress was strongly
116 correlated between species pairs. After 6h of dehydration, for example, 90% of genes that
117 changed expression in *A. thaliana* had also changed expression in the same direction in the
118 other two species (Fig. 1E-G). We thus assume that the direction of gene expression
119 plasticity in *A. thaliana* in response to dehydration is a proxy for the direction of plasticity
120 that existed in the common ancestor of the three species. If the direction in which basal
121 expression levels evolved is independent of such ancestral plasticity, we expect that the
122 direction of interspecific differences in expression and the direction of plasticity will be
123 independent. To test this, we determined the genes in *A. lyrata* and *A. halleri*, whose basal
124 expression level differed significantly from that of *A. thaliana* (there were 3262 and 4634 of
125 these, respectively, FDR<0.05, Supplementary Data 3). Among these, we identified those

126 genes whose basal expression differed from *A thaliana* in the same direction as the plastic
127 reaction to dehydration observed in this outgroup species and called such genetic changes in
128 "orthoplastic" (Fig. 2A). As discussed by Crispo (2007), this term is consistent with the
129 definition proposed by Baldwin (1896). Genetic changes in the opposite direction to the
130 plasticity observed in *A. thaliana* we called "paraplastic" (see Fig. 2A). We note that
131 orthoplastic basal regulatory changes are defined for a given lineage, because they are *de*
132 *facto* paraplastic in the species it is compared to and *vice-versa*. This terminology thus helps
133 describe the evolutionary change in a given species. We observed that a majority of genes in
134 *A. lyrata* (1975/3263, 60.5%, Fig. 2BC) had a basal expression level that was different from *A.*
135 *thaliana* in a direction orthoplastic to the direction of plasticity in *A. thaliana* (Fig. 2D, chi-
136 squared test, p = 2.0e-33). When we compared basal expression levels in *A. halleri* and *A.*
137 *thaliana*, we observed a similar pattern, with 64.4% (2986/4634- Fig2BC, Supplementary
138 Data 3) showing orthoplastic gene expression changes (Figure 2D, chi-squared test, p = 5.2e-
139 86). The predominantly orthoplastic regulatory evolution we observe when comparing *A.*
140 *lyrata* or *A. halleri* to *A. thaliana* was significant at all time points (Supplementary Data 3-7),
141 but was strongest 6h into the stress treatment (Fig. S3A-C, chi-squared tests, minimum p=
142 2.0e-8).
143 The response in *A. thaliana* provides only a proxy for ancestral changes, and it is clear
144 that it may differ from the response that the actual ancestor of *A. lyrata* and *A. halleri*
145 used to deploy. Indeed, *A. thaliana* has a very different ecology and life cycle compared
146 to the other two species and the three species diverged since more than a million years.
147 Thus, we repeated the analysis using the reaction of *A. lyrata* or *A. halleri* for
148 comparison with the evolution of basal gene expression in *A. halleri* and *A. lyrata*,
149 respectively (Fig. S4A-D). The outcome of the analysis was essentially unchanged, a
150 result we expected because the direction of the stress response was largely conserved
151 across species (Fig. 1E-G). We therefore conclude that, for genes responding to
152 dehydration stress, the evolution of basal expression in the Arabidopsis genus is not
153 independent of the direction of plasticity that likely existed before the split between *A.*
154 *lyrata* and *A. halleri*.
155
156 This pattern could plausibly result from a single genetic change, if e.g. species were at
157 different developmental stages or if they differed for only one major regulator [25]. To

158 investigate whether this pattern resulted from more than a few trans-regulatory changes,
159 we used F1 interspecific hybrids to determine how many of these changes were due to cis-
160 acting regulatory modifications. Indeed, in F1 hybrids, alleles are exposed to the same trans-
161 regulatory environment, so that allele-specific difference in expression point to difference in
162 cis-regulation[42]. Cis-acting variation in gene expression can be directly inferred from allele-
163 specific expression differences observed in RNA collected from F1 hybrids between *A.*
164 *thaliana* and either *A. lyrata* or *A. halleri* (Fig. 1B, 2E, S5). The relative expression levels of
165 the alleles within hybrids correlated positively with parental differences in expression (Table
166 S3, Fig. S5). Of the 3262 genes with an altered basal expression in *A. lyrata vs. A. thaliana*,
167 1243 presented a significant cis-acting difference between alleles in the hybrid (FDR 0.05). A
168 majority of these cis-acting modifications (776, 62.4%) were orthoplastic (Odds ratio 1.65,
169 *p*=5.8E-10). In *A. thaliana-A. halleri* F1 hybrids, the total number of basal cis-acting variants
170 distinguishing *A. halleri* from *A. thaliana* was in the same order of magnitude (1112 cis-acting
171 variants at FDR 0.05), 65.6% (730) of which contributed to orthoplastic changes in basal
172 expression (Odds ratio 1.91, *p*=9.6E-14). Therefore, cis-acting regulatory variants were
173 predominantly orthoplastic. Because the allele-specific expression of individual transcripts
174 reveals individual cis-acting variants, we conclude that the orthoplastic evolution of basal
175 gene expression has a polygenic basis, both in *A. lyrata* and in *A. halleri*. The association we
176 observed between the direction of plasticity and the evolution of basal expression levels is
177 the result of many independent regulatory mutations (Fig. S1, Fig. S6A).
178
179
180 **The response is attenuated in *A. lyrata* and *A. halleri*, compared to *A. thaliana***
181 In both *A. lyrata* and *A. halleri,* we further tested whether the direction of genetic change in
182 plasticity was independent of the direction of plasticity in the outgroup species (Fig. 3A). For
183 this, we determined, for each time point *t* of the response, the genes whose plastic response
184 to stress in *A. lyrata* or *A. halleri* had a slope that was significantly different from the
185 response slope measured in *A. thaliana* (Fig. 3B-D). This revealed that for most genes, the
186 plastic responses were attenuated in both *A. halleri* and *A. lyrata*, compared to *A. thaliana*,
187 and, in some rare cases, even reversed (*A. lyrata*: Fig. 3B,D; and *A. lyrata*: Fig. 3C,D). The
188 reaction to stress observed in *A. thaliana* thus tended to be predominantly mitigated in both
189 *A. halleri* and *A. lyrata* (Supplementary Data 3). This pattern was also observed at all other

190 time points in both species (chi-squared test, minimum $p < 1.8E-7$, Fig. S3E-F, Supplementary
191 Data 4-7), confirming that the effect did not result from a delayed stress reaction in one of
192 the species[43]. Interestingly, the pattern was less pronounced in *A. lyrata* (Fig. S3F): we
193 observed 2.5 fold more genes in *A. lyrata* than *A. halleri* with a reaction to stress that was
194 greater than in the outgroup *A. thaliana* after 3-6h of stress (Fig. 3D, Fig, S3E-H).
195 Furthermore, for 578 of the 910 genes whose expression was down-regulated after 6h of
196 stress in *A. halleri*, the slope of the stress reaction was steeper in *A. lyrata* (Fig. S4H). The
197 predominant evolution of a mitigated response to stress in *A. halleri* and the excess of genes
198 showing a magnified response in *A. lyrata* were also observed if we changed the species
199 whose reaction was used as a proxy for the magnitude of ancestral plasticity (Fig. S4E-H).
200
201 As mentioned earlier, interspecific differences in the slope of the reaction to stress could
202 plausibly result from differences in development or from a handful of potentially random
203 changes in regulators of the stress response[25]. We therefore used the interspecific F1
204 hybrids to determine the cis-acting basis of changes in the slope of the plastic response to
205 stress in *A. lyrata* and in *A. halleri*, compared to *A. thaliana*. If the allelic ratio measured
206 within the hybrid were significantly altered after initiation of the stress, we concluded that a
207 cis-acting variant had modified the response of gene expression to stress (Fig. S5). Changes
208 in allelic ratios within the hybrid were positively correlated with parental differences in gene
209 expression plasticity (Fig S5, Table S3). After 6h of dehydration, a significant fold-change
210 difference was apparent between parental species for 2588 genes, 51.4% of which were also
211 observed in hybrids. Of these cis-acting modifications of plasticity in *A. lyrata*, a large
212 majority (72.9%) contributed to mitigating the gene expression response after 6h (Odds ratio
213 2.68, $p<2.2E-16$). In *A. halleri*, the contribution of cis-acting modifications was also strongly
214 skewed towards mitigation (88.1% of 1382, Fig. 3E, Odds ratio 7.37, $p<2.2E-16$). Because the
215 mitigated stress response observed in *A. lyrata* and *A. halleri,* compared to *A. thaliana,* was
216 clearly supported by a large number of cis-acting modifications, we conclude that it is
217 polygenic.
218
219 **Derived** cis-**acting variants reveal interspecific differences in the rate of plasticity**
220 **evolution**

221 A combination of basal expression and plasticity to stress underlies the expression
222 profile of stress responsive genes and thus their molecular effect. Unchanged,
223 orthoplastic or paraplastic basal expression that combine with either unchanged,
224 magnified or mitigated plasticity define eight possible modes of plasticity evolution (Fig.
225 4A). A chi-squared test showed that genes were not distributed uniformly across these
226 modes (Fig. 4A, χ^2=217.4, df = 4, *p* = 6.93e-46, and χ^2= 363.7, df = 4, *p* = 1.89e-77, in *A.*
227 *halleri* and *A. lyrata*, respectively, Supplementary Data 3). However, to understand how
228 the stress response evolves, we must determine how often changes in each of these
229 modes evolve. Our experimental design allows us to compare the rate at which changes
230 in basal expression and slope have evolved in the two sister lineages *A. lyrata* and *A.*
231 *halleri*. In the absence of natural selection, we expect this rate to be similar across
232 lineages. To distinguish derived regulatory changes in each lineage, we assumed
233 parsimony in the evolution of cis-regulation: cis-acting variants detected in only one of
234 the two hybrid types are more likely to have occurred after the split between *A. lyrata*
235 and *A. halleri* (Fig. 4B, Fig. S7). Those detected in both hybrids were called
236 "undetermined" as it is not possible to infer whether they predated the split of the two
237 species or evolved in the outgroup lineage. We note that it is not possible to assign a
238 clear direction of effect for cis-regulatory mutations of undetermined origin. The
239 direction of effect of a derived cis-regulatory variant, instead, can be deduced by
240 comparison with the expression level of the *A. thaliana* allele in the hybrid. We
241 observed that in the sister species, the majority of derived cis-regulatory changes
242 contributed to orthoplastic basal changes in expression and/or to the mitigation of the
243 stress response observed in *A. thaliana* (Fig. 4C, Fig. S6). Cis-regulatory variants,
244 however, tended to remodel plasticity in different ways in *A. halleri* and *A. lyrata*.
245 Grouping genes into the eight modes of plasticity evolution defined previously, we
246 observed that the proportion of derived cis-acting variants in each of the two lineages
247 was not the same across these gene groups (Fig. 4D, χ^2=189.3, df=7 *p*=2.02E-37). The
248 contrast was strongest after 3h and 6h of stress (Fig. S3D,H, Fig. S8). Cis-acting variants
249 in *A. halleri* were proportionately more frequent than in *A. lyrata* among genes
250 combining an orthoplastic basal expression change with a decrease in the slope of the
251 response to stress (Fig. 4D, ortho-mit genes, *p*<1.42E-06 after 6h). This combination
252 corresponds to the genetic assimilation of the plastic response observed by Waddington

253 in his seminal experiment [4,8,11]. We also observed that *A. halleri* genes displaying a
254 paraplastic basal expression change combined with a mitigated response had an excess
255 of derived cis-acting variants (p=3.6E-08). This excess indicates that regulatory
256 modifications opposing the plastic reaction to stress were more favored in *A. halleri*
257 than in *A. lyrata*. The over-representation, in the *A. halleri* lineage, of mutations that
258 reduce plasticity indicates that a greater fraction of the gene expression plasticity
259 observed in the other two species was not adaptive in this lineage.
260
261 In contrast, derived cis-acting variants were enriched in *A. lyrata* among genes
262 displaying a magnification of the stress response, and this was true regardless of
263 whether or not the magnification was combined with a change in basal expression (Fig.
264 4D, partial test χ^2 tests, p=0.003, p=1.4E-19, p=3.9E-08 for ortho-mag, mag-only and
265 para-mag, respectively). Although a magnified response to stress was not the most
266 frequent mode of plasticity evolution identified in this lineage, the comparatively faster
267 rate at which cis-acting variants accumulated among these genes indicates that
268 mutations leading to this mode of plasticity evolution are favored in the *A. lyrata* lineage
269 compared to the *A. halleri* lineage. Interestingly, the functions enriched among them
270 indicate that the regulation of leaf development could be one of the targets of selection
271 for increased plasticity in *A. lyrata*. Gene Ontology categories such as GO:0010207
272 ”Photosystem II Assembly” (p=1.6E-06), GO:009965 ”Leaf Morphogenesis” (p=8.4E-05),
273 GO:0010103 “Stomatal Complex Morphogenesis” (p= 4.5E-06) or GO: 0034968 “Histone
274 Lysine Methylation” (p=4.5E-03) were enriched (Table S4, S5).
275
276 **Genes with magnified plastic responses in *A. lyrata* evolve under increased**
277 **evolutionary constraints**
278 The accumulation of derived cis-acting modifications indicates that the expression of
279 genes displaying magnified plasticity after 6h of stress evolves under positive selection
280 in *A. lyrata*. If such genes with magnified plasticity are subject to stronger selection
281 overall in this lineage, we also might expect that the genes will be exposed to stronger
282 evolutionary constraints than other plastic genes. We grouped *A. lyrata* genes according
283 to their mode of plasticity and assessed the ratio of the divergence at non-synonymous
284 and synonymous sites (*Ka/Ks*) between *A. lyrata* and *A. thaliana*. We additionally

285 included genes that did not change their expression as a control group and used
286 bootstrapping to determine confidence intervals and identify significant differences
287 between groups. For three of the eight modes of regulatory evolution, the number of
288 genes was small, resulting in high bootstrap variance (Fig. 4A, Fig. S9); these modes were
289 therefore excluded from the analysis. We observed that genes of all remaining modes of
290 plasticity evolution showed a significantly lower *Ka/Ks* than the control group (Fig. 5A).
291 Genes with modified plasticity therefore all experienced stronger evolutionary
292 constraints. Interestingly, however, we observed that the *Ks* of control genes was
293 significantly lower than the *Ks* of all gene groups, with the exception of the genes that
294 evolved a magnified response to stress (Fig. 5BC, Fig. S9H-J). To gain further insight into
295 the evolutionary forces operating on these genes in *A. lyrata,* we examined nucleotide
296 polymorphism within a local *A. lyrata* population[44-46]. The average number of pairwise
297 differences did not differ significantly across groups of genes, and was similar to that of
298 the control genes (Fig. 5D). We also used the Tajima's *D* statistic to quantify the
299 contribution of low-frequency variants to nucleotide diversity within each gene group.
300 Mean *D* for non-synonymous sites differed significantly among modes of plasticity
301 evolution (Fig. 5E), but mean *D* did not differ significantly for synonymous sites (Fig. 5F).
302 *A. lyrata* genes that evolved a magnified plastic reaction, but no basal change in
303 expression, displayed the most negative Tajima's *D* of all modes of plasticity evolution at
304 non-synonymous sites (Fig 5EF). We performed the same analysis on the polymorphism
305 for orthologous gene sets within *A. thaliana.* Compared to *A. lyrata*, Tajima's *D* in *A.*
306 *thaliana* was generally lower, presumably due to its history of population expansion[45].
307 Yet, the distinctive Tajima's *D* of *A. lyrata* genes with a magnified plasticity was specific
308 to *A. lyrata,* because the Tajima's *D* of its orthologous *A. thaliana* genes was not lower
309 than that of orthologous genes in the other plasticity groups (Fig. 5A,G). This finding
310 indicates that amino-acid variants in *A. lyrata* tend to segregate at lower frequencies, if
311 they affect genes with a magnified plasticity (Fig. 5G). To confirm that the amino-acid
312 sequence of genes with a magnified plasticity are subject to stronger evolutionary
313 constraints, we estimated the distribution of fitness effects (DFE) of new non
314 synonymous mutations for each gene group, using the software fitδaδi[47,48]. Parameters
315 of a demographic model for the *A. lyrata* population have been previously determined[46].
316 Population data, simulated under this model by fitδaδi, fitted well with the site

317 frequency spectrum of synonymous variation observed in the population (Fig. S10). The
318 DFE was then subsequently estimated by fitting a gamma distribution of fitness effects
319 to the frequency spectrum of non-synonymous variants[47] (Fig. 5H). This analysis showed
320 that, of all modes of plasticity evolution, the genes with a magnified plastic reaction to
321 stress showed the lowest proportion of variants with weak fitness effects. Bootstrapping
322 confirmed that the predicted proportion of nearly neutral deleterious variants was
323 significantly lower among these genes (Fig. S11). This pattern is in agreement with the
324 higher proportion of low-frequency variants indicated by the lower Tajima's *D* statistic
325 of this group of genes, compared to other plastic genes (Fig 5H, Fig. S11). In conclusion,
326 *A. lyrata* genes that evolved a magnified response after 6h of stress not only
327 accumulated an excess of derived cis-acting variants, they also appear to be subject to
328 increased selective constraints at the amino-acid level.
329
330 Waddington proposed that stabilizing selection around the new optimum (canalization)
331 would favor the genetic assimilation of plastic responses[11]. In *A. lyrata*, the constraints
332 detectable on genes with an assimilated stress response that combine the two
333 predominant types of basal and plastic regulatory changes have neither increased nor
334 decreased (Fig. 5A-D). The fitness effects of new mutations in these genes do not differ
335 significantly from those in genes whose regulation remained unchanged (Fig. 5A-D, Fig.
336 S9). This result indicates that the genetic assimilation of the ancestral plastic response is
337 unlikely to associate with increased canalization at the amino-acid level in *A. lyrata*.
338 Future studies will have to examine whether the excess of cis-acting changes leading to
339 a decrease in plasticity in *A. halleri* reflects selection to lower the energetic cost
340 associated with plasticity or whether it is a result of relaxed constraints on the
341 regulation of the stress response[49].
342
343 The rapid and complex plastic responses plants have evolved allow them to tune reactions to
344 ever-changing environmental stressors, with ecological priorities for survival, growth and
345 reproduction[50]. By documenting the cis-regulatory divergence between *A. halleri* or *A. lyrata*
346 and their common outgroup *A. thaliana,* we show that the divergence of plastic stress
347 responses in the *Arabidopsis* genus has a broad polygenic basis. By comparing the direction
348 of cis-acting changes with the direction of the plastic reaction, we demonstrate the profound

349 impact of plasticity on the accumulation of regulatory mutations in each lineage, lending the
350 strongest support to date for the idea that plasticity can be stepping stone for the evolution
351 of transcription[7]. Previous studies concluded that most transcript changes in reaction to
352 stress were maladaptive because they were predominantly lost during adaptation[18,20–22].
353 Here, we challenge these approaches by using two sources of information to infer the
354 adaptive relevance of regulatory changes in the plastic response to stress. First, by
355 comparing allele-specific expression across the two interspecific hybrids, we estimated
356 lineage-specific rates of accumulation of derived cis-acting variants, an approach that has
357 proven successful in revealing the polygenic response to selection on complex traits [29,48].
358 Second, we used the site frequency spectrum of synonymous and non-synonymous
359 mutations in *A. lyrata* to test whether the fitness effects of new mutations in plastic genes
360 depends on the mode of plasticity evolution[29]. In *A. lyrata*, the lineage that best survived the
361 severe dehydration imposed in this experiment (Fig. S1) and displays the strongest drought
362 tolerance [39,51], both approaches point to the sustained action of polygenic selection for an
363 increase in expression plasticity of many genes, rather than its loss. In *A. halleri*, instead, we
364 did observe an excess of cis-acting variants leading to a decreased transcriptional plasticity
365 to stress. We conclude that plasticity can potentiate the adaptation of gene expression, but
366 its selective remodeling depends on the ecological context of each lineage.
367
368 **Methods**
369 **Plant materials and stress treatments**
370 Seeds of *A. thaliana* accession Col-0 and *Arabidopsis lyrata* ssp. *lyrata* genotype MN47 were
371 obtained from the Arabidopsis Biological Resource Center (ABRC, USA). Seeds of *A. halleri*
372 h2-2 (Gorno, Italy) were obtained from Pierre Saumitou-Laprade (University of Lille, France).
373 Based on previous work, these genotypes are representative of the difference in drought
374 tolerance between the three species[39]. F1 crosses were generated by pollinating
375 emasculated *A. thaliana* flowers with pollen of *A. lyrata* (AthxAly) or *A. halleri* (AthxAhal), as
376 described in de Meaux et al. (2006)[52]. Crosses with *A. thaliana* as the pollen parent were
377 unsuccessful and thus no reciprocal F1s were included.
378 Col-0, MN47, AthxAly and AthxAha F1 hybrids were germinated and grown on germination
379 medium containing Murashige and Skoog salts, 1% sucrose, and 0.8% agar. The plants were
380 stratified for 3 days at 4°C, and then transferred to soil. Plants were grown for 4 weeks. *A.*

381 *halleri* plants were multiplied by clonal amplification and grown for three weeks. Plants were
382 all randomly placed and grown in a chamber at 20°C under 14 h light/ 16°C 10 h dark under
383 dim light (100 mmol sec±1 m±2). Dehydration treatment was applied in four independent
384 trials performed at one-week intervals and followed a published protocol [53]. Germination or
385 cuttings were staggered over four weeks to ensure that plant size at harvest was comparable
386 across the four trials. For the dehydration treatment, the aerial part of the plants was cut at
387 the base of the roots to mimic a rupture of the water column, as occurs when the plant wilts,
388 and deposited on absorbent paper for 0, 1.5, 3, 6, 12 and 24 hours in the growth chamber
389 under the same growth conditions. Plants were weighed before and after the stress prior to
390 flash freezing for RNA extraction to verify that the rate of water loss was similar for all three
391 species (Fig. S1). The ecological relevance of this treatment was confirmed in a second
392 experiment by determining the different survival rates of the plants in response to the stress
393 (Fig. S1). *A. lyrata* was the only species to survive 24h of acute dehydration, a result in line
394 with its known ability to withstand several days of wilting[39]. Additionally, there was a
395 significant overlap of stress-responsive genes in this experiment to the stress-responsive
396 genes in Bouzid et al. [39] (hypergeometric test, Table S1), where the authors performed a
397 more progressive dry-down experiment conducted in potted plants.
398
399 **RNA isolation, preparation of cDNA libraries and sequencing**
400 The whole aerial part of parental or hybrid individuals was flash frozen in Eppendorf tubes
401 using liquid nitrogen and homogenized to fine powder in a Homogenizer of Precellys
402 Evolution (Bertin Technologies). Total RNA was extracted in 1 ml of Invitrogen TRIzol Plus
403 RNA Purification System (Thermo Fisher Scientific) and decontaminated with the DNA-free
404 kit (Thermo Fisher Scientific). RNA quality and quantity were examined with the Bioanalyzer
405 2100 (Agilent) and Qubit® 2.0 Fluorometer (Thermo Fisher Scientific). Two microgram of
406 Total RNA was used for library preparation. Library preparation followed the TruSeq®
407 Illumina RNA Sample Preparation v2 Guide. Sequencing was performed on Illumina
408 HiSeq2000 following the manufacturer's protocols, and paired-end 100 bp long reads were
409 obtained. 15.6-21.5 million paired reads for each sample of the parents and 33.4-41.1 million
410 reads for each sample of the hybrids were produced.
411
412 **Read processing**

413 After FastQC quality check (www.bioinformatics.babraham.ac.uk/projects/fastqc/), the
414 FastX-toolkit was used for sequence trimming and filtering. Low-quality nucleotides were
415 removed from the 3' ends of the sequences (fastq_quality_trimmer -Q 33 -t20 -l 50).
416 Sequences were reverse-complemented by fastx_reverse_complement, and 5' ends were
417 cut following the parameter described above, before being reverse-complemented back to
418 the original direction of reads. Reads with less than 90% bases above quality threshold
419 (fastq_quality_filter -Q 33 -q 20 -p 90), and paired-end reads with a single valid end were
420 removed by a custom R script. Trimmed and filtered reads were saved for further analysis.
421 For *A. halleri,* we generated a pseudo-genome using the *A. lyrata* genome and an assembly
422 of the *A. halleri* transcriptome. For this, the *A. halleri* transcriptome was first assembled as
423 described below using the bulk of 24 *A. halleri* transcriptomes yielding a total of 456 million
424 paired-end reads generated in this study. After verifying assembly quality (91% transcripts
425 are single-copy orthologues), we used the assembly to generate a pseudo-reference genome
426 for *A. halleri*.
427
428 The *A. halleri* pseudo-reference genome was generated by aligning the *A. halleri*
429 transcriptome to the *A. lyrata* genome with BLAT[54], calling the SNP variants after the
430 alignment and substituting them on the *A. lyrata* genome with a custom script. The
431 proportion of mapped reads for samples of *A. halleri* and its hybrid using *A. halleri* pseudo
432 genome was similar to the proportion of mapped reads for samples of *A. lyrata* and its
433 hybrid obtained using *A. lyrata* genome as a reference (see Table S6, Fig. S12). When *A.*
434 *lyrata* is used as a reference for *A. halleri* transcriptome, ~70% of the total reads map to the
435 reference. However, 90% of the *A. halleri* reads can be uniquely mapped on the *A. halleri*
436 pseudogenome, a percentage of mapped reads comparable to the percentage obtained for
437 *A. lyrata* reads mapped to the *A. lyrata* reference (~90%). For the hybrids, using a hybrid
438 pseudo-reference genome based on the two parental species increased read mapping from
439 ~80 to ~90% for both the *A. thaliana x A. halleri* and *A. thaliana x A. lyrata* transcriptome,
440 decreased mapping bias and improved the quantification of allele-specific expression (Fig.
441 S12C and below).
442
443 ***A. halleri* transcriptome assembly**

444 An assembly of the transcriptome of *A. halleri* was generated using a total of 456 million
445 paired-end reads generated from all A. halleri transcriptome samples during the desiccation
446 time-course. For this, transcriptome reads were first concatenated into two groups (*_left.fq
447 > all_left_reads.fq; *_right.fq > all_right_reads.fq). These reads were then mapped to the
448 rDNA/TEs/REs plant databases SILVA (https://www.arb-silva.de) and MIPS PlantsDB[55] using
449 STAR version 2.5.0c [56]. All reads mapping to rRNA and TEs were removed using SAMtools
450 version 1.8 (samtools fastq -f 4, Li 2011 Bioinformatics) and read errors were corrected using
451 CORAL version 1.4.1[57]. We further removed low quality reads and unpaired reads using
452 TRIMMOMATIC version 0.36[58]. For the remaining reads, we normalized read counts using
453 the trinity-in-silico-normalize function and then preformed a de novo assembly using
454 TRINITY version 2.2.0 [59].The assembly was then filtered based on IsoPct and length (the
455 longest isoform with the highest IsoPct) using RSEM version 1.3.0[60] and duplicated contigs
456 were removed using CAP3 [61]. Using BUSCO version 1.2 [62], we confirmed the high quality of
457 the assembly we obtained (91% single-copy).
458
459 **Parental and hybrid gene expression**
460 The complete analytical pipeline is summarized in Fig. 1 A,B and S13. For *A. thaliana, A.*
461 *lyrata* and *A. halleri* samples, all the reads were mapped to their own genomes using STAR
462 with default parameters [56]. Read count numbers were calculated by HTSeq-count [63] and
463 differential gene expression was analyzed using DESeq2 [64] with a nested model (Expression ~
464 Species/time). Read counts were computed as number of fragments per kilobase per million
465 reads (FPKM). Variance in standardized read counts did not increase with the duration of
466 stress, thus indicating that the reaction to acute stress remained tightly controlled
467 throughout the stress treatment (Fig. S11). A Spearman correlation coefficient was
468 computed for each gene, based on gene rank for read count, to quantify the among sample
469 correlation in expression level reported in Fig. 1. The significance of gene expression
470 variation over time (plasticity), gene expression variation across species (genetic variation)
471 and gene expression variation as a result of time nested within species (genetic variation in
472 plasticity) was tested with a generalized linear model implemented in DESeq with read count
473 as dependent variable and time nested within species as independent variable (note that
474 DESeq normalizes read counts across samples). Gene expression differences at 0h (e.g. just
475 before initiation of the stress) were assumed to be constitutive and reflect basal expression

476 level (Supplementary Data 3-7). Gene expression plasticity was quantified for each time
477 point *t* of the time series as the slope of the response to stress after *t* hours. For this we
478 computed the ratio of gene expression at time point *t* divided by gene expression at time
479 point t_0= 0h. The significance of the plastic response was determined with the CONTRAST
480 feature of the RESULTS function of DESeq2 [64]. Differences in plasticity between species were
481 further computed as the log2ratio of gene expression plasticity measured in each species,
482 that is the ratio of allele count after *t* h over allele count at t_0= 0h in the first species divided
483 by the ratio of allele count after *t* h over allele count at t_0= 0h in the second species (e.g. log2
484 [($Aly_{t=6h}$/ $Aly_{t=oh}$)/ ($Ath_{t=6h}$/ $Ath_{t=oh}$)] - Supplementary Data 3-7). A negative value of this ratio
485 indicates either a weaker response to the stress or a reversal in the direction of the plastic
486 change, where for example a gene would be up-regulated in one species and down-
487 regulated in the other species. The latter case, however, was rare. More than 90% of the
488 genes that responded to stress in *A. thaliana*, responded in the same direction in the other
489 two species (Fig. 1EG). In *A. lyrata* and *A. halleri*, genes with species-specific differences in
490 plasticity overlapped significantly with plasticity differences reported after exposing plants
491 to progressive soil dehydration [39].
492
493 To define the genetic basis of gene expression change, we determined allele-specific
494 expression levels within the F1 hybrids obtained from crossing each of the two sister species
495 *A. lyrata* and *A. halleri* with their outgroup relative *A. thaliana* as first described in *de Meaux*
496 *et al., 2006*[52] (Fig. 1 A,B, S13). Briefly, allelic read counts were determined by mapping the F1
497 hybrid transcriptome to a hybrid genome concatenating the *A. thaliana* Col-0 reference
498 genome (TAIR10, www.arabidopsis.org) with either the *A. lyrata* MN47 reference genome
499 (Araly1[65]) or with the *A. halleri* pseudogenome. The trimmed and filtered reads were
500 mapped to the hybrid genome using STAR version 2.5.0c [55] with the built-in Bowtie2
501 mapping program[66.] Uniquely and high-quality mapping reads were selected by "samtools
502 view -q 10" (-b: output is bam files, -q 10, mapping quality of phred score that means 90%
503 possibility of the mapping is correct). We focused our analysis on orthologs present in each
504 of the three species, *A. thaliana*, A. *lyrata* and *A. halleri*. The Arsly1 genome annotation
505 detects 17846 orthologous genes in *A. thaliana* and *A.lyrata*. The sequences of these
506 orthologs were blasted against the *A. halleri* draft genome sequences of Aha1.1 (Phytozome
507 10.1, Arabidopsis halleri v1.1). Criteria for orthologous genes was set as hit E-value < 1 e -20)

508 and pairs of genes with reciprocal best BLAST hits were defined to be orthologs. A total of
509 528 genes had no detectable orthologs in the *A. halleri* draft genome, resulting in a set of 17
510 318 orthologous genes in the three species.
511 We added several steps to prevent distortion due to mapping bias. First, unmapped *A. halleri*
512 reads were cut in 30 bp-long read fragments and re-mapped to the reference of *A. lyrata*.
513 Second, we used a set of SNPs fixed in the two sister lineages to re-assign reads that had
514 mapped to the wrong parental genome, because the bowtie2 algorithm sometimes maps
515 reads on the wrong parental allele. For this, the orthologous cDNA sequences of *A. thaliana*
516 and *A. lyrata* were aligned using MAFFT[67] and 1.34 million SNPs were located. A collection of
517 *A. halleri* derived SNPs was provided by P. Novikova (MPI-PZ, Cologne). This collection was
518 used to select the 1.02 M SNPs that were fixed in the common ancestor of *A. lyrata* and *A.*
519 *halleri.* This set of SNPs was used to re-assign the parental origin of each read, based on
520 Samtools SNP callings. We also observed that regions close to intron and/or highly divergent
521 segments, often failed to map properly, causing a mapping bias. We therefore quantified
522 relative allelic abundance focusing on SNPs more than 50 bp away from introns, excluding
523 regions with more than 10 SNPs in 200 bp (greater than the mapping parameter of mismatch
524 number 5 in 100 bp) to minimize mapping bias caused by highly divergent (and/or
525 misannotated) gene regions. Loci with a mean final read count less than 10 were discarded
526 from the analysis to exclude low-expressed genes. Finally, we excluded any SNP position for
527 which the total coverage in the DNA samples was less than 5 reads. After all these filters,
528 252454 SNPs were kept here (14.6 SNPs per gene). Final parental read counts at each SNP
529 included both mapped reads and remapped 30bp fragments, and the median allele ratio SNP
530 ratio was computed for each gene. Finally, each parental read count was divided by a size
531 factor (Total read count for each ortholog/ median total read counts for all orthologs[68]). We
532 used previously published DNAseq data for the hybrids[29] to confirm that these filters
533 allowed an effective control of mapping biases.
534 We then identified genes showing significant allele-specific expression (ASE) with a General
535 Linearized Model (GLM) including a quasi-binomial distribution of error. Under this model,
536 the null hypothesis H_0 assumes no difference between RNA and DNA samples (Allele ratio of
537 $\mu_{RNA} = \mu_{DNA}$), the alternative hypothesis H_1 assumes ASE at the gene considered (ASE, Allele
538 ratio of $\mu_{RNA} \neq \mu_{DNA}$). In other words, the null hypothesis H_0 assumes no differences in the
539 allele ratio between RNA and DNA reads, whereas the alternative H_1 assumes ASE. The GLM

540 model used the read counts of each of the two alleles as dependent variable, and tested for
541 the effect of the 7 sample types (DNA, RNA 0h, RNA 1.5h, RNA 3h, RNA 6h, RNA 12h and
542 RNA 24h) nested within each hybrid type. Loci with a mean final read count < 10 were
543 discarded from the analysis to exclude low-expressed genes. Significant ASE genes were
544 defined by contrasting treatment specific allelic ratios to DNA allelic ratios. P-values were
545 adjusted for controlling the false discovery rate (FDR correction [69]). An FDR threshold of 5%
546 was fixed to call significant ASE genes.
547
548 **Inferring** cis-**acting contribution to parental differences**
549 We analyzed separately constitutive expression difference (i.e. allele or parental expression
550 changes measured at time point 0, before initiation of the stress), and differences in
551 expression plasticity, to account for interspecific differences in the timing and magnitude of
552 gene activation or repression (Fig. 1B, Fig. 2A, Fig. 3A, Fig. S13). Low expression genes in
553 either parent or hybrid (mean count <10) were removed from the analysis.
554
555 Comparing parental and allele-specific expression differences, we defined three categories
556 of gene expression changes, akin to the categories defined by Wittkopp et al. (2004)[42]. We
557 first defined basal expression differences and their genetic architecture (Fig. S13, Fig. S5). If
558 parental genotypes differed significantly in expression (FDR<0.05) while no significant
559 difference in allele-specific expression (ASE) was detected in their hybrid (FDR>0.05), the
560 genetic basis of the expression change was defined as trans-only change. If significant
561 differences were detected both between parental genotypes (FDR>0.05) and between
562 alleles in the hybrid (FDR>0.05), a cis-acting variant was inferred to contribute to the
563 expression change. We did not consider genes where *ASE* was detected in the absence of a
564 significant parental difference (Fig. S5, purple). Indeed, since alleles are expressed in the
565 same environment in F1s, we have greater power to detect allele specific differences than
566 differences between parents. We also did not consider the (rare) genes where the preferred
567 expressed alleles in the hybrid did not belong to the parent expressing the gene at the
568 highest level (Fig. S5).
569 We then determined genes with a plastic cis-acting variant as the genes for which a change
570 in allele-specific expression contributed to change in the plastic response after initiation of
571 the stress (Fig. S5, S13, Table S3). For the analysis of variation in plasticity of gene expression

572 during stress, we used the ratio of plastic expression after 1.5-24h of stress over gene
573 expression at 0h. Significant difference was determined in a single GLM model with quasi-
574 binomial distribution nesting the duration of stress within hybrid type or parental difference.
575 For the hybrids, we used the ratio of the ASE ratio at 1.5-24h of stress vs the ratio of ASE at
576 time point 0h (tp 0, FDR < 0.1). Genetic variants of expression plasticity in response to stress
577 were called significant at FDR<0.1 (Fig. S5 – xh vs 0h).
578
579 **Testing for the impact of plasticity on the direction of gene expression evolution**
580 *A. thaliana* genes with a significantly higher or lower expression after 1.5h-24h compared to
581 0h (FDR<0.05) were defined as plastic and partitioned into up-regulated or down-regulated
582 genes. To test for the impact of the direction of plasticity on the direction of evolutionary
583 change in basal expression level, we used the plastic response of *A. thaliana* as a proxy for
584 the ancestral direction of plastic stress response, since this species forms an outgroup to the
585 sister species *A. halleri* and *A. lyrata.* We then determined whether changes in basal
586 expression in each species were orthoplastic (i.e. in the direction of supposed ancestral
587 plasticity) or paraplastic (i.e. in the direction opposite to supposed ancestral plasticity).
588 For each gene, we plotted the ratio of expression level of *A. lyrata or A. halleri* over the fold
589 change in expression observed in *A. thaliana* (e.g. interspecific evolutionary change in
590 expression significant at FDR 0.05) as a function of the plastic response in *A. thaliana*
591 (significant stress response at FDR = 0.05) (Fig 2A). We then counted the number of genes
592 whose interspecific difference in basal expression (at 0h, before initiation of the stress) was
593 in the direction of the stress response in *A. thaliana* (orthoplastic) or opposite (paraplastic).
594 We subsequently used a chi-squared test with 1 degree of freedom to test whether gene
595 counts were randomly distributed in the 4 quadrants of the plots, thereby estimating the
596 proportion of genes evolving by orthoplasy, given their probability to be up- or down-
597 regulated by stress (Fig 2A-C). We subsequently used a chi-squared test with 1 degree of
598 freedom to test whether the proportion of genes with a detectable cis-acting variant were
599 randomly distributed among these genes. Finally, we compared the distribution in each
600 quadrant in *A. lyrata* with the distribution expected based on the distribution observed in *A.*
601 *halleri,* and used a chi-squared test with 4 degrees of freedom to determine significant
602 differences in the contribution of cis- acting variants to the modification of basal expression
603 levels.

604 A similar approach was used to test for the impact of the direction of plasticity on the
605 direction of evolutionary change in plasticity, which is, as already described above, the ratio
606 of allele count after *t* h over allele count at t_0= 0h in the first species divided by the ratio of
607 allele count after *t* h over allele count at t_0= 0h in the second species. In other term, the
608 change in plasticity was computed as log [($Aly_{t=6h}$/ $Aly_{t=oh}$)/ ($Ath_{t=6h}$/ $Ath_{t=oh}$)]. A positive value
609 indicates an increase in the magnitude of the plastic response (magnification) and a negative
610 value indicates a decrease in magnitude and/or a reversion of the plastic reaction
611 (mitigation). Note, however, that for 90% of the genes, the direction of gene expression
612 change was conserved between species. Significant plasticity changes were taken at FDR 0.1
613 (Fig. 2A-C). In this way, we computed the proportion of genes evolving by a magnified vs.
614 mitigated plastic response as well as the contribution of cis-acting variants to these
615 evolutionary outcomes (Fig. S3G,H, Fig. S5).
616

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

618 For analyzing the strength of selection, whole genome data for 16 *A. lyrata* individuals
619 collected in Plech (Germany) were taken from Takou et al. 2020[46], and 40 *A. thaliana*
620 individuals from Spain were taken from 1001 genomes [70]. Individuals were randomly down-
621 sampled to have the same number of observed alleles for all sites. Vcf files were filtered to
622 remove Indels, sites with more than two alleles, sites with coverage < 10, genotype quality <
623 20 or quality < 30 as well as positions with more than 80% of the individuals missing data, as
624 described in Takou et al. 2020[46]. We focused on orthologous genes in this dataset that had
625 polymorphism information in both species. Synonymous and nonsynonymous positions were
626 retained and folded site frequency spectra were generated for non-overlapping groups of
627 genes defined for each mode of plasticity evolution using R (Fig. S10). As an additional
628 control, we included a group of plastic genes, whose plastic reaction did not change
629 between *A. lyrata* and *A. thaliana.* Additionally, summary statistics (π_n/π_s, Tajima's D, π)
630 were calculated using custom R scripts for each of the categories. Ka and Ks were calculated
631 using an alignment of *A. lyrata* and *A. thaliana* as described in He et al. [29], with the "kaks"
632 function of R package seqinr. For each summary statistic 1000 bootstrap replicates were
633 generated by resampling the genes with replacement, and recalculating the statistics over all
634 genes. An index of interspecific changes was calculated by the ratio between the species

635 ((D_{Aly}+2)/ (D_{Ath}+2)) for the bootstrap replicates. Differences between plasticity groups were
636 assessed by calculating if the difference between the groups significantly differed from 0 for
637 each bootstrap by using the union of the bootstrap values to calculate what proportion of
638 the differences between the groups significantly differed from 0, multiplied by 2, to account
639 for the fact that the test is one-sided. Note that for three mode of plasticity evolution
640 (paraplasy-mitigation, orthoplasy-magnification, paraplasy-magnification), the number of
641 genes was small and bootstraps revealed high variance. Those modes of plasticity evolution
642 were therefore not further considered in the analysis.

643 The program fitδaδi [47] was used to estimate the distribution of fitness effects. This extension
644 to the δaδi program [71], which infers demographic history and infers selection based on
645 genomic data, allows us to specify the demographic model when inferring selection. In short,
646 we are using the simplified demographic model for the *A. lyrata* Plech population based on
647 the demographic model in Takou et al. 2020[46]. The synonymous population scaled mutation
648 rate was estimated using the δaδi function Inference.optimal_sfs_scaling and multiplied by
649 2.76 to get the nonsynonymous mutation rate. The nonsynonymous SFS was then used to
650 estimate the selection parameters which are given by the shape and scale parameter of the
651 gamma distribution. 200 bootstrap replicates of the resampled SFS were used to get 95%
652 confidence intervals for the gamma distribution parameters.
653

654 **Inferring the phylogenetic origin of** cis-**acting variants and their lineage-specific rate of**
655 **accumulation**

656 To infer the phylogenetic origin of cis-acting modifications, we defined *cis* acting changes as
657 ancestral if they were shared between the two types of F1 hybrids, which shared *A. thaliana*
658 as an outgroup. If the cis-acting variant was specific to one of the hybrids, this change was
659 considered to be derived in the corresponding parent, i.e *A. lyrata*- or *A. halleri*-derived.
660 Genes that displayed a change in plasticity contributed by at least one cis-acting
661 modification (e.g. basal cis-change, plastic cis-change or both) were considered as
662 independent variants (although they could have been controlled by more than one
663 mutation) and were assigned to one of the eight combinations of basal change (invariable /
664 Orthoplasy / Paraplasy) and plastic change (invariable /Magnification / Mitigation): Paraplasy
665 Magnification, Paraplasy Mitigation, Paraplasy only, Orthoplasy Magnification, Orthoplasy
666 Mitigation, Orthoplasy only, Magnification only, and Migation only (Table S7). Genes with

667 cis-acting variants that were shared in both comparisons of *A. halleri* or *A. lyrata* to *A.*
668 *thaliana* were separated from the lineage-specific derived cis-acting variants, because the
669 evolutionary direction of their effect could not be determined. A chi-squared test with seven
670 degrees of freedom was performed to test whether the proportion of derived cis-acting
671 variants in one of the lineages was different from either the distribution expected based on
672 the other lineage.
673

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677

678 **Data availability**

679 The RNAseq data generated in this study have been deposited in the Sequence Read Archive
680 (SRA) database under accession code PRJNA640858
681 [https://www.ncbi.nlm.nih.gov/sra/PRJNA640858]. The Transcriptome Shotgun Assembly of
682 *Arabidopsis halleri* has been deposited at DDBJ/EMBL/GenBank under the accession
683 GJCZ00000000. The version described in this paper is the first version, GJCZ01000000. The
684 phenotypic data generated in this study are provided in the Supplementary Information.

685

686 **Author contributions**

687 JDM, PK, AB, FH and KAS designed the study. FH and MB produced the data. FH, KAS, VK, UG,
688 MB, JDM analyzed the data. KAS, FH and JDM wrote the manuscript with input from all
689 authors.
690

691 **Competing interests**

692 The authors declare no competing interests.
693
694

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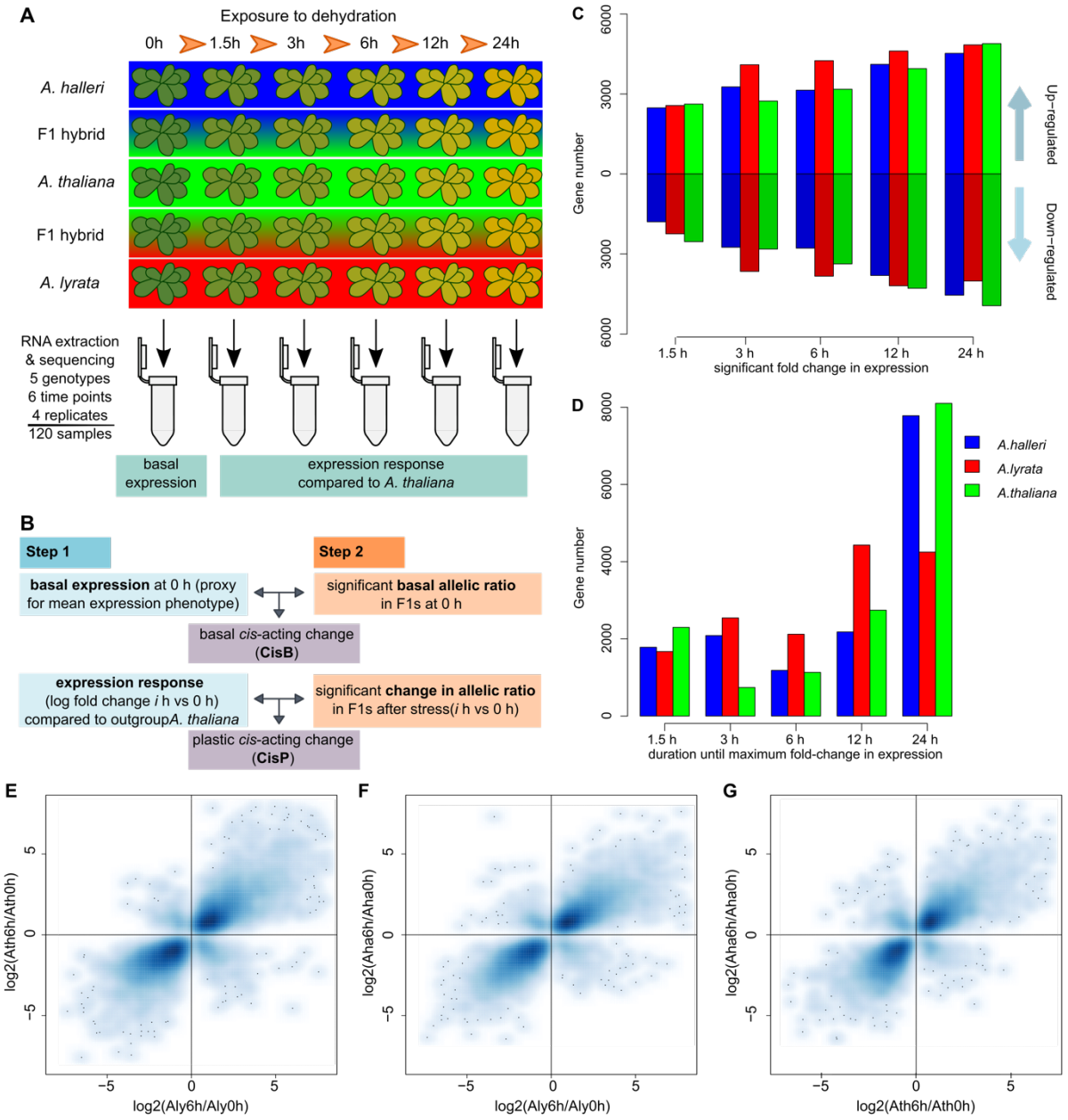


Figure 1: **Overview of the experimental setup and number of genes responding to stress response, the time point of maximal gene expression change**

(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

875 significant change in the allelic ratio in the F1s (step 2), a plastic cis-acting change is
876 contributing to the change in slope. Details for the analytical pipeline in Figure S13.
877 (C) Number of genes showing significant up- and down-regulation at each time point after
878 initiation of the stress in each of the three species. *A. lyrata* has a higher number of up- and
879 down-regulated genes at intermediate time points. D) Number of genes reaching their
880 maximum log-fold change at each time point in each of the three species confirms the
881 stronger response in *A. lyrata* at intermediate points E-G) Strong pairwise correlation in gene
882 expression fold change between 0 and 6 hours into the desiccation stress for each species
883 pairs : (E) *A. thaliana* vs *A. lyrata* (R=0.84, p < 1e-321), (F) *A. halleri* vs *A. lyrata* (R=0.85, p <
884 1e-321) and (G) *A. halleri* vs *A. thaliana* (R=0.83, p < 1e-321). This indicates that the overall
885 response to the stress is similar between the species and that the direction of transcriptional
886 plasticity predates species divergence.
887

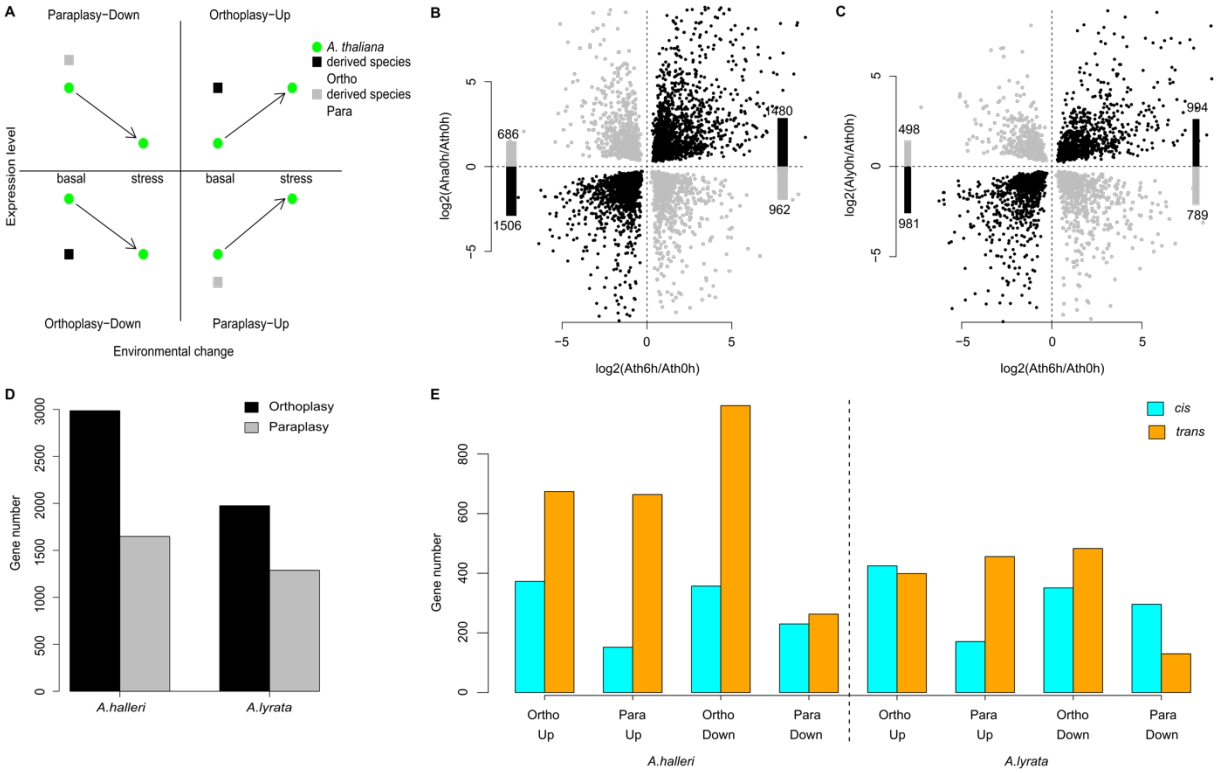


Figure 2: **Basal expression differences between species depend on the direction of plasticity in their common outgroup species. (**A) Sketch illustrating what ortho- or para-plastic changes in the derived species (*A. halleri* or *A. lyrata*) correspond to for up- and down-regulated genes, using *A. thaliana* as a proxy for the plasticity and gene expression level in their common ancestor. 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) 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*).

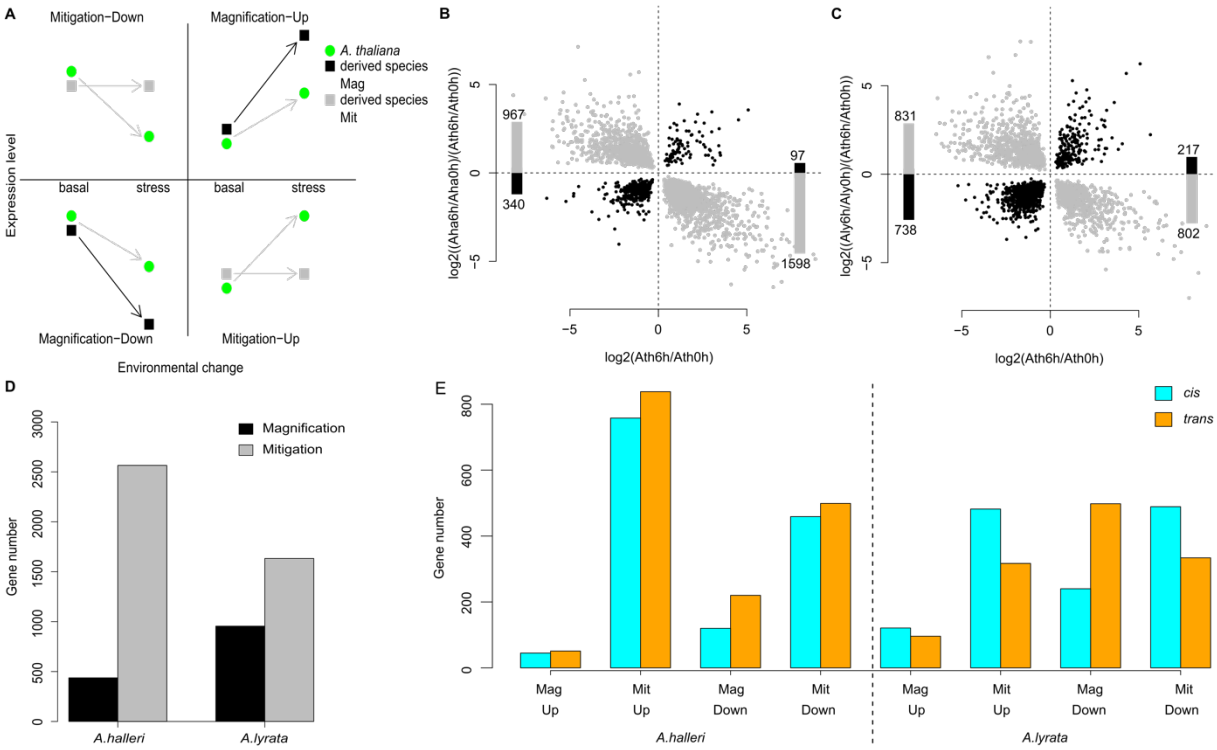


Figure 3: **Attenuated response to the stress compared to the plasticity observed in their outgroup species.**

(A) 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 a comparison. Black arrow: magnified response, grey arrow: mitigated response. Magnified and mitigated response in *A. halleri* (B) and *A. lyrata* (C). 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. (D) Number of genes with a magnified (black) and mitigated (grey) response in *A. halleri* and *A. lyrata,* compared to *A. thaliana*. (E) 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*).

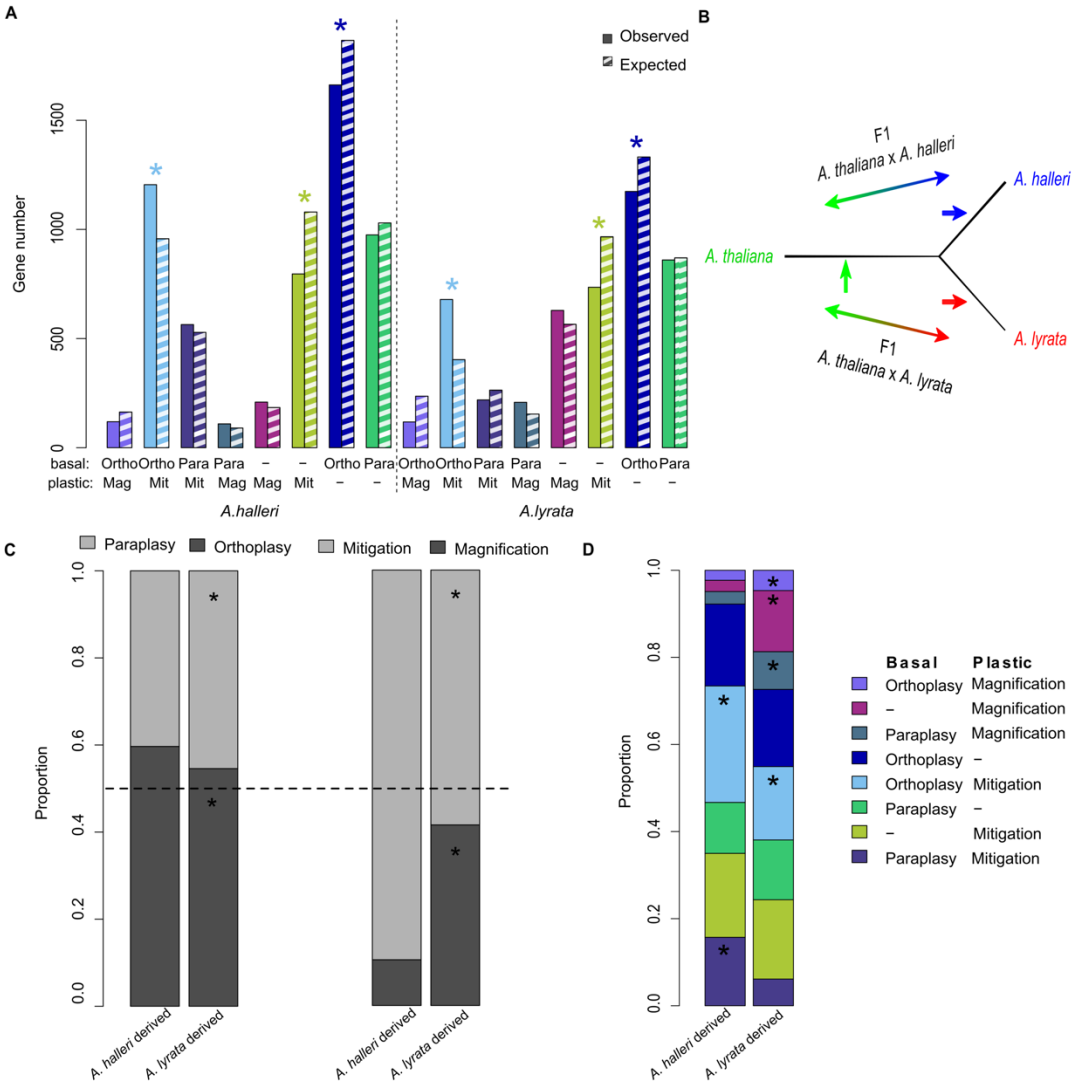


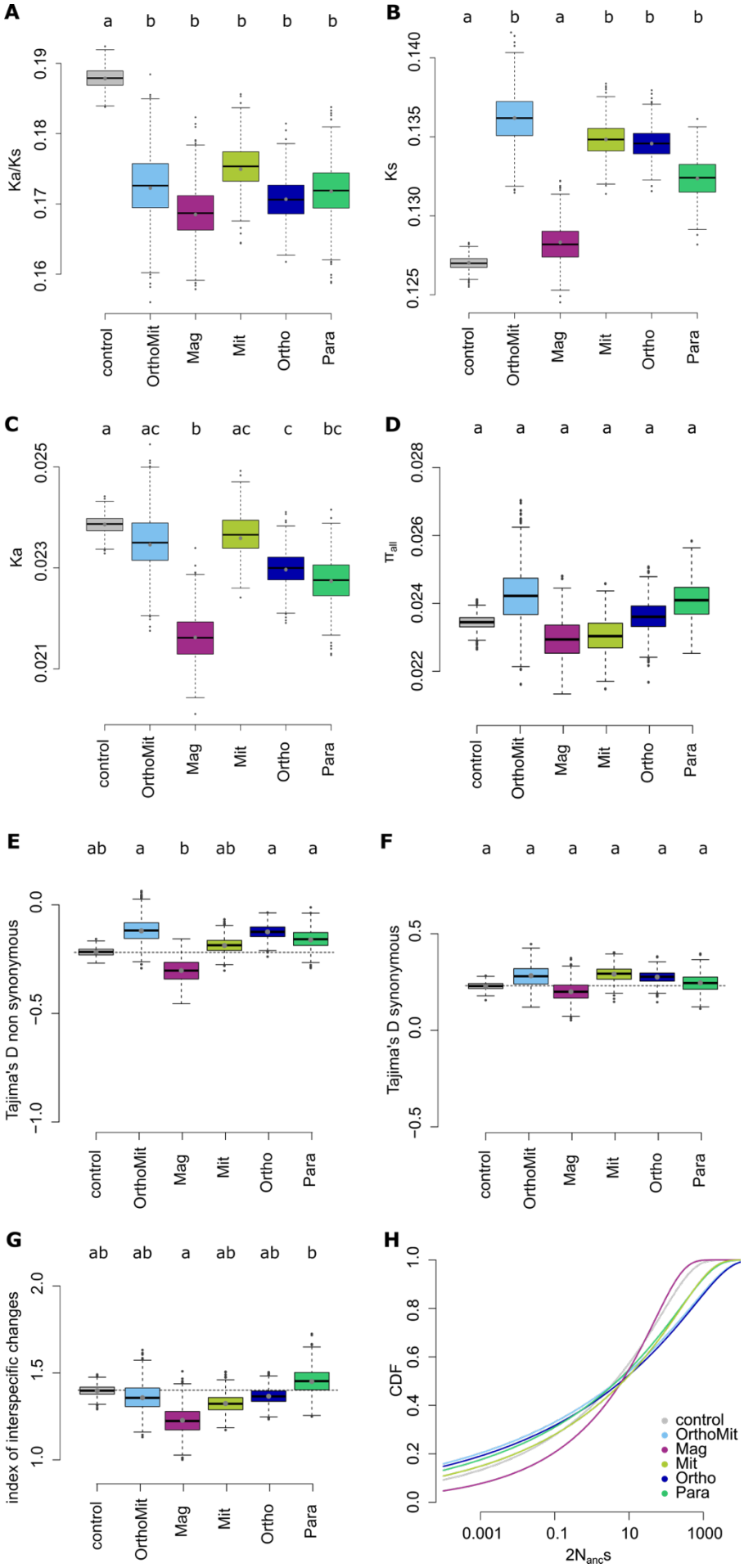
Fig. 4. **Distribution of derived** cis-**acting genes sets defined by their basal and/or plastic expression changes in *A. halleri* or *A. lyrata* compared to *A. thaliana*.** Ortho-Mag: orthoplasy and magnification, Ortho-Mit: orthoplasy and mitigation, Para-mit: paraplasy and mitigation, Para-Mag: 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

937 of a mutational bias. The total number of genes in these groups are shown in Figure S6. "*":
938 Significantly increased ot decreased number of derived changes in *A. halleri* and *A. lyrata*
939 compared to changes predating the separation between the two species (basal: *A. halleri* vs
940 ancestral p=0.09959, *A. lyrata* vs ancestral p=0.0002251; plastic: *A. halleri* vs ancestral
941 p=0.4068, *A. lyrata* vs ancestral p< 2.2e-16).
942 (D) Proportion of derived and cis-acting modifications within each gene set. "*": Significantly
943 increased or decreased number of derived changes in *A. halleri* compared to *A. lyrata*. The
944 association of basal and plastic changes in gene expression is not random (Chi-sq =47.7 df=7
945 p=4.13e-08 and Chi-sq =302.5 df=7 p=1.84e-61).
946


A
Ka/Ks
0.16
0.17
0.18
0.19
a b b b b b
B
Ks
0.125
0.130
0.135
0.140
a b a b b b
C
Ka
0.021
0.023
0.025
a ac b ac c bc
D
π_{all}
0.022
0.024
0.026
0.028
a a a a a a
E
Tajima's D non synonymous
−1.0
−0.5
0.0
ab a b ab a a
F
Tajima's D synonymous
−0.5
0.0
0.5
a a a a a a
G
index of interspecific changes
1.0
1.5
2.0
ab ab a ab ab b
H
CDF
0.0
0.2
0.4
0.6
0.8
1.0
0.001
0.1
10
1000
$2N_{anc}s$
control
OrthoMit
Mag
Mit
Ortho
Para

947

948 Fig. 5. **Tajima's D at synonymous and non-synonymous sites differ between 5 modes of**
949 **plastictiy evolution.** Ortho-Mit: orthoplasy and mitigation, Only-Mag: magnification only,
950 Only-mit: Mitigation only, Only-Ortho: Orthoplasy only, Only-Para: Paraplasy only. The
951 plasticity categories Ortho-Mag, Para-Mit and Para-Mag were excluded due to having too
952 few genes (see Fig. 4A) and therefore too high variance.
953 (A) Ka/Ks ratio, (B) Ks between *A. lyrata* and *A. thaliana*, (C) Ka between *A. lyrata* and *A.*
954 *thaliana,* (D) average **π** per gene, (E) Tajima's D for synonymous sites and (F)
955 nonsynonymous sites in *A. lyrata.* Significance between the groups was estimated based on
956 pairwise comparison of 1000 bootstrap replicates, by using the union of the bootstrap values
957 to calculate what proportion of the differences between the groups significantly differed
958 from 0, multiplied by 2, to account for the fact that the test is one-sided. Box and whiskers
959 depict the 75th and 95th interquantile ranges, respectively, dots show outliers. No shared
960 letters mean that the groups are different with a significance cutoff at $p < 0.05$. Pvalues of all
961 pairwise comparisons can be found in Supplementary data 8. (G) Relative change in mean
962 Tajima's D for non-synonymous sites between *A. lyrata* and *A. thaliana.* ((*A. lyrata* +2) / (*A.*
963 *thaliana* +2)) and (H) Cumulative distribution function of the gamma distribution for the
964 control and the 5 classes of plasticity. OrthoMag, ParaMit and ParaMag were removed due
965 to the low number of genes in these plasticity classes.
966