Vegetative Phase Change Causes Age-Dependent Changes in Phenotypic Plasticity

Authors: Erica H. Lawrence[1,2*], R. Scott Poethig[2] and Jesse R. Lasky[1]

[1] Pennsylvania State University, Department of Biology, University Park, PA
[2] University of Pennsylvania, Department of Biology, Philadelphia, PA
*Corresponding author: lawrence.erica.h@gmail.com

Abstract

1. Phenotypic plasticity allows organisms to optimize traits for their environment. As organisms age, they experience diverse environments that merit varying degrees of phenotypic plasticity. Developmental transitions can control these age-dependent changes in plasticity and as such, the timing of these transitions can determine when plasticity changes in an organism.

2. Here we investigate how the transition from juvenile-to adult-vegetative development known as vegetative phase change (VPC) contributes to age-dependent changes in phenotypic plasticity using both natural accessions and mutant lines in the model plant *Arabidopsis thaliana*. Further, we look at how the timing of this transition and the concordant shifts in plasticity change across accessions and environments.

3. We found that the adult phase of vegetative development has greater plasticity than the juvenile phase and confirmed that this difference in plasticity is caused by VPC using mutant lines. Further, we found that the timing of VPC, and therefore the time when increased plasticity is acquired, varies significantly across genotypes and environments.

4. This genetic and environmental variation in the timing of VPC indicates the potential for population-level adaptive evolution of VPC. The consistent age-dependent changes in plasticity caused by VPC add further support to the hypothesis that VPC is adaptive.

Key words: age-dependent plasticity, developmental timing, ontogeny, phenotypic plasticity, plant development, vegetative phase change

Introduction

Phenotypic plasticity, and its inverse – robustness -- are attributes of development in all organisms. Whether plasticity or robustness in a given trait is most beneficial for fitness depends on the degree of environmental variability, reliability in environmental cues and the costs associated with phenotypic adjustment (Fischer *et al.*, 2014). Although there are many examples of adaptive plasticity, we still have much to learn about what regulates plasticity in traits, including when plasticity changes, which environmental cues bring about plasticity, and the degree of plasticity in a trait.

As organisms age, they transition through different developmental phases that result in changing tradeoffs, while simultaneously experiencing diverse environments during those phases. As such, they might benefit from varying degrees of phenotypic plasticity across their lifespan. For example, cichlid fish display increased plasticity in response to alarm cues to increase morphological defenses (i.e. body size and coloration) when they are most vulnerable—shortly after birth and at the onset of reproduction (Meuthen *et al.*, 2018). Age-dependent changes in plasticity associated with whole plant development are often overlooked, though some have investigated the distinct changes in plasticity associated with the age of a particular organ (e.g., changes in photosynthetic traits associated with aging leaves (Niinemets, 2016)). However, a few studies demonstrate variation in plasticity surrounding reproduction and at different phases of vegetative growth. Two different studies found that *Arabidopsis thaliana* and the aquatic plant *Sagittaria latifolia* have greater phenotypic plasticity in response to changes in nutrients post-flowering compared to pre-flowering (Zhang & Lechowicz, 1994; Dorken & Barrett, 2004). Older but pre-flowering *Plantago lanceolata* individuals show greater plasticity of chemical response to herbivory compared to younger individuals (Barton, 2008). In some cases, the impacts of ontogeny on plasticity causes individuals of *different species* at the same developmental stage to respond more alike to environmental variation than *conspecifics* in different developmental phases (Parrish & Bazzaz, 1985). Despite the significance of developmentally regulated changes in plasticity and its impacts on fitness in animals (Hoverman & Relyea, 2007; Fischer *et al.*, 2014; Nilsson-Örtman *et al.*, 2015; Meuthen *et al.*, 2018; Sebestyén *et al.*, 2020) we know relatively little about developmental changes in plasticity in plants.

In contrast, many studies examine plasticity in the timing of plant developmental transitions, particularly flowering time and germination. Plasticity in the timing of developmental transitions allows plants to optimize their life history for their environment. For example, longer days and warmer temperatures accelerate flowering in *A. thaliana* (Levy & Dean, 1998; Blázquez *et al.*, 2003) to promote flowering in the spring and summer when conditions are favorable, and seeds that mature during short photoperiodic days (i.e. autumn) are more sensitive to cold, delaying germination until after winter (Munir *et al.*, 2001). Furthermore, Zhang & Lechowicz (1994) showed that the timing of developmental transitions and the degree of plasticity in other traits during developmental phases can be interconnected. Specifically, they found that in *A. thaliana* a delay in flowering time was associated with greater plasticity in post-flowering traits (Zhang & Lechowicz, 1994). Together this indicates that the timing of developmental transitions can alter an organism's phenotypic plasticity both by dictating which developmental phase the organism is in, and the degree of trait plasticity within that phase (Fig. **1**).

All plants transition between distinct juvenile and adult phases during vegetative development, which involves a wide-range of changes in physiology and morphology. However, in contrast to reproduction and germination, almost nothing is known about plasticity in the timing of vegetative phase change (VPC) or how it may impact the degree of phenotypic plasticity during vegetative development.

VPC is regulated by a highly conserved microRNA, miR156, and its targets, the Squamosa Promoter Binding-Like (SPL) transcription factors (Wu & Poethig, 2006; Willmann & Poethig, 2007; Wu *et al.*, 2009; He *et al.*, 2018). As individuals transition from a seedling to adult, changes in expression of the miR156/SPL module lead to phase specific differences in leaf morphology, photosynthetic traits, growth strategies and reproductive competence (Poethig, 1990; Bassiri *et al.*, 1992; Bongard-Pierce *et al.*, 1996; Telfer *et al.*, 1997; Wang *et al.*, 2011; Feng *et al.*, 2016; Leichty & Poethig, 2019; Silva *et al.*, 2019; Lawrence *et al.*, 2020, 2021b,a). Specifically, as plants transition from juvenile to adult, leaves become larger with decreased specific leaf area (SLA), and often display increased photosynthetic rates per unit leaf area. These changes in leaf morphology and physiology lead to a switch from a fast- to slow-growth strategy as

96 plants transition from producing low-cost juvenile leaves to expensive adult leaves with
97 long lifespans. Because VPC alters how plants function, the types of structures
98 produced, and the plant's ability to transition to reproduction, the timing of this transition
99 is likely to have significant consequences for fitness. Further, the model presented in
100 Fischer *et al.*, (2014) suggests that age-dependent changes in plasticity that align with
101 the timing of VPC are beneficial for fitness. An increase in plasticity during the adult
102 vegetative phase would provide time during the juvenile phase to collect enough
103 information about the environment before investing in any costly phenotypic adjustment
104 during the adult phase, and is therefore predicted to be an optimal strategy.
105 The timing of VPC is responsive to environmental cues, specifically light, and
106 defoliation (Yang *et al.*, 2011; Leichty & Poethig, 2019; Xu *et al.*, 2021). While the exact
107 effects on the timing of VPC are unknown, expression of the miR156/SPL module is
108 also altered by temperature, and drought (Kong *et al.*, 2010; Lee *et al.*, 2010; May *et al.*,
109 2013; Arshad *et al.*, 2018). Furthermore, the observation that juvenile and adult phases
110 of alfalfa exhibit different tolerance to drought (Arshad *et al.*, 2017) suggests genotypic
111 or species differences in the timing of this transition could be adaptive. However, the
112 validity of this hypothesis is difficult to assess because the range of variation in the
113 timing of VPC across species or even multiple genotypes within a species has only
114 been investigated in a few cases.
115 Intimate relationships between development and plasticity lead us to examine
116 variation in the timing of VPC and explore the effects of this transition on plasticity of
117 vegetative traits. In this study we used ten natural accessions of *A. thaliana* to
118 investigate how much genetic variation and plasticity exists in the timing of VPC
119 (plasticity in developmental timing, Fig. **1**) as well as how VPC impacts leaf trait
120 plasticity (age/phase-dependent plasticity, Fig. **1**). We also examined eight transgenic
121 lines with varying expression levels of the miR156/SPL module, both to confirm the role
122 of this developmental transition in altering juvenile and adult trait plasticity and to further
123 investigate how alterations in the timing of VPC impact plant growth under varying
124 environments, independent of genetic variation at other loci.
125
126 **Materials and Methods**

127 *Plant growth and materials*

128

129 Ten natural accessions of *Arabidopsis thaliana* (Table **S1**)—selected based on a
130 preliminary screen for variation in the timing of VPC—were obtained from the
131 *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH, USA).
132 All mutant lines used in this study were in a Col genetic background. The *mir156a-2*,
133 *mir156c-1*, *mir157c-1*, *35S::MIM156*, *35S::MIR156a*, and *spl9-4 spl13-1* lines have been
134 previously described (Wu & Poethig, 2006; Franco-Zorrilla *et al.*, 2007; Yang *et al.*,
135 2013; Xu *et al.*, 2016; He *et al.*, 2018). The *MIR156A* genomic line developed in the
136 Poethig lab is homozygous for a T-DNA insertion containing a 5.5 kb fragment spanning
137 the region between the genes upstream and downstream of *MIR156A* in a
138 pCAMBIA3301 backbone. These mutant and transgenic lines allowed us to evaluate
139 developmentally juvenile and adult leaves at every leaf position within a single genetic
140 background, thus distinguishing the effects of VPC from other factors such as length of
141 exposure to the environment, and size or age of the plant, which could influence
142 phenotypic plasticity.

143 Seeds were planted in 96 well flats in Fafard-2 growing mix supplemented with
144 Peters 20-10-20 fertilizer. Beneficial nematodes (*Steinernema feltiae*, BioLogic, Willow
145 Hill, PA), Marathon 1% granular insecticide and diatomaceous earth were added to the
146 growing mix to control insects. Plants were placed in 4°C for 5 days before being grown
147 in their respective treatment conditions (Table **S2**) in Percival growth chambers. Short
148 day (10 hrs. light/14 hrs. dark, at 120 or 90 μmol m^{-2} s^{-1}) light conditions were used to
149 prevent flowering so adult vegetative phenotypes were not impacted by the reproductive
150 transition. Plants were grown under 13.5W full spectrum LED lights. A reduction in light
151 intensity for the low light treatment was achieved by increasing the distance between
152 the lights and plants. Plants in all treatments were watered every other day with either
153 700 mL per flat until seeds germinated and throughout the 28-day growth period for
154 control, heat and low light treatments or 350 mL per flat for drought treatment. Soil
155 moisture was recorded with a Delta T HH2 Moisture Meter (Meter Group, Pullman, WA,
156 USA) at a depth of approximately 1.5 cm twice a week both before and after watering at

157 a minimum of two locations within the growth chamber. Temperature, light level and
158 average soil moisture conditions for each treatment are reported in Table **S2**
159
160
161 *Plant Phenotyping*
162
163 Plants were harvested at the soil surface 28 days following transfer to growth chambers.
164 Leaves were removed from the shoot and placed flat between two transparency sheets
165 in the order they were initiated (leaf one is the first leaf to be produced) and scanned
166 using a flatbed scanner (CanoScan LiDE220) at 300 dpi. All shoot material was then
167 transferred to coin envelopes and dried at 60°C until constant mass.
168 Whole plant growth phenotypes were measured to determine the influence of the
169 timing of VPC on plant productivity in the tested environments. Whole shoot mass from
170 dried plants was recorded and shoot area was analyzed from scanned images using
171 FIJI (Fiji software). The number of leaves initiated was counted as the total number of
172 fully expanded leaves and leaf primordia present at the end of the 28-day growing
173 period. This value was used to calculate the average leaf initiation rate across the
174 growing period by dividing the total number of leaves initiated by 28 days.
175 Leaf morphological traits were analyzed from scanned images using FIJI.
176 Specifically, for leaf morphological measures, base leaf angle was measured as the
177 angle between the edges of the leaf on either side of the petiole. Petioles were then
178 removed and the wand tool was used to select each leaf individually from binary images
179 to measure area, perimeter, length, width and circularity and the presence or absence
180 of serrations was noted. These traits describe both leaf shape and size to indicate
181 phenotypic change across development and environment while also being reliably and
182 easily measured from leaf scans.
183
184 *Determination of Developmental Phase*
185
186 Vegetative phase change in *Arabidopsis thaliana* is marked by changes in leaf
187 morphology that include leaf base angle, length/width ratio, circularity, the presence and

188 absence of serrations, and the presence or absence of abaxial trichomes (Telfer *et al.*,
189 1997; Tsukaya *et al.*, 2000; He *et al.*, 2018). While the presence of abaxial trichomes is
190 the most commonly used marker for the onset of the adult phase (Yang *et al.*, 2013; Xu
191 *et al.*, 2016; He *et al.*, 2018), we chose to use a combination of leaf morphological traits
192 more easily measured from leaf scans to determine juvenile and adult phases. This
193 method allows us to avoid the influence of any phase change-independent effects on
194 trichome development (i.e. photoperiodic and UV-B effects on trichome regulators
195 gibberelic acid biosynthesis and GLABRA3, respectively) that could arise in plants
196 grown under different environments (Chien & Sussex, 1996; Olszewski *et al.*, 2002; Yan
197 *et al.*, 2012). Further, by using a combination of traits we can get a more holistic
198 representation of when vegetative phase change occurs, as the onset of different adult
199 traits are often not perfectly synchronized.
200 Leaf stage was determined from leaf scans using a principal component analysis
201 of summarizing phase specific traits (leaf base angle, the length:width ratio of the
202 lamina, the presence vs. absence of serrations, and the circularity of the lamina), and
203 comparing the PC1 value obtained by this approach to the PC1 value for plants over-
204 expressing miR156 (35S::MIR156a, "OX"), in which all leaves are juvenile (Fig. **S1**).
205 Leaves with a PC1 value greater than or equal to the value for the miR156-over
206 expressing line were considered juvenile leaves, and leaves with a PC1 value less than
207 this number were considered adult leaves. Specifically, the minimum PC1 value of all
208 OX leaf samples for each treatment was identified. All leaves with PC1 values below
209 this threshold were considered adult leaves. PC1 threshold values used here were 0 for
210 control, 0.3 for drought, -2 for heat and 0 for low light conditions. Setting independent
211 thresholds for each environment using leaves produced across all leaf positions
212 eliminates the influence of any non-phase-change specific changes in PC1 values (i.e.
213 plant size, age, or environment) in the determination of juvenile and adult leaves.
214
215 *Data Analysis and Statistics*
216
217 All statistical analyses were performed in JMP® Pro v. 15.0.0 (SAS Institute Inc., Cary,
218 NC). Differences in the time of transition among genotypes were compared by two-way

219 ANOVA where genotype and environmental treatment were the main effects. Because
220 treatment had a significant effect ($p < 0.05$) Tukey's HSD was used to determine which
221 treatments were significantly different from each other. Within each genotype, ANOVA
222 was used to determine if environmental treatment had a significant effect on the time of
223 transition measures, total leaves initiated and leaf initiation rate. For genotypes where
224 treatment had a significant effect ($p < 0.05$), Tukey's HSD was used to determine which
225 treatments were significantly different from each other. We used one-way ANOVA with
226 natural accession genotypes as the main effect to estimate broad-sense heritability (R^2
227 from the ANOVA).
228 To understand how the timing of VPC impacts plant growth and productivity
229 across environments, we looked at relationships between the time of transition and
230 whole plant growth phenotypes of shoot mass, shoot area, and leaves initiated in each
231 of the four environments. Significant effects of the timing of VPC on these traits was
232 determined using least square linear regression analysis for both accessions and
233 mutant genotypes. Further, we investigated how the timing of VPC impacts plasticity in
234 these traits using phenotypic plasticity index and the change in each trait between
235 control and each of the three environmental stress treatments. Phenotypic plasticity
236 index was calculated as the absolute difference between the maximum and minimum
237 mean values among all four growth conditions divided by the maximum mean value,
238 following Valladares *et al.*, (2000) and Valladares *et al.*, (2006). While the plasticity
239 index describes the overall plasticity in a trait across all environments, the signed
240 change in trait describes plasticity induced by a specific environment and was
241 calculated as the difference between the mean control value and the mean treatment
242 value. Linear regression was used to evaluate whether there was a significant impact of
243 the timing of VPC on plasticity in plant growth and productivity traits using the mean
244 number of juvenile leaves produced or number of days initiating juvenile leaves for each
245 genotype.
246 To determine if vegetative phase change contributes to age-dependent changes
247 in phenotypic plasticity, we tested whether developmental phase (i.e. juvenile or adult)
248 impacts plasticity of leaf morphology using phenotypic plasticity index and change in
249 trait described above. A student's T-test was used to evaluate whether differences

between juvenile and adult plasticity was significant. Tests were conducted between phenotypic plasticity index for juvenile and adult leaves of all accessions or all leaf positions 1-8 for genotypes in the Col-0 background (i.e. miR156/SPL mutants and Col-0 accession). For evaluating changes in trait data, tests compared the change in trait between control and treatment phenotypes of juvenile and adult leaves for all accessions or all leaf positions 1-8 for genotypes in the Col-0 background for each of the three treatment growth conditions.

To briefly investigate whether there was any relationship between plasticity in the timing of vegetative phase change and an accession's climate of origin, we used the geoclimatic variables from the WorldClim2 data set (Fick & Hijmans, 2017), associated with georeferences for each of the 10 accessions published as part of the 1001 genomes project (Alonso-Blanco *et al.*, 2016). We used linear regression to determine any significant relationships between the bioclimatic variables and the phenotypic plasticity index for the time of transition calculated as described above using the number of juvenile leaves.

Results

The timing of vegetative phase change varies among genotypes and environments

Leaf identity was determined using a principal component analysis of phase specific leaf morphology traits and the timing of VPC was measured in two ways; by the number of juvenile leaves produced and the number of days the plant was initiating juvenile leaves calculated using leaf initiation rate. Both juvenile leaf number and days initiating juvenile leaves significantly differed among genotypes and was altered by abiotic environment (ANOVA: Genotype $p < 0.0001$, Treatment $p < 0.0001$, G x T $p < 0.0001$) (Table 1). Differences in the amount of time plants spent in the juvenile phase were largely due to the effect of environmental conditions on the rate of leaf production, as evident from the differences in the total number of leaves produced by 28 days under these conditions. Among the accessions examined here, VPC occurred as early as leaf 3 or day 6, and as late as leaf 8 or day 18 in "control" conditions. Transgenic genotypes with altered miR156 or *SPL* gene expression in the Col-0 background had VPC that occurred from leaf 1 or day 1 to leaf 25 or the full 28-day period of the experiment (i.e.

281 these plants never transitioned to adult phase). Broad-sense heritability of the timing of
282 VPC was high, ranging between 0.82 in control and 0.64 in low light environments when
283 measured by the number of juvenile leaves and 0.61 in drought and 0.46 in control
284 environments when measured by days initiating juvenile leaves (Table S3).
285 Both measures of the timing of VPC were significantly altered by abiotic
286 environment, and this response differed among genotypes as indicated by a significant
287 ($p < 0.05$) ANOVA interaction term (genotype x treatment). Among the accessions, heat
288 increased the number of juvenile leaves by an average of 1.3 from control and
289 decreased the rate of leaf initiation by 38%, thus increasing the average number of days
290 plants were in the juvenile phase by 11.15 ($p < 0.05$ Tukey's HSD). Low light intensity
291 decreased the average number of juvenile leaves, but decreased the rate of leaf
292 initiation by 49%, which was sufficient to increase the number of days plants were in the
293 juvenile phase by 6.39. Drought caused no significant difference in the number of
294 juvenile leaves or the rate of leaf initiation for most genotypes, and thus had no effect on
295 the days plants were in the juvenile phase (Table 1). All three environmental treatments
296 subjected plants to some degree of stress as indicated by losses of biomass averaging
297 8.4, 7.2 and 3 mg in low light, heat and drought respectively. It should be noted that it is
298 unclear why we observed a decrease in the number of juvenile leaves under low light
299 conditions, given that previous studies have shown that low light intensity increases
300 juvenile leaf number (Leichty & Poethig, 2019; Xu *et al.*, 2021).
301 Plasticity in the number of juvenile leaves and the rate of leaf initiation varied
302 among genotypes in response to abiotic environments, causing variation in the amount
303 of time plants spent in the juvenile phase. This led to variation in the order genotypes
304 transitioned to the adult phase between environments (Fig. **S2**). For example, Strand
305 (ST) is the 10th genotype to transition in control, 7th in drought, 17th in heat and 13th in
306 low light when measured by days initiating juvenile leaves. In the MIM156 target
307 mimicry line (MI) and 35S:*MIR156a* (OX) mutants, where miR156 abundance is highly
308 constrained (functionally non-existent or in excess respectively), there was no plasticity
309 in the days spent in the juvenile phase, although the rate of leaf initiation was
310 significantly affected by heat and light intensity in these genotypes. MI plants produce
311 only adult leaves and OX plants produce only juvenile leaves in all environments (Table

312 1), indicating that variation in miR156 abundance is necessary for the days spent in the
313 juvenile phase to be altered in response to the tested environments.
314 Plasticity in the number of juvenile leaves in the natural accessions across all
315 four environments, measured by phenotypic plasticity index, was significantly related to
316 the temperature of the driest quarter (R^2 = 0.493, $p < 0.05$) and precipitation of the
317 warmest quarter (R^2=0.502, $p < 0.05$) for each accession's climate-of-origin (Fig. **S3**).
318 Specifically, increased plasticity in the timing of VPC was related to higher temperatures
319 during the driest quarter and lower precipitation during the warmest quarter.
320
321 *The timing of vegetative phase change correlates with plant productivity and*
322 *performance among miR156/SPL mutants in the Col-0 background but not across*
323 *natural accessions.*
324
325 To understand how changes in the timing of VPC impacts plant productivity and
326 performance in different environments, we explored the relationships between the
327 number of juvenile leaves or days spent in the juvenile phase and the whole plant
328 growth phenotypes of shoot mass, shoot area, and number of leaves initiated during a
329 28-day period. Among miR156/SPL mutant genotypes, later phase change (i.e. an
330 increase in the number of juvenile leaves or longer time spent initiating juvenile leaves)
331 was significantly associated with increases in all three whole plant phenotypes across
332 most environments (the sole exception being total shoot area vs. days initiating juvenile
333 leaves in heat) (Fig. **2, S4**). The effect of these genotypes on the total number of leaves
334 produced is consistent with previous studies showing that variation in the expression of
335 the mir156/SPL module has a significant effect on the rate of leaf initiation (Schwab *et*
336 *al.*, 2005; Wang *et al.*, 2008; Feng *et al.*, 2016; Lawrence *et al.*, 2021a).
337 By contrast, natural genetic variation in the number of juvenile leaves in
338 accessions showed mostly non-significant ($p > 0.05$) or weak ($R^2 < 0.1$) relationships
339 with whole plant phenotypes within each environment (Fig. **2**). An exception to this was
340 that a more rapid rate of leaf initiation was associated with a larger number of juvenile
341 leaves in all environments, similar to what was observed in the mutants (Fig. **2**).
342 Interestingly, some growth traits had contrasting relationships with the number of days

343 plants were initiating juvenile leaves in natural accessions compared to mutant
344 genotypes. Specifically, shoot mass, and shoot area in control and heat environments,
345 and leaves initiated in the control treatment, were negatively related to the time spent in
346 the juvenile phase in natural accessions (Fig. **S4**). That is, natural accessions that
347 transitioned earlier had greater mass and leaf area in control and heat growth
348 treatments while mutants that transitioned later were bigger. This suggests natural
349 variation in the rate of leaf initiation (the major driver of time spent in the juvenile phase)
350 could be regulated by more than just miR156 abundance or that the impacts of VPC on
351 growth rate vary across genetic backgrounds. However more data are needed to
352 understand why the relationships between the timing of VPC and plant growth differ
353 when the number of juvenile leaves or time spent in the juvenile phase it is altered
354 within a single genetic background (i.e. the isogenic mutants) compared to natural
355 accessions. For example, if greater miR156 abundance leads to increases in vegetative
356 growth (as suggested by our data and other studies (Fu *et al.*, 2012; Wang & Wang,
357 2015; Zheng *et al.*, 2016)) but natural variation in the threshold needed to suppress *SPL*
358 expression exists, then genotypes with comparable levels of miR156 could undergo
359 VPC at different times.
360
361 *The adult vegetative phase has greater phenotypic plasticity than the juvenile phase*
362
363 Plasticity of leaf morphology was greater across the four growth environments for adult
364 leaves than for juvenile leaves. This greater plasticity was true for both accessions and
365 for miR156/SPL mutants (Fig. **3a,b**). Specifically, adult leaves had significantly greater
366 plasticity in leaf base angle, area, perimeter, length/width ratio, and circularity and the
367 average of those traits regardless of leaf position in the Col-0 lines, and significantly
368 greater plasticity in leaf base angle, length/width ratio, and circularity and the average of
369 all measured traits in the accessions (Student's T, $p < 0.05$)(Fig. **3a,b**). The consistency
370 with which adult leaves display greater plasticity across genotypes with different
371 numbers of juvenile leaves (i.e. independent of both exposure time and resources,
372 which has also been associated with increased phenotypic plasticity (Kleunen &
373 Fischer, 2005; Fischer *et al.*, 2014)) confirms the effect of this developmental program

374 on age-dependent changes in plasticity. The greater morphological plasticity of adult
375 leaves compared to juvenile leaves can be visualized for all accessions in Figure **4**.
376 Changes in leaf morphological traits between leaves grown in control and
377 treatment conditions was greater for adult leaves than juvenile leaves in response to
378 heat and low light (Fig. **3c,d**). Across accessions, significantly greater changes in leaf
379 area, perimeter and L/W ratio were observed in adult leaves grown in heat and low light
380 conditions, and in base angle and circularity for leaves grown in heat (Student's T, $p <$
381 0.05). Changes between leaves at positions 1-8 in the Col-0 lines were significantly
382 greater among adult leaves in L/W ratio and circularity in response to both heat and low
383 light, in base angle in response to heat, and in area and perimeter in response to low
384 light (Student's T, $p < 0.05$). Interestingly, the drought treatment—which caused no
385 overall change in the number of juvenile leaves or the amount of time spent in the
386 juvenile phase, and was the least stressful of the three treatments as indicated by
387 biomass loss—lead to no significant differences in the change of morphological traits
388 between juvenile and adult leaves (i.e. juvenile and adult leaf morphology responded
389 similarly to drought). Because miR156 expression has previously been shown to
390 respond to drought (Katiyar *et al.*, 2015; Liu *et al.*, 2019), it is possible that plasticity in
391 VPC would occur in a more severe drought treatment.
392
393 *Plasticity of whole plant growth phenotypes is not related to the timing of vegetative*
394 *phase change*
395
396 Despite differences in plasticity between the juvenile and adult phases of development,
397 the number of juvenile leaves did not impact plasticity of whole plant growth phenotypes
398 (shoot area, shoot mass or leaves initiated) in the accessions across any of the tested
399 environments (Fig. **5**). However, in miR156/SPL mutants, we found that an increase in
400 the number of juvenile leaves resulted in a greater reduction in these phenotypes when
401 grown in heat and low light conditions ($p < 0.05$) (Fig. **5b**). Even within the mutant lines,
402 overall plasticity measured as the phenotypic plasticity index considering all four growth
403 conditions was not related to the number of juvenile leaves (Fig. **5a**).
404

405 **Discussion**

406 Morphological and physiological plasticity and the timing of development are key
407 ways that organisms cope with fluctuating and unpredictable environments. Plasticity is
408 an important element of development, both as it pertains to the timing of transitions as
409 well as its role in dictating the degree of plasticity of an organism. Vegetative phase
410 change (VPC) is a highly conserved developmental transition in plants, but the role of
411 VPC in environmental adaptation is not known. Our study shows how the timing of VPC
412 responds to environmental factors, and demonstrates that the transition alters the
413 degree of phenotypic plasticity in response to environment. The existence of natural
414 genetic variation in VPC and associated plasticity suggests the potential for selection,
415 though the VPC-environment-fitness map remains to be dissected.

416 We found diverse natural accessions consistently showed increases in plasticity
417 with vegetative phase change, that is, the juvenile phase was more robust than the
418 adult phase. This consistency suggests this change in plasticity is adaptive. This
419 increase in plasticity might be favored if it is advantageous to delay high levels of
420 plasticity until after a plant has accumulated a significant amount of information about
421 the environment, but while it still has sufficient time to benefit from a phenotypic
422 adjustment (Fischer *et al.*, 2014). Deviations from this pattern, such as in the OX line
423 which remains in the robust juvenile phase, suggests that there are fitness costs to this
424 strategy, as these OX plants had the greatest reductions in mass, area and leaf
425 initiation in response to environmental stress (Fig **5b**). In addition, Fischer *et al.*, (2014)
426 predicted the initial more robust phenotype would improve fitness if adapted to the most
427 likely environment encountered during that lifestage. Previous studies show the
428 morphology and physiology of the juvenile phase is well suited to the low light
429 environments often encountered by juvenile leaves emerging in a shaded understory
430 and quickly overtopped by newly initiated leaves (Lawrence *et al.*, 2020, 2021b; Xu *et*
431 *al.*, 2021), providing further support for the hypothesis that VPC modulates phenotypes
432 in an adaptive way.

433 Changes in phenotypic plasticity modulated by VPC were consistent regardless
434 of genetic backgrounds or when the transition occurs. Adult leaves had greater plasticity
435 whether they were produced at node one (i.e. in the MI line) or node ten. This confirms

436 that VPC, and even more so, expression levels of miR156, is responsible for these
437 changes in plasticity, rather than simply the length of exposure to environmental cues.
438 High levels of miR156, which are associated with a more robust juvenile phase, likely
439 represent an example of microRNA buffering. MicroRNAs buffer by silencing changes in
440 target gene expression (i.e. in response to environmental cues) when target genes are
441 below a certain threshold. Once transcription of the target genes exceeds this threshold,
442 protein output becomes sensitive to any transcriptional changes (Posadas & Carthew,
443 2014). There are numerous examples of microRNAs contributing to developmental
444 robustness, such as miR164, which buffers its targets and causes robust plant organ
445 development (Sieber *et al.*, 2007). The abundance of miR156 early in the juvenile phase
446 far exceeds the level necessary to repress SPL genes, and is therefore likely to
447 contribute to the more robust phenotypes through this buffering mechanism (He *et al.*,
448 2018). Early studies of VPC noted stable phenotypes that were unresponsive to
449 environmental and hormonal treatments in the first two leaves of *Arabidopsis* plants,
450 where miR156 abundance is highest (Telfer *et al.*, 1997). An example of miR156
451 buffering occurs through the microRNA's role in plant responses to the stress-related
452 phytohormone abscisic acid (ABA). High miR156 levels, as found during the juvenile
453 phase, suppress plant ABA responses through repression of its target SPL genes,
454 which when expressed during the adult phase, interact directly with the transcription
455 factor ABA-INSENSITIVE5 (ABI5) to facilitate ABA signaling (Dong *et al.*, 2021).
456 Because phenotypic plasticity increases between juvenile and adult phases
457 across genotypes, genetic and environmental variation in the timing of VPC determines
458 when increased plasticity is acquired. We found natural variation in the timing of this
459 transition and significant genotype x environment interactions, indicating the time of
460 VPC has the potential to respond to selection. Determining the timing of VPC is not as
461 straightforward as other developmental transitions because it is not marked by any
462 single morphological change, but by multiple traits that do not always appear
463 simultaneously. Because each phase-specific trait can be differentially sensitive to
464 environmental cues, using combinations of traits (as we did) is likely necessary when
465 multiple environments are tested. Despite this increased complexity, understanding how
466 the timing of VPC responds to environments could provide important insights into plant

467 adaptation and acclimation. Here, we add further evidence that VPC impacts plant
468 growth and productivity as increased levels of miR156 leads to greater vegetative
469 growth across environments, consistent with observations in other species (Fu *et al.*,
470 2012; Rubinelli *et al.*, 2013; Wang & Wang, 2015; Zheng *et al.*, 2016), while bringing
471 forth new questions about whether these relationships remain true among plants of
472 different genotypes (Fig **2**). Within the few *Arabidopsis* accessions examined here, we
473 found significant relationships between plasticity in the timing of VPC and multiple
474 climate-of- origin variables (Fig. **S3**), suggesting further exploration of population
475 differences in VPC. Further, trait plasticity can influence a plant's vulnerability to
476 changing climates. For example, plasticity in the leaf traits of 12 perennial plant species
477 was an important determinant of susceptibility to climate change scenarios in a
478 Mongolian steppe (Liancourt *et al.*, 2015). Subsequently, climate can alter selection on
479 plasticity. For instance, end-of-season drought conditions selected for increased
480 plasticity of water use efficiency in *A. thaliana* (Kenney *et al.*, 2014) whereas plasticity of
481 flowering time in response to temperature was maladaptive (Stinchcombe *et al.*, 2004).
482 Modulating age-dependent changes in plasticity might contribute to any role VPC has in
483 adaptation. Additional studies are needed to understand what, if any, type of selection is
484 on VPC and how the timing of vegetative phase change contributes to adaptation and
485 acclimation in plants.
486 The effects of VPC on plasticity found here in *A. thaliana* are likely to be
487 conserved across species. VPC is regulated by miR156 and its sister microRNAs
488 across all land plants, allowing for microRNA buffering to create a consistently more
489 robust juvenile phase. For example, across 40 species of *Passiflora,* juvenile leaf
490 morphology is conserved despite the highly variable morphology among adult leaves
491 (Chitwood & Otoni, 2017), similar to what we observed here between the various
492 genotypes (Fig. **3, 4**). This further suggests the age-dependent changes in plasticity due
493 to VPC could lead to varying degrees of both intra and interspecific trait variation
494 through time among populations. It seems likely many published studies capture
495 patterns of variation driven by VPC within and among species, however are unaware of
496 this transition (i.e. (Mason *et al.*, 2013; Garbowski *et al.*, 2021). Intraspecific variation
497 can play an important role in community- and ecosystem-level processes (Bolnick *et al.*,

498 2011; Madritch *et al.*, 2014; Turner *et al.*, 2020; Westerband *et al.*, 2021), though the
499 functional basis of this variation is usually not understood. To better understand the
500 implications of VPC on these large scale processes, further characterization of the
501 timing of VPC across plant species (of which there is currently few; Maize, (Poethig,
502 1988; Bongard-Pierce *et al.*, 1996); *Arabidopsis thaliana*, (Telfer *et al.*, 1997);
503 *Eucalyptus globulus,* (Jordan *et al.*, 1999); *Nicotiana tabacum*, (Feng *et al.*, 2016);
504 Sorghum, (Hashimoto *et al.*, 2019); Acacia, (Leichty & Poethig, 2019); *Passiflora edulis*,
505 (Silva *et al.*, 2019); Poplar, (Lawrence *et al.*, 2021a) and Rice, (Asai *et al.*, 2002)) and
506 variation among genotypes within species (even fewer; *Eucalyptus globulus*, (Jordan *et*
507 *al.*, 1999); Maize, (Poethig, 1988; Foerster *et al.*, 2015); and *A. thaliana*, (Doody *et al.*,
508 2021)) is necessary.
509 VPC may contribute to plant adaptation by setting up periods of low and high
510 phenotypic plasticity when it is most beneficial. Here we demonstrate that the timing of
511 vegetative phase change varies across genotypes, interacts with the environment, and
512 alters the plasticity of vegetative traits. While more work is needed to fully understand
513 the functional, ecological, and evolutionary significance of VPC, its determination as a
514 modulator of age-dependent changes in plasticity provides new insights for
515 understanding plant environmental interactions.
516
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524 EHL, RSP and JRL contributed to research planning and design, EHL performed the
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527
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Tables

Table 1. Timing of vegetative phase change, measured as number of juvenile leaves produced or the number of days initiating juvenile leaves, and leaf initiation traits during 28-days of growth for natural accessions and miR156/SPL mutants grown in different environments. Values represent the mean ± standard error. Different lowercase letters indicate treatment groups that are significantly different ($p < 0.05$) based on Tukey's HSD, following a significant ANOVA result ($p < 0.05$). Groups significantly different from control are bolded. N represents the number of plants sampled.

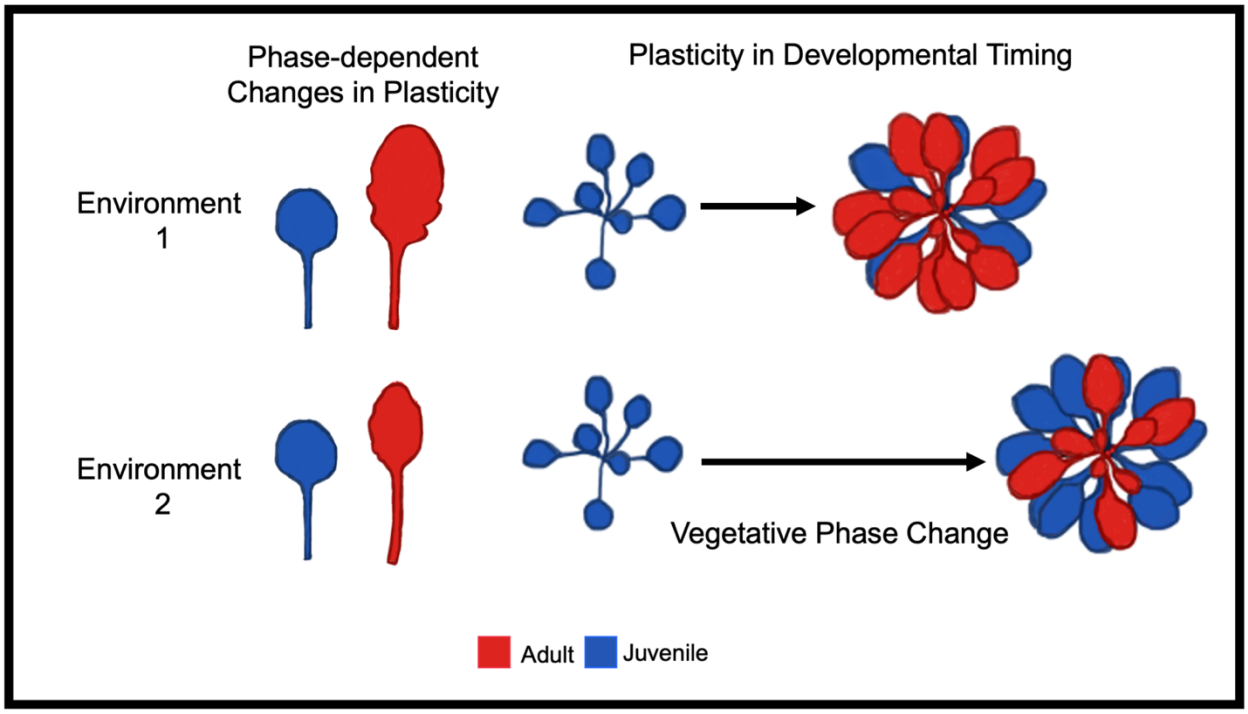
Natural Accessions

Genotype	Treatment	Number of Juvenile Leaves	Days Initiating Juvenile Leaves	Total Leaves Initiated	Leaf Initiation Rate	N
SH	Control	2 ± 0.45 ab	5.6 ± 1.39 b	10.8 ± 0.7 a	0.39 ± 0.03 a	6
	Drought	1 ± 0.52 b	2.4 ± 1.23 b	11.2 ± 0.48 a	0.4 ± 0.02 a	6
	Heat	3.6 ± 0.29 a	**15.8 ± 1.56 a**	**6.4 ± 0.29 b**	**0.23 ± 0.01 b**	9
	Low Light	1 ± 0.63 b	5.6 ± 3.54 b	**5.4 ± 0.24 b**	**0.19 ± 0.01 b**	5
AT	Control	3.3 ± 0.31 ab	8.4 ± 1.5 b	12.1 ± 1.04 a	0.43 ± 0.04 a	8
	Drought	2.5 ± 0.34 b	5 ± 0.73 b	14 ± 0.37 a	0.5 ± 0.01 a	6
	Heat	4.1 ± 0.13 a	**16.6 ± 1.81 a**	**7.4 ± 0.6 b**	**0.26 ± 0.02 b**	8
	Low Light	2.4 ± 0.34 b	10 ± 1.46 b	**6.8 ± 0.27 b**	**0.24 ± 0.01 b**	12
BC	Control	4.1 ± 0.35 b	7.9 ± 0.85 b	14.9 ± 0.58 a	0.53 ± 0.02 a	8
	Drought	3.4 ± 0.6 b	7.1 ± 1.22 b	13.6 ± 1.03 a	0.49 ± 0.04 a	5
	Heat	**5.8 ± 0.36 a**	**17.6 ± 1.42 a**	**9.6 ± 0.73 b**	**0.34 ± 0.03 b**	10
	Low Light	2.6 ± 0.53 b	11.2 ± 2.13 b	**6.3 ± 0.29 c**	**0.22 ± 0.01 c**	7
ST	Control	4.4 ± 0.37 b	11.4 ± 1.74 b	11.6 ± 0.81 a	0.41 ± 0.03 a	7
	Drought	3.8 ± 0.17 b	8.7 ± 0.46 b	12.3 ± 0.21 a	0.44 ± 0.01 a	6
	Heat	**6.4 ± 0.5 a**	**28 ± 0 a**	**6.4 ± 0.5 b**	**0.23 ± 0.02 b**	8
	Low Light	4.5 ± 0.29 b	**22.7 ± 3.12 a**	**5.8 ± 0.48 b**	**0.21 ± 0.02 b**	4
TU	Control	5.6 ± 0.38 ab	11.2 ± 1.19 c	14.5 ± 0.71 a	0.52 ± 0.03 a	8
	Drought	6 ± 0.52 ab	13.9 ± 2.91 bc	13.2 ± 1.14 a	0.47 ± 0.04 a	6
	Heat	6.2 ± 0.22 a	**22.4 ± 1.52 a**	**8.1 ± 0.65 b**	**0.29 ± 0.02 b**	9
	Low Light	4.8 ± 0.36 b	**19.4 ± 1.47 ab**	**6.9 ± 0.29 b**	**0.25 ± 0.01 b**	13
CO	Control	5.9 ± 0.14 a	11.5 ± 0.22 b	14.3 ± 0.47 a	0.51 ± 0.02 a	7
	Drought	5 ± 0.32 ab	10.2 ± 0.72 b	13.8 ± 0.8 a	0.49 ± 0.03 a	5
	Heat	5.5 ± 0.36 a	**18.1 ± 1.37 a**	**8.9 ± 0.71 b**	**0.32 ± 0.03 b**	12
	Low Light	**4.1 ± 0.15 b**	**16.2 ± 1.02 a**	**7.2 ± 0.28 b**	**0.26 ± 0.01 b**	15
VE	Control	6 ± 0 b	13.6 ± 1.08 b	13 ± 1.18 a	0.46 ± 0.04 a	8
	Drought	5.8 ± 0.48 b	12.6 ± 1.12 b	13.2 ± 0.91 a	0.47 ± 0.03 a	6
	Heat	**9 ± 0.8 a**	**25.7 ± 1.18 a**	10 ± 1.07 ab	0.36 ± 0.04 ab	8
	Low Light	5.6 ± 0.69 b	**23.2 ± 2.43 a**	**6.7 ± 0.36 b**	**0.24 ± 0.01 b**	7
CS	Control	6.3 ± 0.16 ab	12.3 ± 1.08 b	14.9 ± 1.01 a	0.53 ± 0.04 a	8
	Drought	5.6 ± 0.24 bc	12.6 ± 0.95 b	12.8 ± 1.24 a	0.46 ± 0.04 a	5
	Heat	7.4 ± 0.46 a	**24.3 ± 1.76 a**	**8.8 ± 0.62 b**	**0.31 ± 0.02 b**	8
	Low Light	**4.3 ± 0.42 c**	**23.2 ± 2.18 a**	**5.5 ± 0.72 b**	**0.2 ± 0.03 b**	6
VO	Control	6.7 ± 0.21	17 ± 2.15 b	11.7 ± 1.12 b	0.42 ± 0.04 b	6
	Drought	6.2 ± 0.17	12.1 ± 1.02 b	**14.7 ± 0.95 a**	**0.52 ± 0.03 a**	6
	Heat	7.3 ± 0.41	**25.1 ± 1.05 a**	**8.4 ± 0.68 c**	**0.3 ± 0.02 c**	11
	Low Light	6.1 ± 0.34	**23.4 ± 1.22 a**	**7.4 ± 0.22 c**	**0.26 ± 0.01 c**	16
BO	Control	7.3 ± 0.18 b	12.3 ± 0.69 c	16.1 ± 1.08 a	0.58 ± 0.04 a	8
	Drought	7 ± 0.37 b	11 ± 0.71 c	18 ± 0.73 a	0.64 ± 0.03 a	6
	Heat	**8.6 ± 0.29 a**	**27.3 ± 0.43 a**	**8.9 ± 0.32 b**	**0.32 ± 0.01 b**	15
	Low Light	6.3 ± 0.25 b	**16.8 ± 1.02 b**	**10.5 ± 0.65 b**	**0.38 ± 0.02 b**	4

Mutants

Genotype	Treatment	Number of Juvenile Leaves	Days Initiating Juvenile Leaves	Total Leaves Initiated	Leaf Initiation Rate	N
MI	Control	0 ± 0	0 ± 0	6 ± 1 a	0.21 ± 0.04 a	4
	Drought	0 ± 0	0 ± 0	4.9 ± 0.48 a	0.17 ± 0.02 a	8
	Heat	0 ± 0	0 ± 0	**2.3 ± 0.31 b**	**0.08 ± 0.01 b**	8
	Low Light	0 ± 0	0 ± 0	**2.1 ± 0.11 b**	**0.08 ± 0 b**	9
ACC	Control	1.4 ± 0.38 ab	5.7 ± 1.65 ab	7.3 ± 0.45 a	0.26 ± 0.02 a	8
	Drought	1.8 ± 0.56 a	7.3 ± 2.62 ab	7.3 ± 0.37 a	0.26 ± 0.01 a	8
	Heat	2.2 ± 0.15 a	10.5 ± 0.96 a	6.1 ± 0.31 a	0.22 ± 0.01 a	14
	Low Light	0.5 ± 0.26 b	3.3 ± 1.72 b	**4.8 ± 0.22 b**	**0.17 ± 0.01 b**	12
SAC	Control	2.3 ± 0.16 ab	7 ± 0.55 b	9.1 ± 0.55 ab	0.33 ± 0.02 ab	8
	Drought	2.3 ± 0.33 ab	6.2 ± 1.25 b	11 ± 0.58 a	0.39 ± 0.02 a	6
	Heat	3.5 ± 0.65 a	**16.7 ± 5.28 a**	7 ± 1.29 bc	0.25 ± 0.05 bc	4
	Low Light	1.8 ± 0.17 b	10.7 ± 1.12 ab	**4.8 ± 0.17 c**	**0.17 ± 0.01 c**	6
M6C	Control	4 ± 0.32 a	9.4 ± 1.2	12.4 ± 1.21 a	0.44 ± 0.04 a	5
	Drought	3.9 ± 0.35 a	9.6 ± 1.11	11.5 ± 0.57 ab	0.41 ± 0.02 a	8
	Heat	4.6 ± 0.29 a	13.4 ± 1.16	**9.8 ± 0.55 b**	**0.35 ± 0.02 b**	9
	Low Light	**2.2 ± 0.24 b**	10.1 ± 1.08	**6 ± 0.21 c**	**0.21 ± 0.01 c**	12
M7C	Control	4.4 ± 0.18 b	10.8 ± 1.14 b	11.9 ± 0.79 a	0.42 ± 0.03 a	8
	Drought	4 ± 0.19 b	9.3 ± 0.82 b	12.4 ± 0.56 a	0.44 ± 0.02 a	8
	Heat	**5.4 ± 0.34 a**	**17.9 ± 1.82 a**	**9 ± 0.79 b**	**0.32 ± 0.03 b**	10
	Low Light	**3.1 ± 0.3 c**	13.6 ± 1.51 ab	**6.6 ± 0.32 b**	**0.24 ± 0.01 b**	8
MA	Control	7 ± 0.37 a	12.7 ± 1.69 b	16.2 ± 1.17 a	0.58 ± 0.04 a	6
	Drought	7.4 ± 0.26 a	15.6 ± 2.02 b	14.3 ± 1.18 a	0.51 ± 0.04 a	8
	Heat	7.3 ± 0.53 a	**22.3 ± 1.53 a**	**9.3 ± 0.62 b**	**0.33 ± 0.02 b**	8
	Low Light	**4.7 ± 0.49 b**	16.7 ± 1.38 ab	**7.8 ± 0.48 b**	**0.28 ± 0.02 b**	6
NT	Control	9.7 ± 0.18 a	15.3 ± 0.61 b	18 ± 0.76 a	0.64 ± 0.03 a	7
	Drought	8.9 ± 0.13 a	16.7 ± 0.93 b	15.3 ± 0.88 a	0.54 ± 0.03 a	8
	Heat	9.2 ± 0.37 a	**23.4 ± 1.92 a**	**11.4 ± 1.21 b**	**0.41 ± 0.04 b**	5
	Low Light	**6.6 ± 0.69 b**	**23.5 ± 2.39 a**	**7.9 ± 0.4 c**	**0.28 ± 0.01 c**	7
OX	Control	24.8 ± 2.04 a	28 ± 0	24.8 ± 2.04 a	0.89 ± 0.07 a	6
	Drought	22.9 ± 1.27 a	28 ± 0	22.9 ± 1.27 a	0.82 ± 0.05 a	8
	Heat	**14.6 ± 2.05 b**	28 ± 0	**14.6 ± 2.05 b**	**0.52 ± 0.07 b**	9
	Low Light	**12.3 ± 0.4 b**	28 ± 0	**12.3 ± 0.4 b**	**0.44 ± 0.01 b**	10

742 **Figures**



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744 **Figure 1.** Illustration of two sources of plasticity involving vegetative phase change,
745 phase-dependent changes in plasticity that describe how the developmental phase of
746 an organ (juvenile or adult) impacts the degree of phenotypic plasticity of that organ,
747 and plasticity in developmental timing describing how the timing of vegetative phase
748 change can be accelerated or delayed in response to environment. In the above
749 example, we find greater plasticity in leaf morphology for adult (red) than juvenile (blue)
750 leaves between environment 1 and 2 as adult leaves change in both shape and size
751 whereas juvenile leaves only change in size. The timing of vegetative phase change is
752 delayed in environment 2 compared to environment 1 as indicated by the longer time
753 arrow and an increased number of juvenile leaves produced.

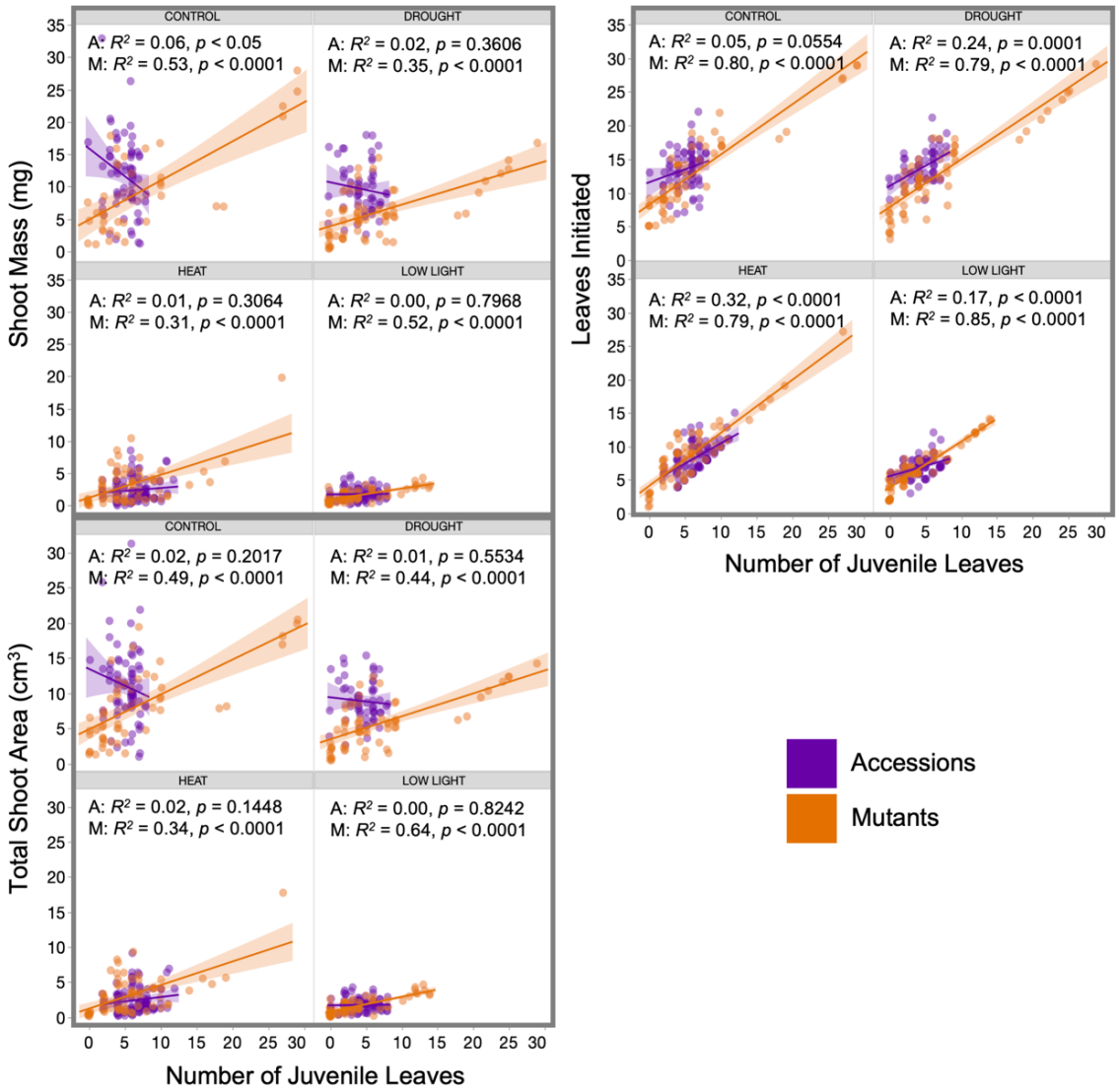

Figure 2. Relationships between the timing of vegetative phase change measured as number of juvenile leaves, and whole plant growth phenotypes of shoot mass, shoot area, and leaves initiated for accessions (purple) and miR156/SPL mutants (orange) in all four environments (clockwise from top left: control, drought, heat and low light). R^2 and p-values for linear regression of accessions (A) and mutants (M) noted at the top of each graph.

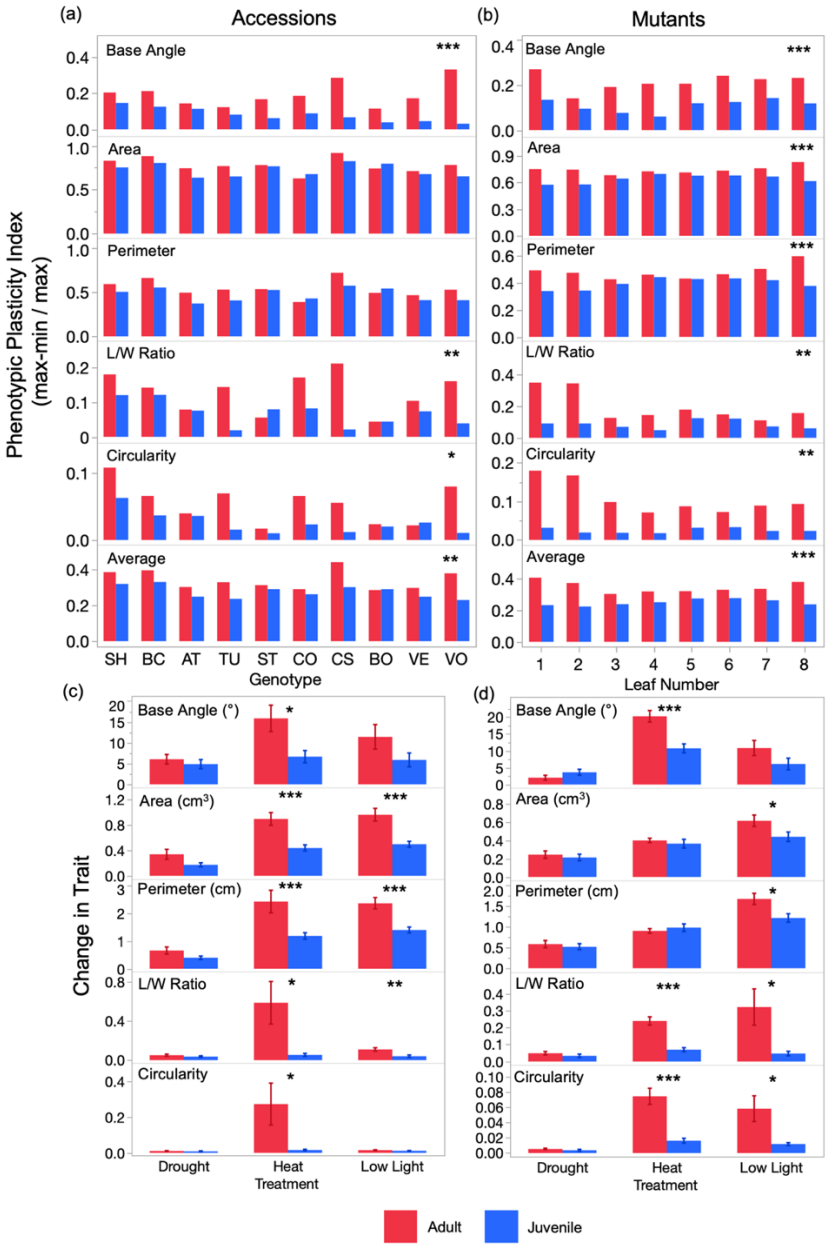

Figure 3. Phenotypic plasticity of juvenile and adult leaf morphology. Phenotypic plasticity index (a, b) and the change in trait between control and treatment conditions (c, d) for leaf base angle, leaf area, leaf perimeter, length/width (L/W) ratio, and leaf circularity for adult (red) and juvenile (blue) leaves of each accession (a, c) or leaf positions 1-8 in plants of the Col-0 genetic background (b, d). Mutations in miR156/SPL pathway cause both juvenile and adult leaves to be produced at each leaf position. Significant differences in phenotypic plasticity index between adult and juvenile leaves across all accessions or leaf positions for each trait denoted by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Significant differences in the change in traits between control and each treatment between adult and juvenile leaves for all accessions or leaf positions determined by Student's t denoted by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

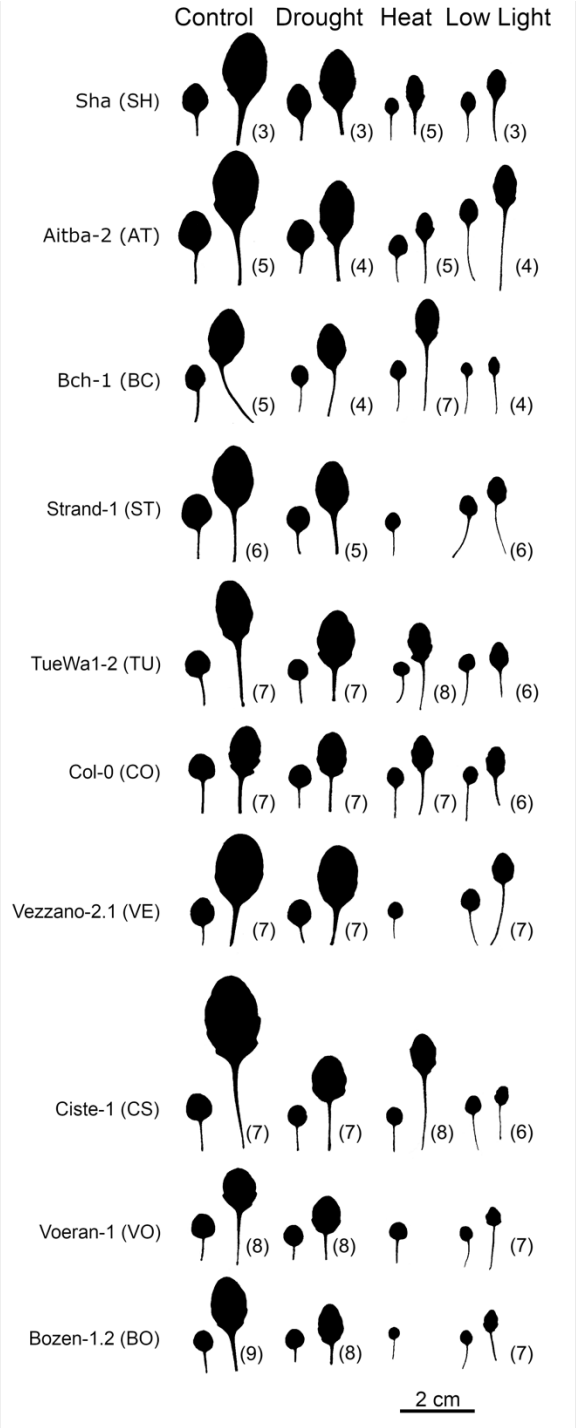

Figure 3. Representative leaf images of juvenile (left) and adult (right) leaves of each accession from control, drought, heat and low light environments. Leaves are of one of the first two positions of their respective developmental phase. Position of adult leaves shown in parenthesis.

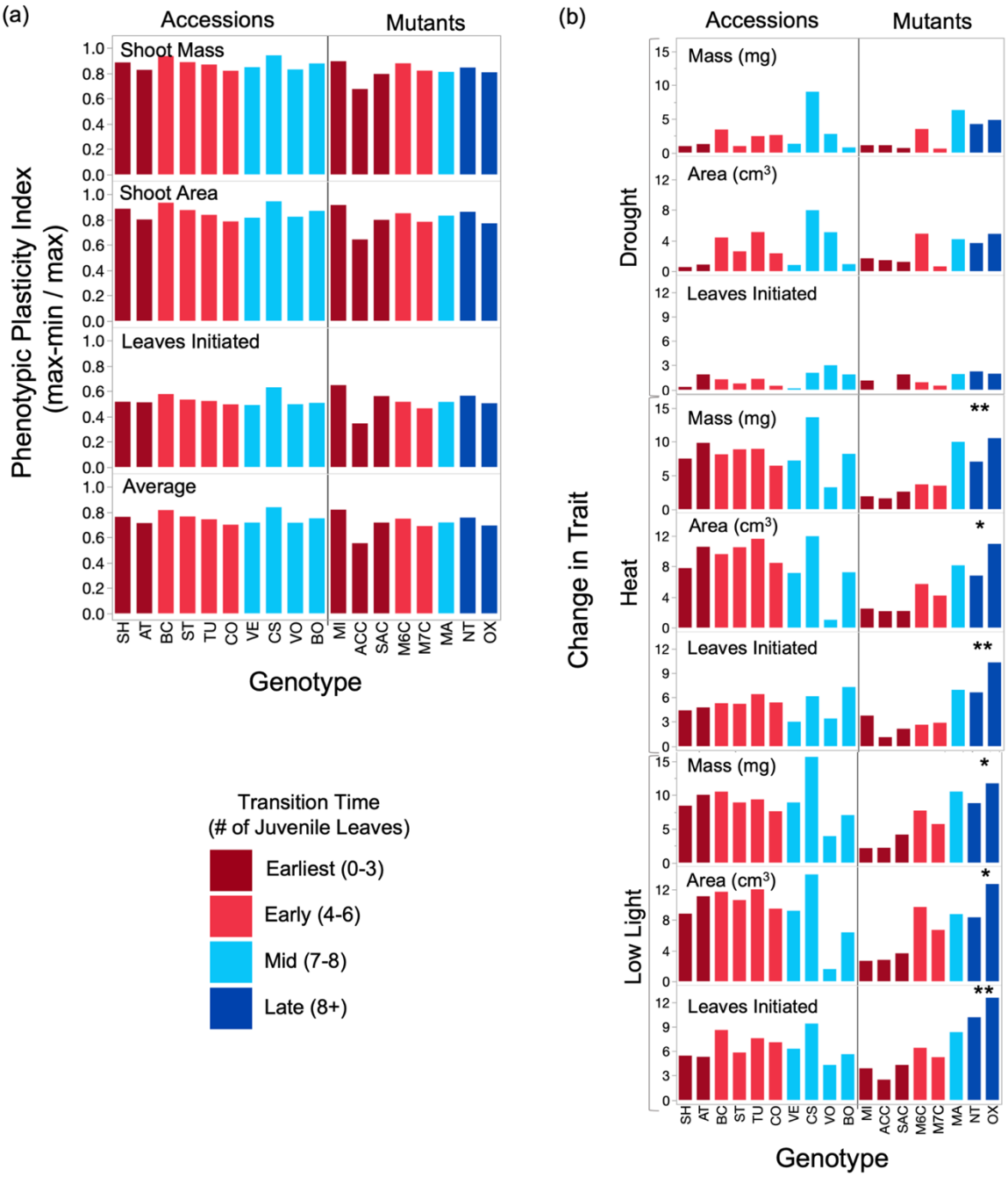

Figure 4. Phenotypic plasticity in the whole plant growth traits of shoot mass, shoot area, and leaves initiated. Phenotypic plasticity index (a) and the change in trait between control and treatment conditions (b) for accessions (left) and miR156/SPL mutants (right). Bar colors indicate time of vegetative phase transition based on number of juvenile leaves produced in control conditions. Significant relationships between number of juvenile leaves and change in trait between treatments were only present among mutant genotypes and are denoted by asterisks (* $p < 0.05$, ** $p < 0.01$).