Vegetative Phase Change Causes Age-Dependent Changes in Phenotypic Plasticity

Authors: Erica H. Lawrence^{1,2*}, R. Scott Poethig² and Jesse R. Lasky¹

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

plants transition from producing low-cost juvenile leaves to expensive adult leaves with long lifespans. Because VPC alters how plants function, the types of structures produced, and the plant's ability to transition to reproduction, the timing of this transition is likely to have significant consequences for fitness. Further, the model presented in Fischer *et al.*, (2014) suggests that age-dependent changes in plasticity that align with the timing of VPC are beneficial for fitness. An increase in plasticity during the adult vegetative phase would provide time during the juvenile phase to collect enough information about the environment before investing in any costly phenotypic adjustment during the adult phase, and is therefore predicted to be an optimal strategy.

The timing of VPC is responsive to environmental cues, specifically light, and defoliation (Yang *et al.*, 2011; Leichthy & Poethig, 2019; Xu *et al.*, 2021). While the exact effects on the timing of VPC are unknown, expression of the miR156/SPL module is also altered by temperature, and drought (Kong *et al.*, 2010; Lee *et al.*, 2010; May *et al.*, 2013; Arshad *et al.*, 2018). Furthermore, the observation that juvenile and adult phases of alfalfa exhibit different tolerance to drought (Arshad *et al.*, 2017) suggests genotypic or species differences in the timing of this transition could be adaptive. However, the validity of this hypothesis is difficult to assess because the range of variation in the timing of VPC across species or even multiple genotypes within a species has only been investigated in a few cases.

Intimate relationships between development and plasticity lead us to examine variation in the timing of VPC and explore the effects of this transition on plasticity of vegetative traits. In this study we used ten natural accessions of *A. thaliana* to investigate how much genetic variation and plasticity exists in the timing of VPC (plasticity in developmental timing, Fig. 1) as well as how VPC impacts leaf trait plasticity (age/phase-dependent plasticity, Fig. 1). We also examined eight transgenic lines with varying expression levels of the miR156/SPL module, both to confirm the role of this developmental transition in altering juvenile and adult trait plasticity and to further investigate how alterations in the timing of VPC impact plant growth under varying environments, independent of genetic variation at other loci.

Materials and Methods

Plant growth and materials

Ten natural accessions of *Arabidopsis thaliana* (Table **S1**)—selected based on a preliminary screen for variation in the timing of VPC—were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH, USA). All mutant lines used in this study were in a Col genetic background. The *mir156a-2*, *mir156c-1*, *mir157c-1*, *35S::MIM156*, *35S::MIR156a*, and *spl9-4 spl13-1* lines have been previously described (Wu & Poethig, 2006; Franco-Zorrilla *et al.*, 2007; Yang *et al.*, 2013; Xu *et al.*, 2016; He *et al.*, 2018). The *MIR156A* genomic line developed in the Poethig lab is homozygous for a T-DNA insertion containing a 5.5 kb fragment spanning the region between the genes upstream and downstream of *MIR156A* in a pCambia3301 backbone. These mutant and transgenic lines allowed us to evaluate developmentally juvenile and adult leaves at every leaf position within a single genetic background, thus distinguishing the effects of VPC from other factors such as length of exposure to the environment, and size or age of the plant, which could influence phenotypic plasticity.

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

a minimum of two locations within the growth chamber. Temperature, light level and average soil moisture conditions for each treatment are reported in Table **S2**

Plant Phenotyping

Plants were harvested at the soil surface 28 days following transfer to growth chambers. Leaves were removed from the shoot and placed flat between two transparency sheets in the order they were initiated (leaf one is the first leaf to be produced) and scanned using a flatbed scanner (CanoScan LiDE220) at 300 dpi. All shoot material was then transferred to coin envelopes and dried at 60°C until constant mass.

Whole plant growth phenotypes were measured to determine the influence of the timing of VPC on plant productivity in the tested environments. Whole shoot mass from dried plants was recorded and shoot area was analyzed from scanned images using FIJI (Fiji software). The number of leaves initiated was counted as the total number of fully expanded leaves and leaf primordia present at the end of the 28-day growing period. This value was used to calculate the average leaf initiation rate across the growing period by dividing the total number of leaves initiated by 28 days.

Leaf morphological traits were analyzed from scanned images using FIJI. Specifically, for leaf morphological measures, base leaf angle was measured as the angle between the edges of the leaf on either side of the petiole. Petioles were then removed and the wand tool was used to select each leaf individually from binary images to measure area, perimeter, length, width and circularity and the presence or absence of serrations was noted. These traits describe both leaf shape and size to indicate phenotypic change across development and environment while also being reliably and easily measured from leaf scans.

Determination of Developmental Phase

Vegetative phase change in *Arabidopsis thaliana* is marked by changes in leaf morphology that include leaf base angle, length/width ratio, circularity, the presence and

absence of serrations, and the presence or absence of abaxial trichomes (Telfer *et al.*, 1997; Tsukaya *et al.*, 2000; He *et al.*, 2018). While the presence of abaxial trichomes is the most commonly used marker for the onset of the adult phase (Yang *et al.*, 2013; Xu *et al.*, 2016; He *et al.*, 2018), we chose to use a combination of leaf morphological traits more easily measured from leaf scans to determine juvenile and adult phases. This method allows us to avoid the influence of any phase change-independent effects on trichome development (i.e. photoperiodic and UV-B effects on trichome regulators gibberelic acid biosynthesis and GLABRA3, respectively) that could arise in plants grown under different environments (Chien & Sussex, 1996; Olszewski *et al.*, 2002; Yan *et al.*, 2012). Further, by using a combination of traits we can get a more holistic representation of when vegetative phase change occurs, as the onset of different adult traits are often not perfectly synchronized.

Leaf stage was determined from leaf scans using a principal component analysis of summarizing phase specific traits (leaf base angle, the length:width ratio of the lamina, the presence vs. absence of serrations, and the circularity of the lamina), and comparing the PC1 value obtained by this approach to the PC1 value for plants over-expressing miR156 (35S::MIR156a, "OX"), in which all leaves are juvenile (Fig. **S1**). Leaves with a PC1 value greater than or equal to the value for the miR156-over expressing line were considered juvenile leaves, and leaves with a PC1 value less than this number were considered adult leaves. Specifically, the minimum PC1 value of all OX leaf samples for each treatment was identified. All leaves with PC1 values below this threshold were considered adult leaves. PC1 threshold values used here were 0 for control, 0.3 for drought, -2 for heat and 0 for low light conditions. Setting independent thresholds for each environment using leaves produced across all leaf positions eliminates the influence of any non-phase-change specific changes in PC1 values (i.e. plant size, age, or environment) in the determination of juvenile and adult leaves.

Data Analysis and Statistics

All statistical analyses were performed in JMP® Pro v. 15.0.0 (SAS Institute Inc., Cary, NC). Differences in the time of transition among genotypes were compared by two-way

ANOVA where genotype and environmental treatment were the main effects. Because treatment had a significant effect ($p < 0.05$) Tukey's HSD was used to determine which treatments were significantly different from each other. Within each genotype, ANOVA was used to determine if environmental treatment had a significant effect on the time of transition measures, total leaves initiated and leaf initiation rate. For genotypes where treatment had a significant effect ($p < 0.05$), Tukey's HSD was used to determine which treatments were significantly different from each other. We used one-way ANOVA with natural accession genotypes as the main effect to estimate broad-sense heritability (R^2 from the ANOVA).

To understand how the timing of VPC impacts plant growth and productivity across environments, we looked at relationships between the time of transition and whole plant growth phenotypes of shoot mass, shoot area, and leaves initiated in each of the four environments. Significant effects of the timing of VPC on these traits was determined using least square linear regression analysis for both accessions and mutant genotypes. Further, we investigated how the timing of VPC impacts plasticity in these traits using phenotypic plasticity index and the change in each trait between control and each of the three environmental stress treatments. Phenotypic plasticity index was calculated as the absolute difference between the maximum and minimum mean values among all four growth conditions divided by the maximum mean value, following Valladares *et al.*, (2000) and Valladares *et al.*, (2006). While the plasticity index describes the overall plasticity in a trait across all environments, the signed change in trait describes plasticity induced by a specific environment and was calculated as the difference between the mean control value and the mean treatment value. Linear regression was used to evaluate whether there was a significant impact of the timing of VPC on plasticity in plant growth and productivity traits using the mean number of juvenile leaves produced or number of days initiating juvenile leaves for each genotype.

To determine if vegetative phase change contributes to age-dependent changes in phenotypic plasticity, we tested whether developmental phase (i.e. juvenile or adult) impacts plasticity of leaf morphology using phenotypic plasticity index and change in 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.

these plants never transitioned to adult phase). Broad-sense heritability of the timing of VPC was high, ranging between 0.82 in control and 0.64 in low light environments when measured by the number of juvenile leaves and 0.61 in drought and 0.46 in control environments when measured by days initiating juvenile leaves (Table S3).

Both measures of the timing of VPC were significantly altered by abiotic environment, and this response differed among genotypes as indicated by a significant ($p < 0.05$) ANOVA interaction term (genotype x treatment). Among the accessions, heat increased the number of juvenile leaves by an average of 1.3 from control and decreased the rate of leaf initiation by 38%, thus increasing the average number of days plants were in the juvenile phase by 11.15 ($p < 0.05$ Tukey's HSD). Low light intensity decreased the average number of juvenile leaves, but decreased the rate of leaf initiation by 49%, which was sufficient to increase the number of days plants were in the juvenile phase by 6.39. Drought caused no significant difference in the number of juvenile leaves or the rate of leaf initiation for most genotypes, and thus had no effect on the days plants were in the juvenile phase (Table 1). All three environmental treatments subjected plants to some degree of stress as indicated by losses of biomass averaging 8.4, 7.2 and 3 mg in low light, heat and drought respectively. It should be noted that it is unclear why we observed a decrease in the number of juvenile leaves under low light conditions, given that previous studies have shown that low light intensity increases juvenile leaf number (Leichty & Poethig, 2019; Xu *et al.*, 2021).

Plasticity in the number of juvenile leaves and the rate of leaf initiation varied among genotypes in response to abiotic environments, causing variation in the amount of time plants spent in the juvenile phase. This led to variation in the order genotypes transitioned to the adult phase between environments (Fig. S2). For example, Strand (ST) is the 10th genotype to transition in control, 7th in drought, 17th in heat and 13th in low light when measured by days initiating juvenile leaves. In the MIM156 target mimicry line (MI) and 35S:*MIR156a* (OX) mutants, where miR156 abundance is highly constrained (functionally non-existent or in excess respectively), there was no plasticity in the days spent in the juvenile phase, although the rate of leaf initiation was significantly affected by heat and light intensity in these genotypes. MI plants produce only adult leaves and OX plants produce only juvenile leaves in all environments (Table

1), indicating that variation in miR156 abundance is necessary for the days spent in the juvenile phase to be altered in response to the tested environments.

Plasticity in the number of juvenile leaves in the natural accessions across all four environments, measured by phenotypic plasticity index, was significantly related to the temperature of the driest quarter ($R^2 = 0.493$, $p < 0.05$) and precipitation of the warmest quarter ($R^2=0.502$, $p < 0.05$) for each accession's climate-of-origin (Fig. **S3**). Specifically, increased plasticity in the timing of VPC was related to higher temperatures during the driest quarter and lower precipitation during the warmest quarter.

The timing of vegetative phase change correlates with plant productivity and performance among miR156/SPL mutants in the Col-0 background but not across natural accessions.

To understand how changes in the timing of VPC impacts plant productivity and performance in different environments, we explored the relationships between the number of juvenile leaves or days spent in the juvenile phase and the whole plant growth phenotypes of shoot mass, shoot area, and number of leaves initiated during a 28-day period. Among miR156/SPL mutant genotypes, later phase change (i.e. an increase in the number of juvenile leaves or longer time spent initiating juvenile leaves) was significantly associated with increases in all three whole plant phenotypes across most environments (the sole exception being total shoot area vs. days initiating juvenile leaves in heat) (Fig. **2**, **S4**). The effect of these genotypes on the total number of leaves produced is consistent with previous studies showing that variation in the expression of the mir156/SPL module has a significant effect on the rate of leaf initiation (Schwab *et al.*, 2005; Wang *et al.*, 2008; Feng *et al.*, 2016; Lawrence *et al.*, 2021a).

By contrast, natural genetic variation in the number of juvenile leaves in accessions showed mostly non-significant ($p > 0.05$) or weak ($R^2 < 0.1$) relationships with whole plant phenotypes within each environment (Fig. **2**). An exception to this was that a more rapid rate of leaf initiation was associated with a larger number of juvenile leaves in all environments, similar to what was observed in the mutants (Fig. **2**). Interestingly, some growth traits had contrasting relationships with the number of days

plants were initiating juvenile leaves in natural accessions compared to mutant genotypes. Specifically, shoot mass, and shoot area in control and heat environments, and leaves initiated in the control treatment, were negatively related to the time spent in the juvenile phase in natural accessions (Fig. **S4**). That is, natural accessions that transitioned earlier had greater mass and leaf area in control and heat growth treatments while mutants that transitioned later were bigger. This suggests natural variation in the rate of leaf initiation (the major driver of time spent in the juvenile phase) could be regulated by more than just miR156 abundance or that the impacts of VPC on growth rate vary across genetic backgrounds. However more data are needed to understand why the relationships between the timing of VPC and plant growth differ when the number of juvenile leaves or time spent in the juvenile phase it is altered within a single genetic background (i.e. the isogenic mutants) compared to natural accessions. For example, if greater miR156 abundance leads to increases in vegetative growth (as suggested by our data and other studies (Fu *et al.*, 2012; Wang & Wang, 2015; Zheng *et al.*, 2016)) but natural variation in the threshold needed to suppress *SPL* expression exists, then genotypes with comparable levels of miR156 could undergo VPC at different times.

The adult vegetative phase has greater phenotypic plasticity than the juvenile phase

Plasticity of leaf morphology was greater across the four growth environments for adult leaves than for juvenile leaves. This greater plasticity was true for both accessions and for miR156/*SPL* mutants (Fig. **3a,b**). Specifically, adult leaves had significantly greater plasticity in leaf base angle, area, perimeter, length/width ratio, and circularity and the average of those traits regardless of leaf position in the Col-0 lines, and significantly greater plasticity in leaf base angle, length/width ratio, and circularity and the average of all measured traits in the accessions (Student's T, $p < 0.05$)(Fig. **3a,b**). The consistency with which adult leaves display greater plasticity across genotypes with different numbers of juvenile leaves (i.e. independent of both exposure time and resources, which has also been associated with increased phenotypic plasticity (Kleunen & Fischer, 2005; Fischer *et al.*, 2014)) confirms the effect of this developmental program

on age-dependent changes in plasticity. The greater morphological plasticity of adult leaves compared to juvenile leaves can be visualized for all accessions in Figure 4.

Changes in leaf morphological traits between leaves grown in control and treatment conditions was greater for adult leaves than juvenile leaves in response to heat and low light (Fig. 3c,d). Across accessions, significantly greater changes in leaf area, perimeter and L/W ratio were observed in adult leaves grown in heat and low light conditions, and in base angle and circularity for leaves grown in heat (Student's T, $p < 0.05$). Changes between leaves at positions 1-8 in the Col-0 lines were significantly greater among adult leaves in L/W ratio and circularity in response to both heat and low light, in base angle in response to heat, and in area and perimeter in response to low light (Student's T, $p < 0.05$). Interestingly, the drought treatment—which caused no overall change in the number of juvenile leaves or the amount of time spent in the juvenile phase, and was the least stressful of the three treatments as indicated by biomass loss—lead to no significant differences in the change of morphological traits between juvenile and adult leaves (i.e. juvenile and adult leaf morphology responded similarly to drought). Because miR156 expression has previously been shown to respond to drought (Katiyar *et al.*, 2015; Liu *et al.*, 2019), it is possible that plasticity in VPC would occur in a more severe drought treatment.

Plasticity of whole plant growth phenotypes is not related to the timing of vegetative phase change

Despite differences in plasticity between the juvenile and adult phases of development, the number of juvenile leaves did not impact plasticity of whole plant growth phenotypes (shoot area, shoot mass or leaves initiated) in the accessions across any of the tested environments (Fig. 5). However, in miR156/SPL mutants, we found that an increase in the number of juvenile leaves resulted in a greater reduction in these phenotypes when grown in heat and low light conditions ($p < 0.05$) (Fig. 5b). Even within the mutant lines, overall plasticity measured as the phenotypic plasticity index considering all four growth conditions was not related to the number of juvenile leaves (Fig. 5a).

Discussion

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

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

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

that VPC, and even more so, expression levels of miR156, is responsible for these changes in plasticity, rather than simply the length of exposure to environmental cues. High levels of miR156, which are associated with a more robust juvenile phase, likely represent an example of microRNA buffering. MicroRNAs buffer by silencing changes in target gene expression (i.e. in response to environmental cues) when target genes are below a certain threshold. Once transcription of the target genes exceeds this threshold, protein output becomes sensitive to any transcriptional changes (Posadas & Carthew, 2014). There are numerous examples of microRNAs contributing to developmental robustness, such as miR164, which buffers its targets and causes robust plant organ development (Sieber *et al.*, 2007). The abundance of miR156 early in the juvenile phase far exceeds the level necessary to repress SPL genes, and is therefore likely to contribute to the more robust phenotypes through this buffering mechanism (He *et al.*, 2018). Early studies of VPC noted stable phenotypes that were unresponsive to environmental and hormonal treatments in the first two leaves of *Arabidopsis* plants, where miR156 abundance is highest (Telfer *et al.*, 1997). An example of miR156 buffering occurs through the microRNA's role in plant responses to the stress-related phytohormone abscisic acid (ABA). High miR156 levels, as found during the juvenile phase, suppress plant ABA responses through repression of its target SPL genes, which when expressed during the adult phase, interact directly with the transcription factor ABA-INSENSITIVE5 (ABI5) to facilitate ABA signaling (Dong *et al.*, 2021).

Because phenotypic plasticity increases between juvenile and adult phases across genotypes, genetic and environmental variation in the timing of VPC determines when increased plasticity is acquired. We found natural variation in the timing of this transition and significant genotype x environment interactions, indicating the time of VPC has the potential to respond to selection. Determining the timing of VPC is not as straightforward as other developmental transitions because it is not marked by any single morphological change, but by multiple traits that do not always appear simultaneously. Because each phase-specific trait can be differentially sensitive to environmental cues, using combinations of traits (as we did) is likely necessary when multiple environments are tested. Despite this increased complexity, understanding how the timing of VPC responds to environments could provide important insights into plant

adaptation and acclimation. Here, we add further evidence that VPC impacts plant growth and productivity as increased levels of miR156 leads to greater vegetative growth across environments, consistent with observations in other species (Fu *et al.*, 2012; Rubinelli *et al.*, 2013; Wang & Wang, 2015; Zheng *et al.*, 2016), while bringing forth new questions about whether these relationships remain true among plants of different genotypes (Fig 2). Within the few *Arabidopsis* accessions examined here, we found significant relationships between plasticity in the timing of VPC and multiple climate-of- origin variables (Fig. S3), suggesting further exploration of population differences in VPC. Further, trait plasticity can influence a plant's vulnerability to changing climates. For example, plasticity in the leaf traits of 12 perennial plant species was an important determinant of susceptibility to climate change scenarios in a Mongolian steppe (Liancourt *et al.*, 2015). Subsequently, climate can alter selection on plasticity. For instance, end-of-season drought conditions selected for increased plasticity of water use efficiency in *A. thaliana* (Kenney *et al.*, 2014) whereas plasticity of flowering time in response to temperature was maladaptive (Stinchcombe *et al.*, 2004). Modulating age-dependent changes in plasticity might contribute to any role VPC has in adaptation. Additional studies are needed to understand what, if any, type of selection is on VPC and how the timing of vegetative phase change contributes to adaptation and acclimation in plants.

The effects of VPC on plasticity found here in *A. thaliana* are likely to be conserved across species. VPC is regulated by miR156 and its sister microRNAs across all land plants, allowing for microRNA buffering to create a consistently more robust juvenile phase. For example, across 40 species of *Passiflora*, juvenile leaf morphology is conserved despite the highly variable morphology among adult leaves (Chitwood & Otoni, 2017), similar to what we observed here between the various genotypes (Fig. 3, 4). This further suggests the age-dependent changes in plasticity due to VPC could lead to varying degrees of both intra and interspecific trait variation through time among populations. It seems likely many published studies capture patterns of variation driven by VPC within and among species, however are unaware of this transition (i.e. (Mason *et al.*, 2013; Garbowski *et al.*, 2021). Intraspecific variation can play an important role in community- and ecosystem-level processes (Bolnick *et al.*,

2011; Madritch *et al.*, 2014; Turner *et al.*, 2020; Westerland *et al.*, 2021), though the functional basis of this variation is usually not understood. To better understand the implications of VPC on these large scale processes, further characterization of the timing of VPC across plant species (of which there is currently few; Maize, (Poethig, 1988; Bongard-Pierce *et al.*, 1996); *Arabidopsis thaliana*, (Telfer *et al.*, 1997); *Eucalyptus globulus*, (Jordan *et al.*, 1999); *Nicotiana tabacum*, (Feng *et al.*, 2016); Sorghum, (Hashimoto *et al.*, 2019); Acacia, (Leichty & Poethig, 2019); *Passiflora edulis*, (Silva *et al.*, 2019); Poplar, (Lawrence *et al.*, 2021a) and Rice, (Asai *et al.*, 2002)) and variation among genotypes within species (even fewer; *Eucalyptus globulus*, (Jordan *et al.*, 1999); Maize, (Poethig, 1988; Foerster *et al.*, 2015); and *A. thaliana*, (Doody *et al.*, 2021)) is necessary.

VPC may contribute to plant adaptation by setting up periods of low and high phenotypic plasticity when it is most beneficial. Here we demonstrate that the timing of vegetative phase change varies across genotypes, interacts with the environment, and alters the plasticity of vegetative traits. While more work is needed to fully understand the functional, ecological, and evolutionary significance of VPC, its determination as a modulator of age-dependent changes in plasticity provides new insights for understanding plant environmental interactions.

Acknowledgments

We thank Erin Doody for providing preliminary data regarding the timing of vegetative phase change in *Arabidopsis thaliana* accessions. This research was funded by NSF grant DGE-1845298 awarded to EHL, NSF DEB-1927009 and NIH 1R35GM138300-01 grants awarded to JRL, and NIH GM51893 awarded to RSP.

Author Contributions

EHL, RSP and JRL contributed to research planning and design, EHL performed the experiments and statistical analysis, EHL wrote the initial version of the manuscript and all authors revised and provided comments.

References

Alonso-Blanco C, Andrade J, Becker C, Bemm F, Bergelson J, Borgwardt KM, Cao J, Chae E, Dezwaan TM, Ding W, et al. 2016. 1,135 Genomes reveal the global pattern of polymorphism in *Arabidopsis thaliana*. *Cell* **166**: 481–491.

Arshad M, Feyissa BA, Amyot L, Aung B, Hannoufa A. 2017. MicroRNA156 improves drought stress tolerance in alfalfa (*Medicago sativa*) by silencing SPL13. *Plant Science* **258**: 122–136.

Arshad M, Gruber MY, Hannoufa A. 2018. Transcriptome analysis of microRNA156 overexpression alfalfa roots under drought stress. *Scientific Reports* **8**: 9363.

Asai K, Satoh N, Sasaki H, Satoh H, Nagato Y. 2002. A rice heterochronic mutant, *mori1*, is defective in the juvenile-adult phase change. *Development* **129**: 265–273.

Barton KE. 2008. Phenotypic plasticity in seedling defense strategies: compensatory growth and chemical induction. *Oikos* **117**: 917–925.

Bassiri A, Irish EE, Scott PR. 1992. Heterochronic effects of Teopod 2 on the growth and photosensitivity of the maize shoot. *The Plant Cell* **4**: 497–504.

Blázquez MA, Ahn JH, Weigel D. 2003. A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. *Nature genetics* **33**: 168–171.

Bolnick DI, Amarasekare P, Araújo MS, Bürger R, Levine JM, Novak M, Rudolf VHW, Schreiber SJ, Urban MC, Vasseur DA. 2011. Why intraspecific trait variation matters in community ecology. *Trends in Ecology & Evolution* **26**: 183–192.

Bongard-Pierce DK, Evans MMS, Poethig RS. 1996. Heteroblastic features of leaf anatomy in maize and their genetic regulation. *International Journal of Plant Sciences* **157**: 331.

Chien JC, Sussex IM. 1996. Differential regulation of trichome formation on the adaxial and abaxial leaf surfaces by gibberellins and photoperiod in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiology* **111**: 1321–1328.

Chitwood DH, Otoni WC. 2017. Morphometric analysis of *Passiflora* leaves: the relationship between landmarks of the vasculature and elliptical Fourier descriptors of the blade. *GigaScience* **6**: 1–13.

Dong H, Yan S, Jing Y, Yang R, Zhang Y, Zhou Y, Zhu Y, Sun J. 2021. MIR156-Targeted SPL9 is phosphorylated by SnRK2s and interacts with ABI5 to enhance ABA responses in *Arabidopsis*. *Frontiers in Plant Science* **12**: 708573.

Doody E, Zha Y, He J, Poethig S. 2021. QTL analysis of vegetative phase change in natural accessions of *Arabidopsis thaliana*. *bioRxiv*: 2021.10.27.465806.

Dorken ME, Barrett SCH. 2004. Phenotypic plasticity of vegetative and reproductive traits in monoecious and dioecious populations of *Sagittaria latifolia* (*Alismataceae*): a clonal aquatic plant. *Journal of Ecology* **92**: 32–44.

Feng S, Xu Y, Guo C, Zheng J, Zhou B, Zhang Y, Ding Y, Zhang L, Zhu Z, Wang H, et al. 2016. Modulation of miR156 to identify traits associated with vegetative phase change in tobacco (*Nicotiana tabacum*). *Journal of Experimental Botany* **67**: 1493–1504.

Fick SE, Hijmans RJ. 2017. WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology* **37**: 4302–4315.

Fischer B, van Doorn GS, Dieckmann U, Taborsky B. 2014. The evolution of age-dependent plasticity. *American Naturalist* **183**: 108–125.

Foerster JM, Beissinger T, de Leon N, Kaeppler S. 2015. Large effect QTL explain natural phenotypic variation for the developmental timing of vegetative phase change in maize (*Zea mays* L.). *Theoretical and Applied Genetics* **128**: 529–538.

Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, Leyva A, Weigel D, García JA, Paz-Ares J, et al. 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nature Genetics* **39**: 1033–1037.

Fu C, Sunkar R, Zhou C, Shen H, Zhang J-YY, Matts J, Wolf J, Mann DGJ, Stewart CN, Tang Y, et al. 2012. Overexpression of miR156 in switchgrass (*Panicum virgatum* L.) results in various morphological alterations and leads to improved biomass production. *Plant Biotechnology Journal* **10**: 443–452.

Garbowski M, Johnston DB, Brown CS. 2021. Leaf and root traits, but not relationships among traits, vary with ontogeny in seedlings. *Plant and Soil*.

Hashimoto S, Tezuka T, Yokoi S. 2019. Morphological changes during juvenile-to-adult phase transition in *sorghum*. *Planta* **250**.

He J, Xu M, Willmann MR, McCormick K, Hu T, Yang L, Starker CG, Voytas DF, Meyers BC, Poethig RS. 2018. Threshold-dependent repression of SPL gene expression by miR156/miR157 controls vegetative phase change in *Arabidopsis thaliana*. *PLOS Genetics* **14**: e1007337.

Hoverman JT, Relyea RA. 2007. How flexible is phenotypic plasticity? Developmental windows for trait induction and reversal. *Ecology* **88**: 693–705.

Jordan GJ, Potts BM, Wiltshire RJE. 1999. Strong, independent, quantitative genetic control of the timing of vegetative phase change and first flowering in *Eucalyptus globulus* ssp. *globulus* (Tasmanian Blue Gum). *Heredity* **83**: 179–187.

Katiyar A, Smita S, Muthusamy SK, Chinnusamy V, Pandey DM, Bansal KC. 2015. Identification of novel drought-responsive microRNAs and trans-acting siRNAs from *Sorghum bicolor* (L.) Moench by high-throughput sequencing analysis. *Frontiers in Plant Science* **6**: 506.

Kenney AM, McKay JK, Richards JH, Juenger TE. 2014. Direct and indirect selection on flowering time, water-use efficiency (WUE, $\delta^{13}C$), and WUE plasticity to drought in *Arabidopsis thaliana*. *Ecology and Evolution* **4**: 4505–4521.

Kleunen M Van, Fischer M. 2005. Constraints on the evolution of adaptive phenotypic plasticity in plants. *New Phytologist* **166**: 49–60.

Kong Y, Elling A, Chen B, Deng XW. 2010. Differential expression of microRNAs in maize inbred and hybrid lines during salt and drought stress. *American Journal of Plant Sciences* **01**: 69–76.

Lawrence EH, Leichty AR, Doody EE, Ma C, Strauss SH, Poethig RS. 2021a. Vegetative phase change in *Populus tremula x alba*. *New Phytologist* **231**: nph.17316.

Lawrence EH, Springer CJ, Helliker BR, Poethig RS. 2020. MicroRNA156-mediated changes in leaf composition lead to altered photosynthetic traits during vegetative phase change. *New Phytologist*: nph.17007.

Lawrence E, Springer C, Helliker B, Poethig S. 2021b. The carbon economics of vegetative phase change: why plants make juvenile and adult leaves. *Research Square*: 10.21203/rs.3.rs-448939/v1.

Lee H, Yoo SJ, Lee JH, Kim W, Yoo SK, Fitzgerald H, Carrington JC, Ahn JH. 2010. Genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs in *Arabidopsis*. *Nucleic acids research* **38**: 3081–93.

Leichty AR, Poethig RS. 2019. Development and evolution of age-dependent defenses in ant-acacias. *Proceedings of the National Academy of Sciences* **116**: 15596–15601.

- Levy YY, Dean C. 1998.** Control of flowering time. *Current Opinion in Plant Biology* **1**: 49–54.
- Liancourt P, Boldgiv B, Song DS, Spence LA, Helliker BR, Petraitis PS, Casper BB. 2015.** Leaf-trait plasticity and species vulnerability to climate change in a Mongolian steppe. *Global Change Biology* **21**: 3489–3498.
- Liu X, Zhang X, Sun B, Hao L, Liu C, Zhang D, Tang H, Li C, Li Y, Shi Y, et al. 2019.** Genome-wide identification and comparative analysis of drought-related microRNAs in two maize inbred lines with contrasting drought tolerance by deep sequencing. *PLoS ONE* **7**: e0219176.
- Madritch MD, Kingdon CC, Singh A, Mock KE, Lindroth RL, Townsend PA. 2014.** Imaging spectroscopy links aspen genotype with below-ground processes at landscape scales. *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**.
- Mason CM, Mcgaughey SE, Donovan LA. 2013.** Ontogeny strongly and differentially alters leaf economic and other key traits in three diverse *Helianthus* species. *Journal of Experimental Botany* **64**: 4089–4099.
- May P, Liao W, Wu Y, Shuai B, Richard McCombie W, Zhang MQ, Liu QA. 2013.** The effects of carbon dioxide and temperature on microRNA expression in *Arabidopsis* development. *Nature Communications* **4**: 2145.
- Meuthen D, Baldauf SA, Bakker TCM, Thünken T. 2018.** Neglected patterns of variation in phenotypic plasticity: age-and sex-specific antipredator plasticity in a cichlid fish. *The American Naturalist* **191**: 475–490.
- Munir J, Dorn LA, Donohue K, Schmitt J. 2001.** The effect of maternal photoperiod on seasonal dormancy in *Arabidopsis thaliana* (*Brassicaceae*). *American Journal of Botany* **88**: 1240–1249.
- Niinemets Ü. 2016.** Leaf age dependent changes in within-canopy variation in leaf functional traits: a meta-analysis. *Journal of Plant Research* **129**: 313–338.
- Nilsson-Örtman V, Rogell B, Stoks R, Johansson F. 2015.** Ontogenetic changes in genetic variances of age-dependent plasticity along a latitudinal gradient. *Heredity* **2015** **115**: 366–378.
- Olszewski N, Sun T, Gubler F. 2002.** Gibberellin signaling biosynthesis, catabolism, and response pathways. *The Plant Cell* **14**: S61–S80.

Parrish JAD, Bazzaz FA. 1985. Ontogenetic niche shifts in old-field annuals. *Ecology* **66**: 1296–1302.

Poethig RS. 1988. Heterochronic mutations affecting shoot development in maize. *Genetics* **119**: 959–73.

Poethig RS. 1990. Phase change and the regulation of shoot morphogenesis in plants. *Science* **250**: 923–930.

Posadas DM, Carthew RW. 2014. MicroRNAs and their roles in developmental canalization. *Current Opinion in Genetics and Development* **27**: 1–6.

Rubinelli PM, Chuck G, Li X, Meilan R. 2013. Constitutive expression of the *Corngrass1* microRNA in poplar affects plant architecture and stem lignin content and composition. *Biomass and Bioenergy* **54**: 312–321.

Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D. 2005. Specific effects of microRNAs on the plant transcriptome. *Developmental Cell* **8**: 517–527.

Sebestyén F, Miklós M, Iván K, Tökölyi J. 2020. Age-dependent plasticity in reproductive investment, regeneration capacity and survival in a partially clonal animal (*Hydra oligactis*). *Journal of Animal Ecology* **89**: 2246–2257.

Sieber P, Wellmer F, Gheyselinck J, Riechmann JL, Meyerowitz EM. 2007. Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. *Development* **134**: 1051–1060.

Silva PO, Batista DS, Henrique J, Cavalcanti F, Koehler AD, Vieira LM, Fernandes AM, Hernan Barrera-Rojas C, Ribeiro DM, Nogueira FTS, et al. 2019. Leaf heteroblasty in *Passiflora edulis* as revealed by metabolic profiling and expression analyses of the microRNAs miR156 and miR172. *Annals of Botany* **123**: 1191–1203.

Stinchcombe JR, Dorn LA, Schmitt J. 2004. Flowering time plasticity in *Arabidopsis thaliana*: a reanalysis of Westerman & Lawrence (1970). *Journal of Evolutionary Biology* **17**: 197–207.

Telfer A, Bollman KM, Poethig RS. 1997. Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development* **124**: 645–654.

Tsukaya H, Shoda K, Kim G-T, Uchimiya H. 2000. Heteroblasty in *Arabidopsis thaliana* (L.) Heynh. *Planta* **210**: 536–542.

Turner KG, Lorts CM, Haile AT, Lasky JR. 2020. Effects of genomic and functional diversity on stand-level productivity and performance of non-native *Arabidopsis*. *Proceedings of the Royal Society B* **287**: 20202041.

Valladares F, Sanchez-Gomez D, Zavala MA. 2006. Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. *Journal of Ecology* **94**: 1103–1116.

Valladares F, Wright SJ, Lasso E, Kitajima K, Pearcy RW. 2000. Plastic phenotypic response to light of 16 congeneric shrubs from a panamanian rainforest. *Ecology* **81**: 1925–1936.

Wang JW, Park MY, Wang LJ, Koo Y, Chen XY, Weigel D, Poethig RS. 2011. MiRNA control of vegetative phase change in trees. *PLoS Genetics* **7**: e1002012.

Wang J-WW, Schwab R, Czech B, Mica E, Weigel D. 2008. Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in *Arabidopsis thaliana*. *Plant Cell* **20**: 1231–1243.

Wang H, Wang H. 2015. The miR156/SPL module, a regulatory hub and versatile toolbox, gears up crops for enhanced agronomic traits. *Molecular Plant* **8**: 677–688.

Westerband AC, Funk JL, Barton KE. 2021. Intraspecific trait variation in plants: a renewed focus on its role in ecological processes. *Annals of botany* **127**: 397–410.

Willmann MR, Poethig RS. 2007. Conservation and evolution of miRNA regulatory programs in plant development. *Current Opinion in Plant Biology* **10**: 503–511.

Wu G, Park MY, Conway SR, Wang JW, Weigel D, Poethig RS. 2009. The sequential action of miR156 and miR172 regulates developmental timing in *Arabidopsis*. *Cell* **138**: 750–759.

Wu G, Poethig RS. 2006. Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. *Development* **133**: 3539–3547.

Xu M, Hu T, Poethig RS. 2021. Low light intensity delays vegetative phase change. *Plant Physiology* **kiab243**.

Xu M, Hu T, Zhao J, Park M-Y, Earley KW, Wu G, Yang L, Poethig RS. 2016. Developmental functions of miR156-regulated SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes in *Arabidopsis thaliana*. *PLOS Genetics* **12**: e1006263.

Yan A, Pan J, An L, Gan Y, Feng H. 2012. The responses of trichome mutants to

enhanced ultraviolet-B radiation in *Arabidopsis thaliana*. *Journal of Photochemistry and Photobiology B: Biology* **113**: 29–35.

Yang L, Conway SR, Poethig RS. 2011. Vegetative phase change is mediated by a leaf-derived signal that represses the transcription of miR156. *Development* **138**: 245–249.

Yang L, Xu M, Koo Y, He J, Poethig RS. 2013. Sugar promotes vegetative phase change in *Arabidopsis thaliana* by repressing the expression of MIR156A and MIR156C. *eLife* **2**: e00260.

Zhang J, Lechowicz MJ. 1994. Correlation between time of flowering and phenotypic plasticity in *Arabidopsis thaliana* (Brassicaceae). *American Journal of Botany* **81**: 1336–1342.

Zheng Q-L, Liu J, Goff BM, Dinkins RD, Zhu H. 2016. Genetic manipulation of miR156 for improvement of biomass production and forage quality in Red Clover. *Crop Science* **56**: 1–7.

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

Figures

Figure 1. Illustration of two sources of plasticity involving vegetative phase change, phase-dependent changes in plasticity that describe how the developmental phase of an organ (juvenile or adult) impacts the degree of phenotypic plasticity of that organ, and plasticity in developmental timing describing how the timing of vegetative phase change can be accelerated or delayed in response to environment. In the above example, we find greater plasticity in leaf morphology for adult (red) than juvenile (blue) leaves between environment 1 and 2 as adult leaves change in both shape and size whereas juvenile leaves only change in size. The timing of vegetative phase change is delayed in environment 2 compared to environment 1 as indicated by the longer time 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 (* $p < 0.05$, ** $p < 0.01$).