Vegetative Phase Change Causes Age-Dependent Changes in Phenotypic Plasticity
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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 developmentally 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; Leichty & 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 µmol m⁻² 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 gibberellic 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 $p < 0.0001$). 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, 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 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; Garbowsk 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; Westerband 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, (Leichy & 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


### 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.

<table>
<thead>
<tr>
<th>Genotype</th>
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</tbody>
</table>
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).