

Low light intensity delays vegetative phase change

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Abstract

Plants that develop under low light (LL) intensity often display a phenotype known as the “shade tolerance syndrome (STS)”. This syndrome is similar to the phenotype of plants in the juvenile phase of shoot development, but the basis for this similarity is unknown. We tested the hypothesis that the STS is regulated by the same mechanism that regulates the juvenile vegetative phase by examining the effect of LL on rosette development in *Arabidopsis* (*Arabidopsis thaliana*). We found that LL prolonged the juvenile vegetative phase and that this was associated with an increase in the expression of the master regulators of vegetative phase change, miR156 and miR157, and a decrease in the expression of their SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) targets. Exogenous sucrose partially corrected the effect of LL on seedling development and miR156 expression. Our results suggest that the response of *Arabidopsis* to LL is mediated by an increase in miR156/miR157 expression and by factors that repress SPL gene expression independently of miR156/miR157, and is caused in part by a decrease in carbohydrate production. The effect of LL on vegetative phase change does not require the photoreceptors and transcription factors responsible for the shade avoidance syndrome, implying that light intensity and light quality regulate rosette development through different pathways.

Introduction

Over the last 50 years, research on the response of plants to light has largely focused on the effect of the presence versus absence of light and the effect of light quality on early seedling development (Smith, 1982; Chory, 1997; Roig-Villanova and Martinez-Garcia, 2016; Ballare and Pierik, 2017). Extensive research on the effect of red (R), far-red (FR), and blue light on hypocotyl elongation in *Arabidopsis* has provided a deep understanding of the molecular mechanism by which specific wavelengths of light regulate this developmental process. In contrast, the effect of light intensity on plant development has received relatively little attention, despite the fact that in nature plants can experience dramatic variation in light intensity on

both short (e.g. seconds or minutes) and long (hours, months, years) time scales.

Plants adapted for growth under low light (LL) intensity, as well as shoots exposed to a prolonged period of LL, often exhibit a phenotype known as the shade tolerance syndrome (STS; Valladares and Niinemets, 2008). This phenotype consists of a large number of physiological, morphological, and anatomical traits that optimize carbon gain; indeed, it is generally assumed that the primary, if not the only, function of the STS is to maximize photosynthesis under LL. However, some of the anatomical and morphological traits associated with the STS have no known function in photosynthesis (Valladares and Niinemets, 2008), raising the question of why the expression of these traits is associated

with LL. Additionally, many of the anatomical/morphological features associated with the STS are distinct from, and in some cases opposite to, the traits that characterize the shade avoidance syndrome (SAS; Valladares and Niinemets, 2008; Ballare and Pierik, 2017), a phenotype generated by a low R:FR light ratio or exposure to FR light near the end of the day. This is important because in nature, LL is typically caused by the presence of surrounding plants, which both decrease light intensity and decrease the R:FR ratio by absorbing red light during photosynthesis (Chory, 1997). This suggests that plants respond to LL and a decreased R:FR ratio by different mechanisms and are able to “choose” the mechanism that is appropriate for the specific light conditions in which they find themselves.

It has been recognized for over a hundred years that leaves grown in LL resemble leaves produced during the juvenile phase of shoot development (Nordhausen, 1912; Schramm, 1912; Njoku, 1956). However, the basis for this similarity is unknown. One possibility is that the LL delays the juvenile-to-adult transition (vegetative phase change) or reactivates the juvenile vegetative phase in adult plants. An alternative possibility is that LL intensity affects some of the traits associated with the juvenile phase, without actually affecting the timing of vegetative phase change (Jones, 1995, 1999). Both hypotheses imply that the STS is regulated in whole or in part by the vegetative phase change pathway, and recent evidence suggests that juvenile leaves are photosynthetically more efficient under LL intensity than are adult leaves. Lawrence et al., (2020) provides intriguing support for this hypothesis. To investigate the role of the vegetative phase change pathway in the STS, we characterized the effect of LL on vegetative phase change in Arabidopsis.

Vegetative phase change is regulated by a decline in the level of the related miRNAs, miR156/157, and the resulting increase in the expression of their targets, SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) transcription factors (Wu and Poethig, 2006; Chuck et al., 2007; Wu et al., 2009). In Arabidopsis, miR156/157 directly regulate 10 SPL genes (Rhoades et al., 2002; Xu et al., 2016; He et al., 2018). These SPL genes are responsible for many aspects of adult shoot development, including adult leaf morphology, abaxial trichome production, reduced branching, a low propensity for adventitious root initiation and shoot regeneration, low anthocyanin production, decreased insect resistance, and increased thermo sensitivity (Chuck et al., 2007; Schwarz et al., 2008; Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009; Yu et al., 2010; Gou et al., 2011; Padmanabhan et al., 2013; Stief et al., 2014; Zhang et al., 2015; Xu et al., 2016b; Mao et al., 2017; Nguyen et al., 2017).

Approximately 90% of the miR156 in the rosette leaves of Arabidopsis is produced by MIR156A and MIR156C (He et al., 2018). The downregulation of these genes in successive rosette leaves is largely dependent on changes in their chromatin structure (Pico et al., 2015; Xu et al., 2016a, 2016b, 2018). Early in shoot development, MIR156A/MIR156C contain elevated levels of the active histone modification,

H3K27ac (Xu et al., 2016a), as well as high levels of H2A.Z, which facilitates the deposition of the active histone modification, H3K4me3 (Xu et al., 2018). The subsequent repression of these genes is mediated by the deposition of H3K27me3 in a process modulated by Polycomb Repressive Complex 1 (PRC1; Pico et al., 2015), PRC2 (Xu et al., 2016a), and the chromatin remodelers PICKLE (PKL; Xu et al., 2016a) and BRAHMA (Xu et al., 2016c). In addition to being post-transcriptionally repressed by miR156/157, the expression of SPL genes is regulated by several processes that operate independently of miR156/157, including H2A monoubiquitination (H2Aub) by AtRING1A/AtRING1B (Kim et al., 2015; Li et al., 2017), histone acetylation by SAGA-like histone acetyltransferase (HAG1; Kim et al., 2015), and O-linked N-acetylglucosamine modification of SPL proteins (Xu et al., 2017). However, it is currently unknown if these modifications control the temporal and spatial pattern of SPL gene expression.

Most of the research on the response of Arabidopsis to shade has focused on the mechanism of the SAS (Casal, 2013; Roig-Villanova and Martinez-Garcia, 2016; Ballare and Pierik, 2017). As noted above, this syndrome is produced by exposure to FR-enriched light and a decrease in the intensity of blue light, leading to an increase in the expression of the transcription factors PIF4, 5, and 7, the primary mediators of this syndrome. It has been suggested that some aspects of the SAS are mediated by the miR156/SPL pathway based on the observation that miR156 levels decline by 80% while SPL transcript levels increase up to four-fold when plants are briefly exposed to FR light at the end of the day (Xie et al., 2017). However, this result has yet to be reconciled with the observation that the primary sources of miR156 in Arabidopsis—MIR156A and MIR156C—are not targets of the PIF transcription factors that mediate this end of day FR response (Xie et al., 2017).

Here, we show that the juvenile-to-adult transition in Arabidopsis is delayed by LL, and that this response is mediated by both an increase in the level of miR156/miR157, and by a miR156-independent decrease in the level of SPL transcription factors. Phytochrome- and cryptochrome-mediated signaling are not required for this response, suggesting that the response of plants to light quality and their response to LL are mediated by different regulatory pathways. Along with the evidence that juvenile leaves are photosynthetically more efficient under LL than adult leaves (Lawrence et al., 2020), these results suggest that the STS is under the regulation of the miR156/SPL vegetative phase change pathway.

Results

Vegetative phase change is delayed by LL

To study the role of light intensity in vegetative phase change without the complicating effect of a difference in flowering time, we grew wild-type Col in short days under high light (HL; 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and LL (62 $\mu\text{mol m}^{-2} \text{s}^{-1}$) conditions. This was accomplished by varying the number

of fluorescent light bulbs in otherwise identical growth chambers. Plants grown in LL were lighter green and slower to develop than plants grown in HL (Figure 1, A and B), and their leaves were rounder and less serrated than HL plants (Figure 1C). Additionally, abaxial trichome production was significantly delayed in LL plants compared to plants grown in HL (Figure 1C). These results demonstrate that LL delays vegetative phase change.

To determine if the effect of light intensity on vegetative phase change is attributable to changes in the levels of miR156/157, we measured the abundance of these miRNAs in plants grown in short days under HL and LL. Because plants grow more slowly in LL (Figure 1B), we sampled shoot apices according to their developmental stage. Samples were collected when leaves 1, 3, 5, 7, or 9 were 1 mm in length, and consisted of this leaf primordium and all younger leaf primordia and the shoot apical meristem (L1+, L3+, L5+, L7+, and L9+).

The temporal pattern of miR156 expression was similar in HL and LL, but the amount of miR156 in LL shoot apices was significantly higher than the amount of miR156 in HL apices at every stage of development (Figure 1D). There was no significant difference in the abundance of miR157 in L1+ apices grown in LL and HL. In HL, the abundance of miR157 began to decline at L3+, whereas in LL it began to decline at L5+ (Figure 1E). This slight delay in the downregulation of miR157 relative to miR156 suggests that light intensity regulates the expression of miR156 and miR157 by different mechanisms. We then analyzed the effect of LL on the primary transcripts of *MIR156A* and *MIR156C*, the major sources of miR156 in Arabidopsis (He et al., 2018). Both transcripts were present at significantly higher levels at early stages of development (L1+, L3+, L5+) in LL- than in HL-grown plants (Figure 1, F and G), but declined to the same level in older (L9+) plants. This result is consistent with the expression pattern of the mature miR156 transcript, and suggests that these genes are primarily responsible for this expression pattern. Consistent with the elevated levels of miR156, the *SPL9*, *SPL13*, and *SPL15* transcripts were less abundant in LL than in HL plants (Figure 1H; Supplemental Figure S1A).

To determine if miR156 and miR157 are responsible for the delay in vegetative phase change under LL, we compared the phenotype of stocks deficient for miR156 (*mir156a-2 mir156c-1*; hereafter *mir156a mir156c*) or miR157 (*mir157a-1 mir157c-1*, hereafter *mir157a mir157c*), and stocks deficient for both of these miRNAs (*mir156a mir156c mir157a mir157c* and plant overexpressing miR156 target mimic (35S::MIM156) under HL and LL. Abaxial trichome production was significantly delayed by LL in both wild-type Col and in the *mir156a mir156c* and *mir157a mir157c* double mutants. However, LL had a slightly smaller effect on abaxial trichome production in these mutants than in Col, and had no significant effect on abaxial trichome production in the *mir156a mir156c mir157a mir157c* quadruple mutant or in 35S::MIM156 (Figure 1I). These results demonstrate that

miR156 and miR157 both contribute to the effect of LL on abaxial trichome production.

A miR156-independent pathway is involved in the LL response

Although LL did not affect abaxial trichome production in the *mir156a mir156c mir157a mir157c* and 35S::MIM156 lines (Figure 1I), it did have a significant effect on leaf morphology in these miR156/157-deficient lines (Figure 2A; Supplemental Figure S1B). In Arabidopsis, juvenile leaves have a smaller angle between the petiole and the leaf blade than adult leaves (Yang et al., 2011; He et al., 2018). We found that LL significantly decreased the leaf blade to petiole angle of leaves 1 and 2 in both Col and in mutants deficient for miR156/157 (Figure 2B). This result demonstrates that some of the effects of LL on leaf morphology do not require miR156/157.

To determine if the effect of LL on leaf morphology might be mediated by a miR156/157-independent effect of LL on *SPL* gene expression, we measured the abundance of the *SPL9*, *SPL13*, and *SPL15* transcripts in Col and 35S::MIM156 seedlings under HL and LL (Figure 2C). As expected (He et al., 2018), under HL, all three transcripts were elevated in 35S::MIM156 seedlings relative to Col. LL reduced the expression of all three transcripts in both Col and 35S::MIM156 plants, but appeared to have a slightly bigger relative effect on *SPL9* and *SPL13* in Col than in 35S::MIM156, which is consistent with the elevated level of miR156 in LL Col plants. miR156 represses *SPL* gene expression primarily at a translational level (He et al., 2018). Therefore, to obtain a more accurate picture of the relative importance of miR156-dependent versus miR156-independent regulation of *SPL* activity, we examined the effect of light intensity on the expression of miR156-sensitive and miR156-resistant *pSPL13::SPL13-GUS* translational reporters. LL nearly completely repressed the expression of the miR156-sensitive *pSPL13::SPL13-GUS* reporter, and reduced the expression of the miR156-resistant *pSPL13::rSPL13-GUS* to about 0.4 of the level in HL plants (Figure 2D). These results demonstrate that light intensity regulates *SPL* expression through both miR156-dependent and miR156-independent pathways.

The response to light intensity is partially mediated by carbohydrates

Mutations in the chlorophyll oxygenase gene, *CHLORINA1* (*CH1*; Oster et al., 2000), produce yellow-green plants that undergo delayed vegetative phase change (Yang et al., 2013), and thus resemble wild-type plants grown in LL. Wild-type plants grown in LL have about 60% of the amount of chlorophyll present in HL plants (Figure 3A), whereas the *ch1-4* mutant has about 30% of the wild-type level chlorophyll (Figure 3B). Previous work has suggested that *ch1-4* delays vegetative phase change because it reduces sugar production (Yang et al., 2013). To determine if the effect of LL on vegetative phase change is attributable to a reduction

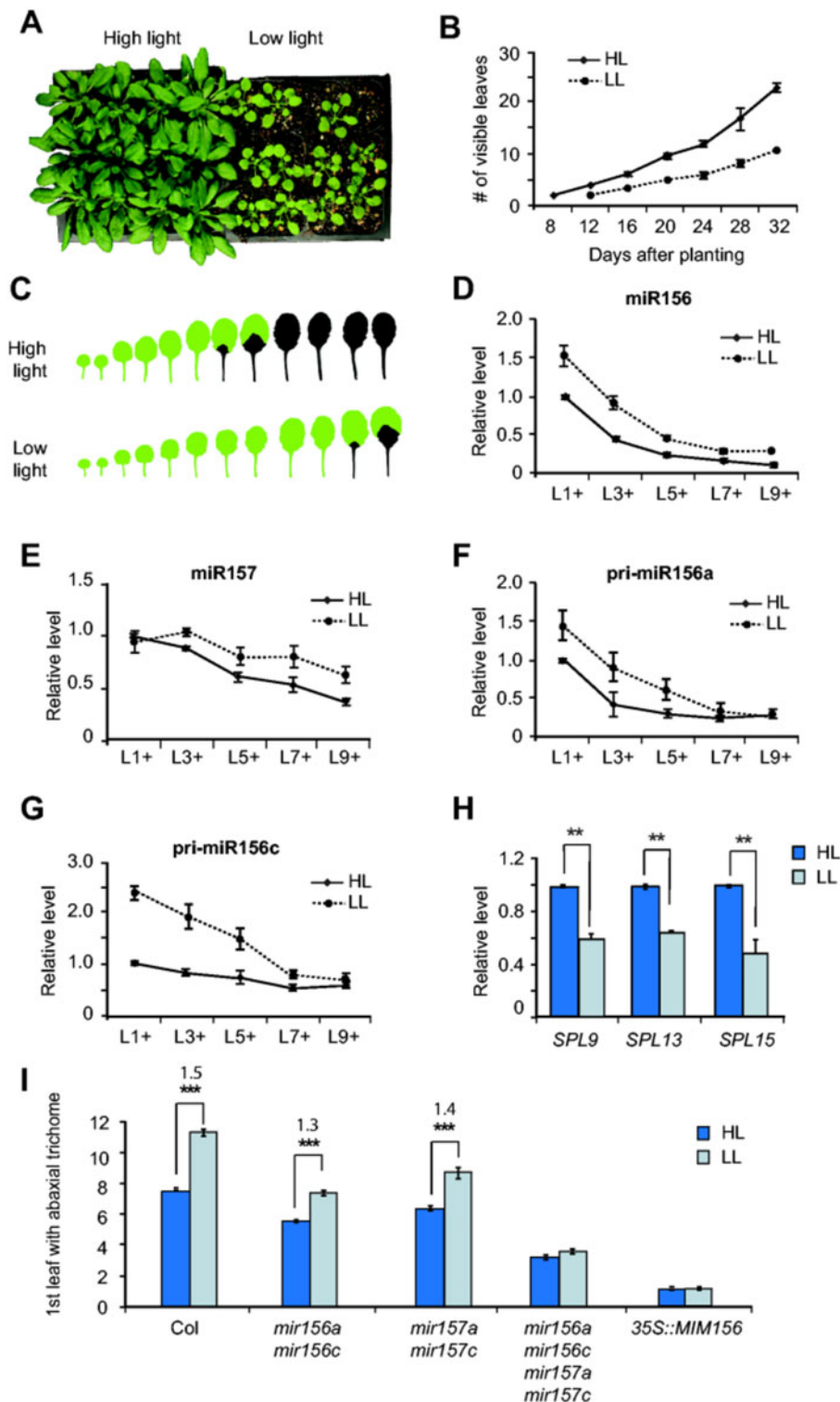


Figure 1 LL delays vegetative phase change via miR156/157. **A**, Col plants grown under HL (left) are greener and have more leaves than plants grown under LL (right). **B**, Rate of leaf initiation in Col grown in HL and LL. $n = 32$ for each condition. $P < 0.001$, one-way analysis of variance (ANOVA). **C**, LL delays the production of rosette leaves with abaxial trichomes in Col. Green, no abaxial trichomes; Black, abaxial trichomes. Images of leaves were digitally extracted for comparison. The images are the same as the images of Col in [Supplemental Figure S1B](#). **D–G**, RT-qPCR of miR156 (**D**), miR157 (**E**), pri-miR156a (**F**), and pri-miR156c (**G**) in the shoot apices of Col plants grown in HL and LL. Samples were harvested according to their developmental stage. The overall levels of miR156, miR157, pri-miR156a, and pri-miR156c in LL conditions (**D–G**) are significantly different from their levels in HL conditions, $P < 0.05$, two-way ANOVA. **H**, RT-qPCR analysis of the transcripts of miR156/157-targeted genes in L3+ shoot apices. In **D–H**, values are the mean $\pm$ SE from three biological replicates. **I**, The first leaf with abaxial trichomes in Col and mutant plants deficient for miR156, miR157, miR156 and miR157, and 35S::MIM156 under HL and LL conditions. $n = 20–24$ for each genotype. Significant difference: ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA. Error bars are SE.

in the amount of sugar, we grew Col in HL and LL on media containing different concentrations of sucrose. Sucrose of 1% and 2% partially corrected the effect of LL on seedling growth, and 3% sucrose did not produce further changes (Figure 3C). Sucrose of 1% and 2% significantly accelerated abaxial trichome production in LL conditions, but not accelerate abaxial trichome production in HL conditions (Figure 3D). Consistent with this difference in abaxial trichome production, sucrose reduced the level of

miR156/miR157 to a greater extent in LL than in HL (Figure 3, E and F). Specifically, plants grown on 2% sucrose in LL had approximately 0.7 times of the amount of miR156 present in plants grown without sucrose; plants grown in HL on sucrose had 0.8 times the amount of miR156 present in plants grown without sucrose, and similar results were obtained for miR157 (Figure 3E). Consistent with its effect on miR156, sucrose reduced the level of *pri-miR156a* and *pri-miR156c* more in LL than in HL (Figure 3F). These results

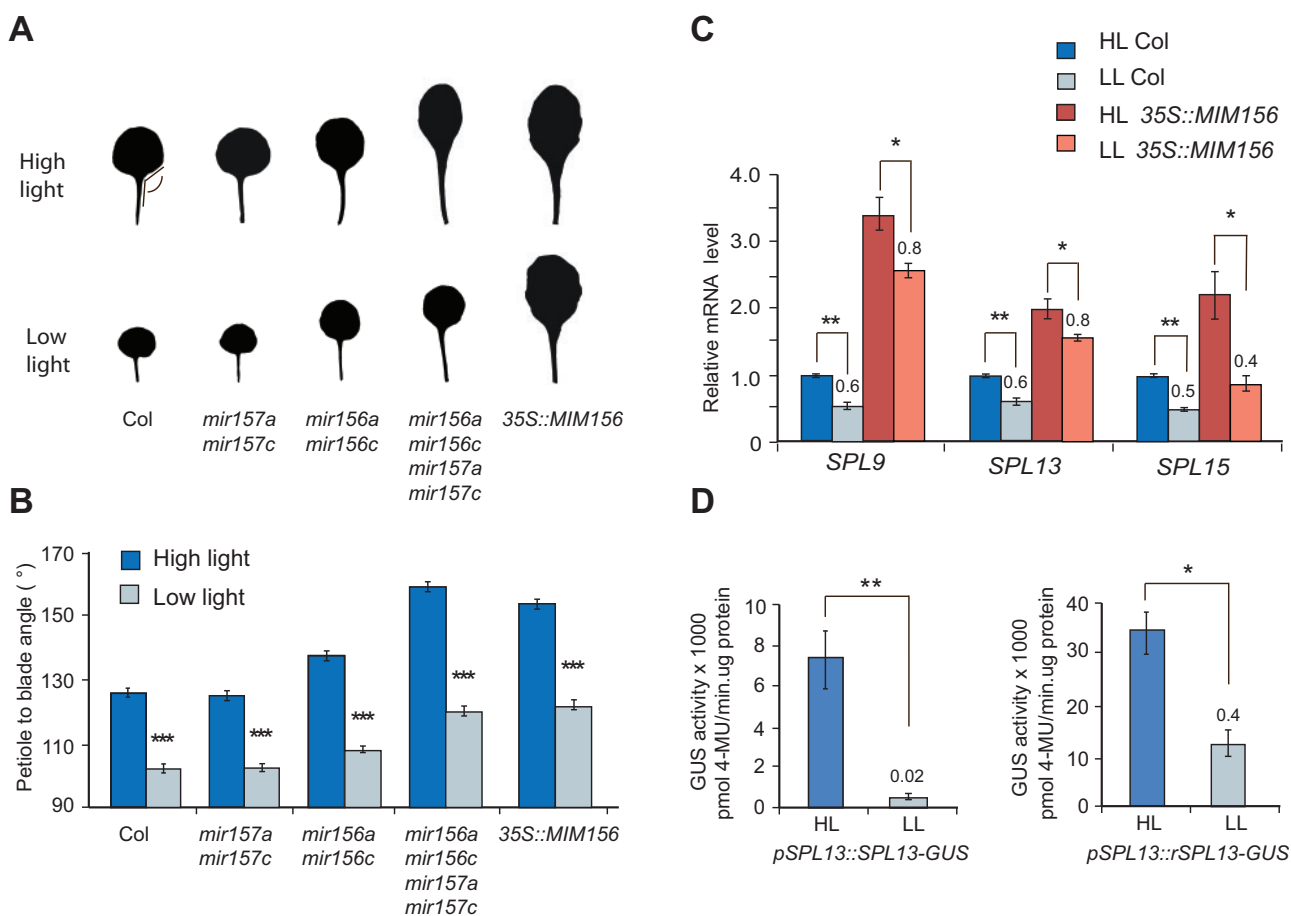


Figure 2 LL affects leaf morphology and SPL expression independently of miR156/157. A, The morphology of leaf 1 under HL and LL in wild-type Col, in mutants lacking miR156 and/or miR157, and in plants constitutively expressing a miR156/157 target mimic (*35S::MIM156*). Images of leaves were digitally extracted for comparison. The leaf 1 of different genotypes are the same as leaf 1 in Supplemental Figure S1B (B) The petiole:blade angle (measured by ImageJ) of leaves 1 and 2 under HL and LL in wild-type Col and in genotypes deficient for miR156, miR157, and both miR156 and miR157. Values are mean ± SE. ***P < 0.001, one-way ANOVA. C, The relative amount of SPL9, SPL13, and SPL15 transcripts in Col and *35S::MIM156* plants grown in HL and LL. Values are normalized to the transcript level in Col grown under HL. The ratio of the transcript abundance in LL relative to HL in each genotype is indicated. D, 4-Methylumbelliferyl-β-D-glucuronide hydrate (MUG) assay of GUS activity in transgenic plants expressing miR156-sensitive (left) and miR156-resistant (right) genomic constructs of SPL13-GUS under HL and LL. Numbers above bars represents the ratio of LL/HL MUG activity. Note the difference in the scale of the Y axes. C–D, ***P < 0.001, **P < 0.01, *P < 0.05, one-way ANOVA. RT-qPCR analyses and MUG assays are mean ± SE from three biological replicates

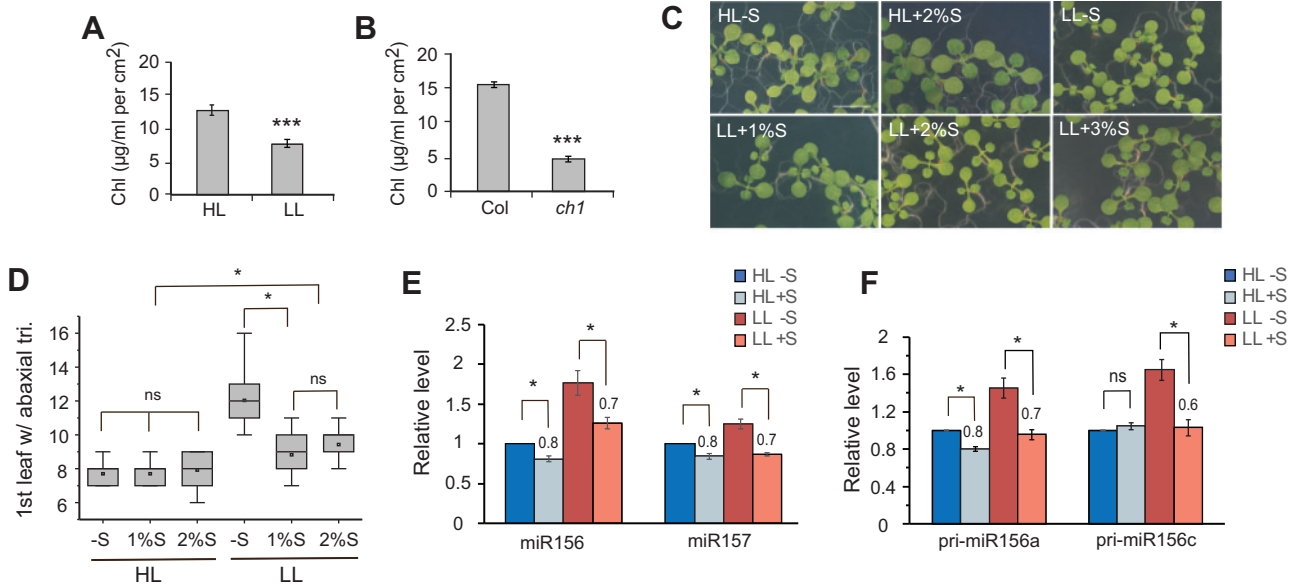


Figure 3 Sucrose partially corrects the effect of LL. A, B, Chlorophyll concentration in Col under HL and LL (A), and in Col and *ch1-4* under HL (B). Values are mean $\pm$ SE from four biological replicates. *** $P < 0.001$, one-way ANOVA. C, Col seedlings grown under HL and LL in the absence or presence of exogenous sucrose. D, The first leaf with abaxial trichomes in Col grown on 1/2MS without sucrose (–S) or with 1%S or 2%S in HL or LL conditions. Center line, median; box limits, upper and lower quartiles; square, mean; whiskers, $1.5\times$ interquartile range. * $P < 0.05$, two-way ANOVA. NS denotes not significant difference, $P > 0.05$, two-way ANOVA. E, The effect of exogenous sucrose on the abundance of miR156 and miR157 in Col seedlings grown in HL and LL. Values were normalized to the transcript levels in Col under HL. Numbers represent the amount of transcript in samples grown in the presence of sugar relative to samples grown in the absence of sugar under the same light conditions. F, The effect of exogenous sucrose on the abundance of pri-miR156a and pri-miR156c in Col seedlings grown in HL and LL. Values were normalized to the transcript levels in Col under HL. Numbers represent the amount of transcript in samples grown in the presence of sugar relative to samples grown in the absence of sugar under the same light conditions. * $P < 0.05$, one-way ANOVA. NS denotes not significant difference, $P > 0.05$. Values are mean $\pm$ SE from three biological replicates.

suggest that the effect of LL on the expression of miR156/miR157 is partially explained by the effect of LL on carbohydrate production.

Light intensity probably regulates miR156 expression independently of H3K27me3

Under HL, the downregulation of *MIR156A* and *MIR156C* is mediated by the deposition of H3K27me3 at these loci (Pico et al., 2015; Xu et al., 2016a). To determine if light intensity affects this process, we used chromatin immunoprecipitation (ChIP) to measure the level of H3K27me3 at *MIR156A*/*MIR156C* in HL and LL plants. The level of H3K27me3 at *MIR156A*/*MIR156C* was significantly lower in 2-week-old LL plants than in 2-week-old HL plants (Figure 4, A and B). However, in the case of *MIR156A*, this is probably explained by developmental delay caused by LL since 3-week-old LL plants—which were at the same developmental stage as 2-week-old HL plants—had nearly the same level of H3K27me3 at *MIR156A* as HL plants (Figure 4A). In contrast, 3-week-old LL plants had significantly less H3K27me3 at *MIR156C* than 2-week-old HL plants (Figure 4B). Thus, light intensity has a more significant effect on the deposition of H3K27me3 at *MIR156C* than at *MIR156A*. To determine if light intensity modulates the deposition of H3K27me3 by affecting carbohydrate production, we used ChIP to measure the level of H3K27me3 at *MIR156A*/*MIR156C* in 2-week-old

plants growing in LL with or without sucrose. Sucrose significantly elevated the level of H3K27me3 at *MIR156A* (Figure 4C) but had no effect on the level of H3K27me3 at *MIR156C* (Figure 4D). In aggregate, these results indicate that LL and sugar can affect the level of H3K27me3 at *MIR156A* or *MIR156C* in ways that are consistent with the effect of these factors on the expression of these genes. However, the biological relevance of these results is unclear because LL and sugar had different effects on the deposition of H3K27me3 at *MIR156A* and *MIR156C*, even though these factors affected the expression of these genes in the same way.

We next asked if the effect of LL on vegetative phase change requires H3K27me3. This repressive histone modification is deposited on target chromatin by the activity of PRC2 (Hennig and Derkacheva, 2009; Ito and Sun, 2009; Bouyer et al., 2011), one component of which is the histone methyl transferase, SWINGER (SWN; Zhou et al., 2018; Shu et al., 2019). The CHD3-like chromatin remodeler, PKL, also promotes the deposition of H3K27me3, although the mechanism by which it does this is still unknown (Zhang et al., 2008, 2012; Xu et al., 2016a). Plants mutant for both *swn* and *pk1* have reduced levels of H3K27me3 at *MIR156A*/*MIR156C* and display elevated expression of both genes (Xu et al., 2016a). To determine if changes in H3K27me3 are responsible for the effect of light intensity on vegetative phase

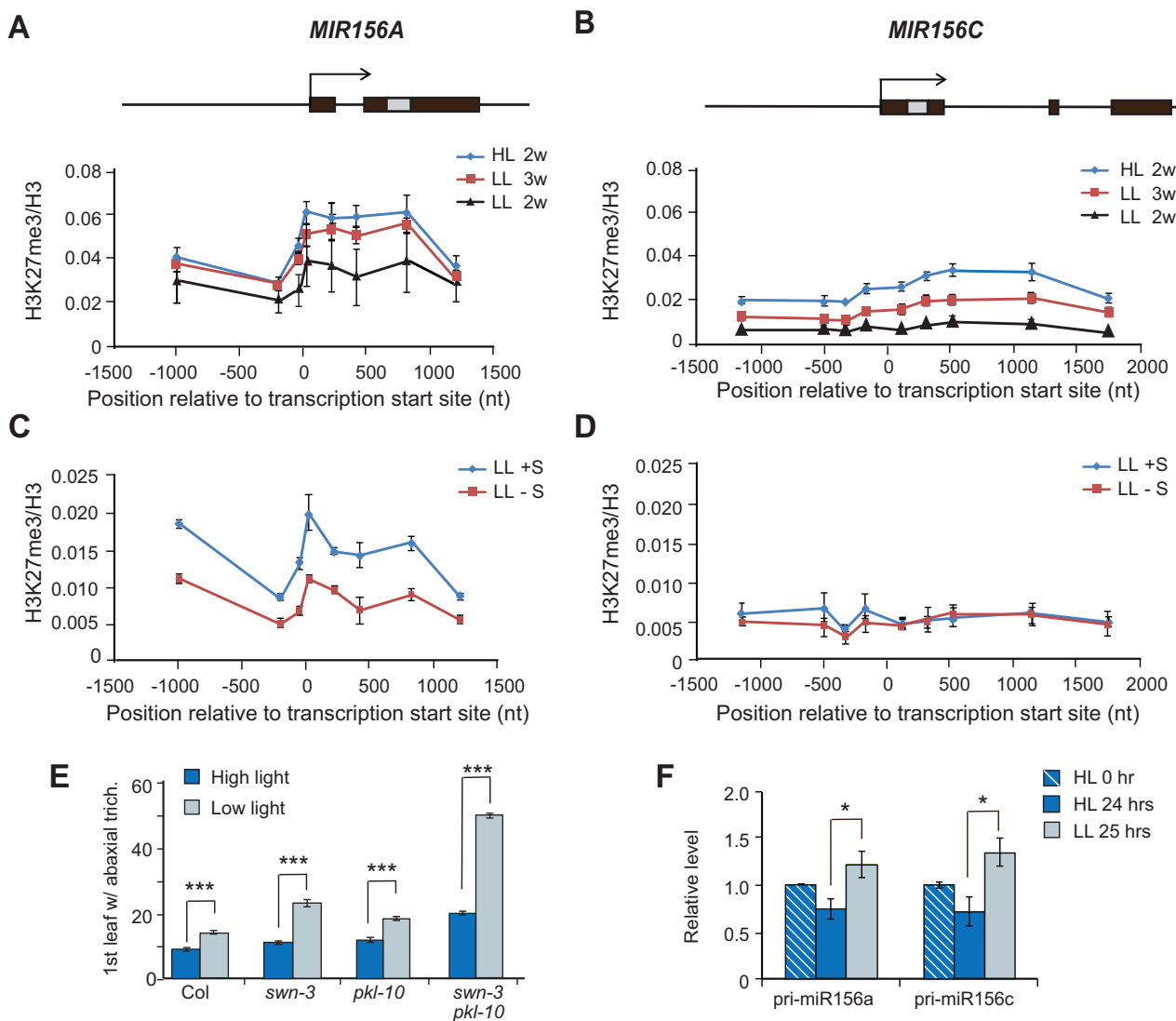


Figure 4 LL does not act by decreasing the deposition of H3K27me3 at *MIR156A* and *MIR156C*. A, B, Schematic diagram of the genomic structures of *MIR156A* and *MIR156C* and ChIP-qPCR analysis of H3K27me3 at these loci in 2- or 3-week-old Col plants grown in HL or LL. Black boxes represent exons, the grey box represents the miR156 hairpin, and arrows indicate transcription start site. H3K27me3 levels in 2 week (2w) HL are significantly different from levels in 2w LL ($P < 0.05$), two-way ANOVA (A). H3K27me3 levels in 2w HL are significantly different from levels in 2w LL and 3w LL ($P < 0.01$), two-way ANOVA (B). C, D, ChIP-qPCR analysis of H3K27me3 at *MIR156A* (C) and *MIR156C* (D) in Col plants grown in LL with (+S) or without (-S) sucrose. H3K27me3 levels are significantly different between HL and LL conditions at *MIR156A*, $P < 0.05$, one-way ANOVA. Data are expressed as relative to SHOOT MERISTEMLESS (STM): $(\text{H3K27me3}/\text{H3})_{\text{MIR156A}}/(\text{H3K27me3}/\text{H3})_{\text{STM}}$ (A and C) or $(\text{H3K27me3}/\text{H3})_{\text{MIR156C}}/(\text{H3K27me3}/\text{H3})_{\text{STM}}$ (B and D). E, The first leaf with abaxial trichomes in Col, *swn-3*, *pkl-10*, and *swn-3 pkl-10* plants grown in HL and LL. Values are mean $\pm$ SE. $n = 24$ for each group. F, RT-qPCR analysis of the abundance of pri-miR156a and pri-miR156c transcripts in Col seedlings 24 h after the transfer from HL to LL. * $P < 0.05$, one-way ANOVA. Values in A–D and F are mean $\pm$ SE from three biological replicates.

change, we compared the phenotype of *swn-3 pkl-10* mutants under HL and LL. Previously, we reported that under HL *swn-3*, *pkl-10*, and the *swn-3 pkl-10* double mutant undergo delayed vegetative phase change (Xu et al., 2016a), and we were able to replicate this result (Figure 4E). We also found that LL further delayed vegetative phase change in these mutants. This was particularly dramatic in the case of *swn-3 pkl-10*. In HL, the appearance of abaxial trichomes was delayed by 11 leaves in *swn-3 pkl-10* (19.4 ± 0.3) compared to Col (7.8 ± 0.2); however, in LL, *swn-3 pkl-10* produced abaxial trichomes over 37 leaves later than Col

(51.0 ± 0.9 versus 13.1 ± 0.3 ; Figure 4E). This result demonstrates that a reduction in H3K27me3 makes plants hypersensitive to the effect of LL, but also suggests that the effect of LL on vegetative phase change may not be mediated by a LL-induced reduction in H3K27me3 since *swn-3 pkl-10* already has low levels of H3K27me3 at *MIR156A* and *MIR156C* (Xu et al., 2016a).

H3K27me3 is usually deposited at a locus over several days or weeks (Jiang et al., 2008; Angel et al., 2011; Coustham et al., 2012; Yang et al., 2014; Xu et al., 2016a). We reasoned that if LL de-represses *MIR156A/MIR156C* by

reducing the deposition of H3K27me₃, is it unlikely that a brief exposure to LL would cause a significant increase in the expression of these genes. To test this hypothesis, we grew Col in HL for 7 d and then compared the abundance of *pri-miR156a* and *pri-miR156c* in the shoot apices of plants grown in HL for an additional 24 h with the abundance in plants transferred to LL for 24 h. Plants grown in HL for an additional 24 h had less *pri-miR156a* and *pri-miR156c* than 7-d-old HL plants, which is expected because the expression of these transcripts declines early in shoot development. In contrast, plants transferred to LL for 24 h had significantly more *pri-miR156a* and *pri-miR156c* than HL plants (Figure 4F). Thus, LL has a relatively rapid effect on the transcription of *MIR156A/MIR156C*, suggesting that this effect is unlikely to be due to a decrease in H3K27me₃ deposition.

The response to LL intensity is not mediated by phytochrome or cryptochrome signaling

It has been reported that the SAS is regulated in part by a decrease in expression of *miR156*, mediated by exposure to FR light at the end of the day (Xie et al., 2017). We therefore decided to investigate if the R:FR and blue light signaling pathways contribute to the response of plants to LL. In Arabidopsis, the main photoreceptors for R and FR light are *phyA* and *phyB*, and the main blue light photoreceptors are *CRY1* and *CRY2* (Casal, 2013). To determine if these photoreceptors are required for the response to LL, we examined the phenotype of plants deficient for *CRY1* and *CRY2* (*hy4-B104 cry2-1*) and plants deficient for *PHYA* and *PHYB* (*phyA-211 phyB-9*; Figure 5A). The vegetative morphology, as well as the timing of abaxial trichome production, in the *cry1 cry2* double mutant was essentially identical to WT under both HL and LL conditions (Figure 5A). Consistent with previous reports (Reed et al., 1994), under HL, *phyA phyB* double mutants were light green, and had an elongated hypocotyl, elongated internodes, small narrow leaves with elongated petioles, and flowered much earlier than WT. These double mutants also produced abaxial trichomes significantly earlier than WT plants under HL (Figure 5A). *phyA phyB* mutants had a similar if not identical morphology under LL, but produced abaxial trichomes significantly later under LL than under HL. However, the relative delay in abaxial trichome production in *phyA phyB* mutants under LL versus HL was slightly less than in WT plants. These results indicate that *CRY1* and *CRY2* may play no significant role in the response of plants to LL, and also suggest that *PHYA* and *PHYB* may have little or no role in this response.

To further explore the role of blue and red light in the STS, we examined the effect of LL on genes that mediate the effect of these stimuli. Red and blue light control plant growth by regulating the expression of a family of basic helix–loop–helix transcription factors known as PHYTOCHROME-INTERACTING FACTORS (PIFs) (Pedmale et al., 2016). PIF proteins accumulate in the presence of a low R:FR ratio and low-intensity blue light, and promote the

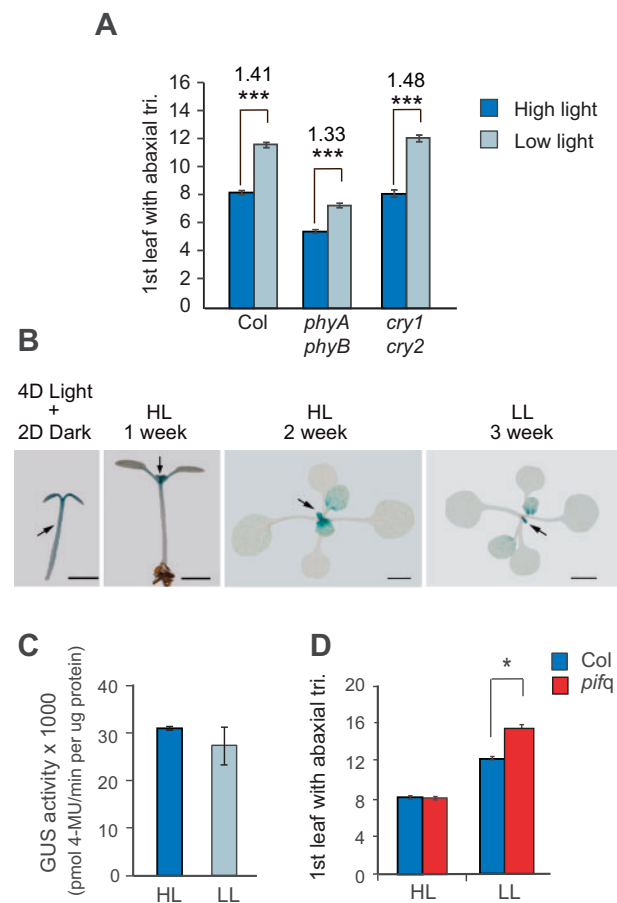


Figure 5 The red and blue light signaling pathways are not required for the effect of LL on vegetative phase change. A, The effect of HL and LL on the first leaf with abaxial trichomes in Col and plants deficient for the red-light photoreceptors, *phyA* and *phyB*, and the blue light photoreceptors, *CRY1* and *CRY2*. $n = 24$ for each group. *** $P < 0.001$, one-way ANOVA. B, Expression of *PIF5::PIF5-GUS* in seedlings and rosette leaves in HL and LL. Arrows in the hypocotyl or shoot apex. Scale bars = 2 mm. Arrows identify the hypocotyl or shoot apex. C, Quantitative MUG assay of *PIF5::PIF5-GUS* activity in the HL and LL rosettes shown in (B). Values are mean $\pm$ se from three biological replicates. D, First leaf with abaxial trichome production in Col and *pifq*. Values are mean $\pm$ se. $n = 33$ for HL Col, 23 for LL Col, 23 for HL *pifq*, 20 for LL *pifq*. * $P < 0.05$, one-way ANOVA.

expression of genes that generate the SAS. Plants mutant for *pif4* and *pif5*, as well as the *pif1 pif3 pif4 pif5* quadruple mutant (*pifq*), do not display the SAS under low R:FR light or low-intensity blue light, and resemble plants grown in HL (Leivar et al., 2012). If PIF proteins promote the STS as well as the SAS, the expectation is that that these proteins will be overexpressed under LL intensity and that loss of PIF gene expression will accelerate the onset of the adult phase.

To test these hypotheses, we first examined the effect of light intensity on the expression of *PIF5* in seedlings and rosette leaves. Consistent with previous observations (Pedmale et al., 2016), the *PIF5::GUS* transcriptional reporter was highly expressed in the hypocotyl, shoot apex, and cotyledons of seedlings germinated in HL for 4 d and transferred to the

dark for 2 d, but was expressed only in the shoot apex of seedlings grown continuously in HL (Figure 5B); thus, *PIF5::GUS* expression in the hypocotyl is repressed by HL. In 2-week-old plants grown continuously in HL, *PIF5::GUS* was strongly expressed in young leaf primordia but was restricted to the vascular tissue of fully expanded leaves. It had a similar expression pattern in 3-week-old plants grown in LL, which were developmentally comparable to the 2-week-old HL plants (Figure 5B). 4-Methylumbelliferyl- β -D-glucuronide hydrate assays in 2-week-old HL grown plants and 3-week-old LL grown plants revealed no significant difference in the amount of GUS activity in these plants (Figure 5C). Therefore, in contrast to its expression in the hypocotyl–*PIF5* expression in rosette leaves is insensitive to light intensity. We next examined the effect of the *pifq* mutant on abaxial trichome production under HL and LL conditions. There was no significant difference in the timing of abaxial trichome production in wild type and *pifq* mutant in HL; however, abaxial trichome production was delayed more significantly in LL in the *pifq* mutant than in Col (Figure 5D). This latter result implies that PIF proteins repress the response of plants to LL, which is the opposite of their function in the response to low R:FR light (i.e. the SAS), which they promote. Together, these results indicate that LL delays vegetative phase change by a mechanism that is distinct from the mechanism involved in the SAS.

Discussion

The STS is typically considered to consist of a set of traits that enable a plant to fix carbon above the photosynthetic compensation point under LL intensity (5); in other words, it is viewed as a physiological adaptation to LL intensity. This begs the question of how LL promotes the expression of these traits, and why the STS includes traits that have no obvious connection to photosynthesis. Our results suggest that the answer to these questions lies in the effect of LL on the expression of genes that regulate vegetative phase change, specifically miR156/miR157 and their targets, the SPL family of transcription factors. This conclusion is supported by the observation that juvenile leaves of Arabidopsis, maize (*Zea mays*), and poplar (*Populus tremula* $\times$ *alba*) are photosynthetically more efficient under LL than adult leaves (Lawrence et al., 2020), and by the results of this study.

We found that LL prolongs the duration of the juvenile phase and that it does so by both increasing the abundance of miR156 and by decreasing the transcription of the SPL genes regulated by miR156. These effects appear to be mediated by a reduction in photosynthesis, as well as by other unknown factors. Importantly, the effect of LL on vegetative phase change does not require the photoreceptors that mediate the response of Arabidopsis to red and blue light, specifically, phyA, phyB, CRY1, and CRY2, or the PIF transcription factors that mediate the effects of these photoreceptors. This supports the conclusion that LL regulates vegetative phase change by a different mechanism, or set of

mechanisms, than the mechanism responsible for the SAS. Further supporting this conclusion, the traits associated with the SAS are opposite to the traits associated with the STS. Specifically, in Arabidopsis, exposure to a low R:FR ratio or end-of-day FR light produces elongated internodes, and light green elongated leaves with long petioles, and accelerated abaxial trichome production, whereas LL produces normal length internodes, and rounded, dark green leaves with normal length petioles and delayed abaxial trichome production.

Evidence that LL operates through both miR156-dependent and miR156-independent mechanisms is provided by the effect of LL on plants deficient for miR156. Specifically, we found that miR156 is essential for the effect of LL on abaxial trichome production, but is not essential for its effect on leaf shape. We also found that the expression of the direct targets of miR156 is reduced by LL, even in a genetic background with little or no functional miR156 and miR157. Finally, we found that LL reduces the expression of a miR156-insensitive translational reporter for SPL13. Together, these observations suggest that specific phenotype of plants grown in LL may depend on the SPL genes that regulate particular traits and on the relative importance of post-transcriptional (i.e. miR156-dependent) versus transcriptional regulation for the expression of these SPL genes. This scenario could explain why the phenotype of plants growing under LL intensity is similar but not necessarily identical to the phenotype of juvenile shoots growing under HL (Jones, 1995).

The observation that exogenous sucrose partially corrects the effect of LL on the expression of *MIR156A* is consistent with previous studies demonstrating that sugar accelerates vegetative phase change (Yang et al., 2013; Yu et al., 2013), and supports the hypothesis that LL delays vegetative phase change through its effect on photosynthesis. However, this is not the only explanation for the effect of LL on vegetative phase change because sugar did not correct the LL-induced increase in the expression of *MIR156C* or the LL-induced reduction in H3K27me3 at this gene. Although this decrease in H3K27me3 may contribute to the increase in *MIR156C* expression, it is unlikely to be the only factor involved in this response because *MIR156C* expression increases relatively quickly in response to LL. Together, these results suggest that LL regulates the expression of miR156 by reducing photosynthesis, and by several light-sensitive processes or pathways that operate independently of photosynthesis. The identity of these pathways is difficult to predict because so little is known about the factors that operate upstream of miR156, and because of the complexity of the processes involved in both carbohydrate metabolism and sensing. One possibility are pathways that depend on trehalose-6-phosphate synthase, as mutations in this gene have been shown to affect the vegetative phase change, flowering time and the level of miR156 (Wahl et al., 2013; Ponnu et al., 2020).

The observation that shade intolerant species are able to display features characteristic of the STS when grown in LL

suggests that the genes responsible for the STS are not unique to shade tolerant species. Interspecific comparisons of plants with strongly differentiated juvenile and adult phases have suggested that the morphology of juvenile shoots is an adaptation to LL environments (Day, 1998), and this is supported by studies in species with less differentiated juvenile and adult phases (Lusk, 2004; Lusk et al., 2008). Additional support for this conclusion comes from the observation that the photosynthetic properties of juvenile leaves in English ivy (*Hedera helix*) resemble those of leaves adapted for growth in LL (Bauer and Bauer, 1980), and from a recent study demonstrating that juvenile leaves, as well as leaves overexpressing miR156, have a substantially higher maximum rate of photosynthesis under LL than adult leaves, and that the rate of photosynthesis in these leaves is less sensitive to LL than the rate of photosynthesis in adult leaves (Lawrence et al., 2020). These studies, and the results presented here, suggest that the juvenile phase is naturally adapted for LL conditions, and further suggest that the STS is regulated by the vegetative phase change pathway.

Materials and methods

Plant material and growth conditions

All mutants used in this study were in a Columbia background. *mir156a-2*, *mir156c-1*, *mir157a-1*, *mir157c-1*, *35S:MIM156*, *chlorina1-4*, *swn-3*, *pkl-10*, and *swn3 pkl-10* have been described previously (Wang et al., 2009; Yang et al., 2013; Xu et al., 2016a; He et al., 2018). Seeds of the *phyA-211 phyB-9* and *cry1(hy4-B104) cry2-1* double mutants were provided by Dr Meng Chen (University of California, Riverside). The *phyB-9* lines lack the mutation in the *VENOSA-4* gene that is present in the original *phyB-9* stock. The *pif1-1 pif3-7 pif4-2 pif5-3 (pifq)* quadruple mutant was obtained from the Arabidopsis Resource Center and the *PIF5::GUS* line was provided by Dr Joanne Chory (Salk Institute, La Jolla, CA, USA). *pSPL13::SPL13-GUS* and *miR156-resistant pSPL13::rSPL13-GUS* transgenic plants have been described previously (Xu et al., 2016b). Plants were grown in Conviron growth chambers in short days (10-h light:14-h dark) at 22°C, with illumination provided by a combination of Sylvania warm white (4000 K) and Interlectric WS Gro-lite T8 fluorescent bulbs. High light conditions consisted of an overall intensity of 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ [15.7 $\mu\text{mol m}^{-2} \text{s}^{-1}$ blue (450–550 nm), 59 $\mu\text{mol m}^{-2} \text{s}^{-1}$ red (610–740 nm), 9.4 $\mu\text{mol m}^{-2} \text{s}^{-1}$ far red (710–850 nm)]. LL conditions consisted of an overall intensity of 62 $\mu\text{mol m}^{-2} \text{s}^{-1}$ [5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ blue (450–550 nm), 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ red (610–740 nm), 3.75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ far red (710–850 nm)]. For sugar treatment, plants were grown in 1/2 MS plates with or without 1%, 2%, 3% (w/v) sucrose as indicated.

Reverse transcription quantitative polymerase chain reaction

RNA for reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis was isolated from shoot apices containing leaves <1 mm in length. HL- or LL-grown plants

were compared at the same developmental stage. For example, the SL1+ apices were harvested by removing the two cotyledons when leaf primordia 1 and 2 had just emerged. SL3+ apices were harvested when leaf primordium 3 was just visible, and the cotyledons and leaves 1 and 2 were removed. Total RNA was isolated using TRIzol method, followed by DNase treatment (Ambion) according to the manufacturer's instructions. The primers used for this study were the same as the primers in Xu et al. (2016).

Chlorophyll concentration

Leaf punches were collected from the middle of lamina (leaf 3 or 4) and ground in a mortar and pestle. The ground tissue was suspended in 80% (v/v) acetone and centrifuged briefly. The optical density of the supernatant was measured at 645 nm and 663 nm using a spectrophotometer, and chlorophyll concentration was calculated as $(\text{OD}_{645} \times 20.2 + \text{OD}_{663} \times 8) / \text{mL} \cdot \text{cm}^2$.

ChIP

Chromatin was isolated from Col plants at the same developmental stage and immunoprecipitated with antibodies to H3 or H3K27me3, as previously described (Xu et al., 2016a). DNA sequences were analyzed by qPCR, using the primers for *MIR156A* and *MIR156C* described in (Xu et al., 2016a).

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: AT2G25095 (*MIR156A*), AT4G31877 (*MIR156C*), AT2G42200 (*SPL9*), AT5G50570 (*SPL13*), AT3G57920 (*SPL15*), AT2G25170 (*PKL*), AT4G02020 (*SWN*), AT1G09570 (*phyA*), AT2G18790 (*phyB*), and AT3G59060 (*PIF5*).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. LL delays vegetative phase change.

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Conflict of interest statement. The authors declare no conflict of interest.

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