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Plant proximity reduces seed yield in Arabidopsis plants by decreasing the number of ovule primordia

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Abstract

Proximity of vegetation, which is influenced by planting density, significantly impacts plant development. In *Arabidopsis thaliana*, it is well established that simulated shade, which mimics the proximity of other plants, triggers hypocotyl and petiole elongation, accelerates flowering and suppresses axillary bud growth. Although there is evidence that simulated shade affects reproduction beyond accelerating flowering, its impact on the development of reproductive tissues after plant architecture establishment (i.e., once flowering has begun) remains poorly explored. Here, we report that simulated shade promotes silique and pedicel elongation while reducing seed production, primarily by decreasing ovule number formation. Shade perception triggers rapid changes in gene expression in reproductive tissues, with some genes showing tissue-specific responses and others being induced in both seedlings and reproductive tissues, highlighting a conserved core of shade-responsive genes associated with light perception, photosynthesis and hormone regulation. However, while shade-induced elongation responses occur rapidly, reduction in ovule number requires prolonged shade exposure, suggesting distinct regulatory pathways for these responses. These findings shed light on the complex interplay between common (e.g., elongation and core gene expression) and tissue-specific responses (e.g., ovule formation and specialized gene expression) to shade, contributing to the developmental plasticity of Arabidopsis. Furthermore, they enhance our understanding of how external signals, indicative of vegetation proximity, can modulate seed production, a genetically determined process.

1 | INTRODUCTION

Light provides plants with the energy needed for photosynthesis and essential environmental information, such as the presence of nearby vegetation. When plants are too close to each other, neighboring plants filter and deplete sunlight intensity, compromising photosynthetic activity, growth and overall thriving. To deal with mutual shading, plants activate responses even before the actual reduction in light quantity occurs. These responses help to effectively and continuously adjust growth and development to the ever-changing proximity of other plants.

Plants have evolved two major ways to deal with nearby vegetation or shade: avoidance and tolerance (Martinez-Garcia and Rodriguez-Concepcion, 2023). Shade-tolerant species use the resources more conservatively, showing low growth and elongation rates, thinner leaves, reduced apical dominance and increased branching (Smith, 1982; Gommers et al., 2013). On the contrary, in shade-avoider (or sun-loving) species, vegetation proximity perception activates a set of responses known as the shade avoidance syndrome (SAS) that strongly affects plant development and architecture to overcome the negative effects of the proximity of vegetation. In the early stages of development, shade typically promotes elongation growth, such as that of hypocotyls or epicotyls. In adult plants, shade exposure also promotes leaf petioles and internode elongation, and apical dominance, hence reducing the number of branches. It also reduces root and leaf growth as well as the defense response against herbivores and pathogens, compromising viability (Ballare and Pierik, 2017; Roig-Villanova and Martinez-Garcia, 2022).

These responses rely on the perception of light quality information, as vegetation proximity and shade reduce the red (R) to far-red light (FR) ratio (R:FR). These changes are detected by phytochrome photoreceptors, which absorb in the R and FR regions and are encoded by multi-member gene families (Smith, 2000). In *Arabidopsis thaliana*, a shade-avoider species, phytochrome B (phyB) is the main phytochrome controlling the SAS responses, although other phytochromes, such as the photolabile phyA or photostable phyD and phyE, also contribute to different degrees (Casal, 2012; Martinez-Garcia et al., 2014). Active phyB interacts with and inactivates the PHYTOCHROME INTERACTING FACTOR (PIF) family of transcription factors. Inactivation of phyB by low R:FR results in PIF4, PIF5 and PIF7 accumulation and/or promotion of their transcriptional activity, which initiates a transcriptional cascade including the *YUCCA* (*YUC*) genes (Casal, 2012; Martinez-Garcia et al., 2014). Induction of *YUCs* contributes to the rapid but transient increase in the levels of the auxin indole-3-acetic acid (IAA) in cotyledons and leaf lamina production, shown as essential for the shade-induced hypocotyl and petiole elongation, respectively, the most conspicuous and studied SAS responses (Bou-Torrent et al., 2014; de Wit et al., 2015; Pucciariello et al., 2018). Although flowering is unaffected or even delayed in response to shade in some herbaceous species, in many other species, such as *A. thaliana*, it is strongly accelerated from the PIF-mediated induction of *FLOWERING LOCUS T* (*FT*) and its close homolog *TWIN SISTER OF FT* (*TSF*) expression (Halliday et al., 2003; Procko et al., 2014; Galvao et al., 2019; Lorenzo et al., 2019). Once the plant enters the reproductive phase, shade is generally considered to reduce seed set and truncate fruit development (Smith and Whitelam, 1997; Procko et al., 2014), although there is not much information available. In pepper (*Capsicum annuum*), simulated shade had no impact on seed number per fruit, although it reduced fruit set and stimulated flower and fruit abortion, probably through enhanced competition for assimilates between apices and flowers, which limits assimilate import into flowers (Chen et al., 2024). In soybean, shade reduced seed yield (weight of seeds per plant), an effect attributed primarily to reductions in the number of branches and pods per plant as there was no effect in seed number per pod and seed weight (Green-Tracewicz et al., 2011). In the specific case of two mustards, *A. thaliana* and *Brassica rapa*, simulated shade treatments of adult plants also reduce seed number (Procko et al., 2014). The molecular or cellular basis of this reduction has not yet been explored.

Seeds are the reproductive bodies of both angiosperms (flowering plants) and gymnosperms (e.g., conifers, cycads and ginkgos). Also, they are the foundation of human diet and bear the genetic potential of crop species and their varieties as a consequence of their breeding and selection over time (https://www.fao.org/seeds/en). Therefore, seed yield and quality factors are of key importance for agricultural production. In angiosperms, seeds are protected within the fruits, which ensures their dispersal (Knapp, 2002). Fruits derive mostly from the fertilized mature gynoecium, although, especially in fleshy fruits, additional floral components are frequently recruited. The gynoecium (or pistil), the female reproductive part of a flower, is surrounded by petals, sepals and stamens, and may consist of a single carpel or a number of carpels that have merged. Carpels are essential for sexual plant reproduction because they house the ovules that, once fertilized, will give rise to the seeds.

General characteristics of fruits and seeds, such as shape, size or seed number, are genetically determined. Homeotic genes are known to control flower organ identity in angiosperms. In *A. thaliana* those homeotic regulatory components have been well described. This is the case of *AGAMOUS* (*AG*), which controls stamen, carpel and ovule identity (Bowman et al., 1989). *AG* is a member of a monophyletic clade of MADS-box genes that include *SHATTERPROOF1* (*SHP1*), *SHP2* and *SEEDSTICK* (*STK*). Genetic analyses indicate that these four genes, together with other MADS-box genes such as *SEPALLATA1* (*SEP1*), *SEP2*, *SEP3* and the homeodomain gene *BELL1* (*BEL1*), control ovule identity (Pinyopich et al., 2003; Brambilla et al., 2007). Once ovule identity is determined, *AINTEGUMENTA* (*ANT*), *CUP-SHAPED COTYLEDON* (*CUC*) 1 and *CUC2*, which respectively encode an APETALA2 (AP2)-like and two NAC transcription factors, play additive but distinct roles in the establishment of ovule primordia number: while ANT promotes cell proliferation, CUCs redundantly control the proper definition of the primordia boundaries (Galbiati et al., 2013). The endogenous mechanisms that regulate ovule primordia initiation and development, including gene regulatory networks involving transcription factors as well as complex hormonal communication, have been nicely reviewed (Cucinotta et al., 2020). Upon fertilization, ovules develop into seeds, and the gynoecium that contains them becomes fruit. Indeed, the final seed number within the fruit is clearly

dependent on the efficiency of pollination and fertilization, which has been described to be genetically and physically controlled by a very complex network (Lord and Russell, 2002; Pajoro et al., 2014; Jiang et al., 2020). However, pollination and fertilization are also clearly dependent on exogenous factors such as the presence of pollinators (in non-cleistogamic species, i.e., those that do not self-pollinate) or the environment. Thus, the influence of environmental factors on these processes, particularly in fruit and seed development, and the mechanisms of their control still remain pretty unknown. One of these external factors is vegetation proximity.

It is still unclear what aspects of fruit and seed development are affected by vegetation proximity and how the perception of vegetation proximity and seed- and fruit-set are connected. In this manuscript, we characterize the SAS responses at the fruit level in *A. thaliana* and analyze whether vegetation proximity affects seed production. Furthermore, we analyze the cellular and molecular changes in the inflorescences in response to shade and compare these expression analyses with the molecular networks previously defined in the control of SAS responses in seedlings.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Arabidopsis thaliana mutants *phyB-9*, *phyA-501*, *ant-4*, *pPIL1:GUS* line #28 (expressing *GUS* under the control of 2 kbp of the *PIL1* promoter in Col-0 background), *DR5:GUS* and MB2 (p*AP1:AP1-GR* in *ap1 cal* background) plants (in L*er* background) have been described before (Krizek, 2009; Hornitschek et al., 2012; Martinez-Garcia et al., 2014; Pajoro et al., 2014). The sequences of the primers used for genotyping these lines by PCR are provided in Table S1). Arabidopsis plants for seed production were grown in the greenhouse as previously described (Martinez-Garcia et al., 2014; Gallemi et al., 2016).

Shade treatments were provided by enriching W produced by cool-white fluorescent tubes with different intensities of FR produced by LED lamps (www.philips.com/horti) to produce the indicated R:FR without altering photosynthetic active radiation (PAR). Fluence rates were measured with a Spectrosense2 meter associated with a 4-channel sensor (Skye instruments Ltd.), which measures PAR (400–700 nm) and 10 nm windows in the blue (464–473 nm), R (664–673 nm) and FR (725–734 nm) regions (Martinez-Garcia et al., 2014). In seedlings, W was produced by vertical fluorescent tubes (weak Photosynthetic Photon Flux Density, or PPFD, of 20–24 μmol m^{-2} s^{-1}, R:FR of 2.5) that was enriched with FR (R:FR of 0.05) (Molina-Contreras et al.,2019). In the experiments using adult plants, plants were grown under short day (SD, 8 h light +16 h dark) and/or long day photoperiods (LD, 16 h light +8 h dark). In both photoperiods, W was generated by horizontal fluorescent tubes that provided higher amounts of light (strong PPFD of ∼100 μmol m^{-2} s^{-1}, R:FR of ∼3.0); for shade treatments, W was supplemented with FR (W + FR, PPFD of ∼100 μmol m^{-2} s^{-1}, R:FR of ∼0.05–0.06).

All experiments were performed with surface-sterilized seeds sown on Petri dishes with solid growth medium (without sucrose, GM-) as described by Roig-Villanova et al. (2006). In seedlings experiments, seeds were stratified for 3–5 days at 4°C. After this, plates were incubated in a growth chamber at 22°C under continuous W and/or W + FR (weak PPFD; see above). In all the experiments performed on adult plants, seeds were first germinated and grown for 7 days under continuous W (weak PPFD). Next, selected well-grown seedlings were transferred into individual pots placed at 22°C under SD for two weeks (unless otherwise stated). Following this period, plants were transferred to LD for the indicated days. Transfer to W + FR is detailed in each experiment. In the RNA-seq experiments, simulated shade treatments were initiated at Zeitgeber Time 3 (ZT3).

2.2 | Dexamethasone treatments

Dexamethasone (DEX, Sigma-Aldrich) was dissolved in absolute ethanol at 50 mM. Stock solution was kept at −20°C until use. Inflorescences of 20 day-old MB2 plants were induced with a drop of 25 μL of 2 μM DEX [in 0.02% (v/v) EtOH and 0.01% (v/v) Silwet L-77] and were harvested at days 23 and 24 to identify the pistil and ovule primordia formation stage.

2.3 | Phenotypic analyses of morphological traits

Analyses of plant height and number of rosette leaves, rosette branches and cauline branches were performed on soil-grown plants. Flowering time was scored as the mean value of the number of rosette leaves. Plant, silique and silique pedicel length was measured using Image J software on digital images.

Seed number, seed abortions and non-fertilized ovules within the silique were visualized (stereomicroscope) and counted in about 20 siliques.

Ovule number per pistil was quantified using inflorescences fixed and cleared as described (Ceccato et al., 2013). Pistils were dissected and observed at an optical microscope (AxioPhot ZEISS) equipped with differential interface contrast (DIC) optics. Images were recorded with a DP70 Olympus digital camera.

Seed area was measured using SMARTGRAIN software (Tanabata et al., 2012) on digital images taken with a NIKON digital camera.

2.4 | Auxin quantification

About 14–15 young inflorescences per biological replica (that ranged from 80–120 mg) were immediately frozen in liquid nitrogen. Hormone extraction and analysis were performed as described (Simura et al., 2018) with a few modifications, as described elsewhere (Pastor-Andreu et al., 2024). Briefly, around 100 mg of fresh material was extracted in 1 mL of 50% acetonitrile (v/v) prepared with ultrapure water, adding 2.5 ng of [$^2H_5$]IAA as internal standard in a ball mill (MillMix 20, Domel) for 10 min at 17 rps, followed by 5 min of sonication. After sonication, the samples were centrifuged at 4000x*g* at 4°C for 10 min. Finally, supernatants were filtered through SPE columns

(OASIS HLB 30 mg 1 cc, Waters), recovering the eluent. Finally, 0.5 mL of 30% acetonitrile (v/v) prepared in ultrapure water was added to SPE columns and the eluent was recovered and added to the previous ones.

Chromatographic separations were performed on a reverse-phase C18 column (50 × 2.1 mm, 1.6-μm particle size, Luna-Omega, Phenomenex) using a acetonitrile:water (both supplemented with 0.1% formic acid) gradient at a flow rate of 300 μL/min. IAA was detected with a triple quadrupole mass spectrometer connected online to the output of the column through an orthogonal Z-spray electrospray ion source (Xevo TQ-S). Finally, IAA content was quantified by interpolation in a standard curve prepared with commercial IAA (Sigma) using the Masslynx v4.2 software.

2.5 | Expression analysis

Quantitative reverse transcription PCR (RT–qPCR) was carried out to analyze changes in gene expression of inflorescences. Total RNA was extracted from a pool of a minimum of 4 inflorescences with the RNeasy Plant Mini Kit (Qiagen) or the Maxwell 16 LEV simplyRNA Tissue Kit (Promega). Reverse transcriptase and RT-qPCR analyses of gene expression were performed as described (Gallemi et al., 2017). The *ACTIN8* gene (At1g49240) was used as a control for normalizations (Balanza et al., 2016). Three biological replicas for each sample were assayed. Primer sequences can be found in Table S1.

2.6 | GUS assays

Histochemical GUS assays were performed essentially as described (Roig-Villanova et al., 2006). Seedlings, inflorescences and cauline leaves were cleared with 70% (v/v) ethanol washes to improve contrast. Finally, whole-mount preparations were made in 50% (v/v) glycerol to visualize GUS activity using a Leica DMS1000 digital microscope, Leica MZFLIII stereoscopicmicroscope and a Leica DC200 digital camera (Leica Microsistemas).

2.7 | Microarray data analysis from published information

Published microarray data from Affymetrix microarrays (GEO accession number for the microarray sequence data GSE28297) were used to identify sets of genes that transcriptionally respond after 1, 3 and 24 h of W + FR treatment in young seedlings (two-day-old maintained under constant W) of Col-0 (Leivar et al., 2012). Published data were analyzed as described and detailed somewhere else (Bou-Torrent et al., 2014). Briefly, data were imported into the Resolver gene expression data analysis system version 7.1 (Rosetta Biosoftware, http://www.rosettabiosoftware.com) and processed as described (Wellmer et al., 2006). Resolver uses a platform-specific error model-based approach to stabilize the variance estimation to improve the specificity and sensitivity in differential gene expression detection (Weng et al., 2006). Data from the biological replicates of each condition were combined, resulting in an error-model weighted average of the replicates. The P-values for differential expression calculated by Resolver were adjusted for multi-hypothesis testing using the Benjamini & Hochberg procedure, as implemented in the Bioconductor multtest package in R (http://www.bioconductor.org/packages/bioc/stable/src/contrib/html/multtest.html). Genes for which the Benjamini & Hochberg-adjusted P-value (BH) was <0.05 and an absolute fold-change (FC) cutoff of 1.5 were considered as Differentially Expressed Genes (DEGs) in response to increasing times of 1, 3 or 24 h of W + FR treatment.

2.8 | mRNA library preparation and sequencing

Total RNA was extracted as indicated before. Total RNA was assayed for quantity and quality using Qubit® RNA BR Assay kit (Thermo Fisher Scientific) and RNA 6000 Nano Assay on a Bioanalyzer 2100 (Agilent). The RNA-seq libraries were prepared from total RNA using KAPA Stranded mRNA-Seq Kit Illumina Platforms (Kapa Biosystems) with minor modifications. Briefly, from 500 ng of total RNA, the poly-A mRNA was enriched by oligo-dT magnetic beads and fragmented. The second strand cDNA synthesis was performed in the presence of dUTP instead of dTTP to achieve the strand specificity. The blunt-ended double-stranded cDNA was 3-adenylated and Illumina indexed adapters (Illumina) were ligated. The ligation product was enriched with 15 PCR cycles and the final library was validated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay. Each library was sequenced using TruSeq SBS Kit v4-HS in paired-end mode with a read length of 2x76bp on HiSeq2000 (Illumina) following the manufacturer's protocol. Images analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA 1.18.64) and followed by the generation of FASTQ sequence files by CASAVA. RNA-seq data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO accession GSE230448.

2.9 | RNA-seq data processing and analysis

RNA-sequencing reads were mapped against the *Arabidopsis thaliana* reference genome (TAIR10) with STAR/2.5.3a (Dobin et al., 2013) using ENCODE parameters. Genes and isoforms were quantified with RSEM/1.3.0 (Li and Dewey, 2011) with default parameters and using the TAIR10.36 annotation. The overall variation in transcriptomic profiles of wild-type Col-0 flower buds under different light treatments (W vs. W + FR) and time points was evaluated by performing a Principal Component Analysis (PCA). Differential expression analysis was performed with the R package DESeq2/1.18 with default parameters (Love et al., 2014). Genes with FDR <5% and |log2FC| > 0.58 were considered significantly differentially expressed (DEGs).

For the creation of the gene-coexpression modules, WGCNA package was used in RStudio (Langfelder and Horvath, 2008). Tutorials from the authors were followed, using the automatic, one-step network construction and module detection with a power of 18, according to the recommendations of the developers (our sample size was less than 20), TOMtype = "signed", maxBlockSize = 20000, minModule- Size = 30, reassignThreshold = 0, mergeCutHeight = 0.25 and verbose = 3 arguments for the blockwiseModules function. For the WGCNA, only samples of the WT genotype were used, and the "WT_24h_WFR_3" sample was removed as it was identified as an outlier in the sample Tree clustering. The visualization was generated using RStudio (RStudio Team, 2020) and the most used packages were those included Tidyverse package (Wickham et al., 2019) such as dplyr or ggplot2 (Wickham et al., 2021).

2.10 | Statistical analysis

Differences in the different parameters recorded were analyzed by Student's *t*-test to verify variances ($p < 0.05$) (Microsoft Excel). Analyses of variance (ANOVA) were performed to assess the effects of genotype, shade treatments, and their interaction on the silique length and seed production of wild-type and mutant plants treated with simulated shade. The analysis was conducted using the lm function from the stats package in R (R Core Team, 2019). Data transformations (log and square root) were applied in some cases to ensure the assumptions of ANOVA. The Kruskal-Wallis test was applied when the assumptions of normality and homogeneity of variance could not be met. Levene's Test and the Shapiro–Wilk test were utilized to assess the assumptions of homogeneity of variance and normality, respectively, prior to and after transformations.

3 | RESULTS

3.1 | Simulated shade reduces seed production (seed number per silique)

Exposure to simulated shade (low R:FR) of adult Arabidopsis plants is reported to strongly drop seed yield (from 14.1 mg of seeds per plant in high R:FR to 7.1 in low R:FR) (Procko et al., 2014). Indeed, in adult Arabidopsis plants, acceleration of the onset of flowering is one of the most dramatic effects of low R:FR exposure (Cerdan and Chory, 2003; Halliday et al., 2003). This shade-induced response shortens generation time and promotes apical dominance, hence altering plant architecture (i.e., the organization of plant body in the three-dimensional space). Changes in architecture strongly reduce the amount of flowers produced per plant and may indirectly account for the observed drop in seed yield. However, we aimed to specifically study whether low R:FR directly affected seed production rather than indirectly because of its impact on plant architecture.

To minimize the possible interference of the changes in plant architecture caused by simulated shade, wild-type (Col-0) adult plants of different ages (21, 28 and 35 days after germination, from now on indicated as DAG) growing under high R:FR (provided by white light, W) were transferred to low R:FR (W enriched in FR, W + FR) (Roig-Villanova et al., 2019) (Figure 1). On 44 DAG, all plants were flowering, even a control group kept under W during the whole period (Figure 1A). Simulated shade (W + FR) treatments promoted petiole length when applied to rosette plants before flowering was induced (Figure S1A), as also previously reported (de Wit et al., 2015; Pantazopoulou et al., 2017; Molina-Contreras et al., 2019). Flowering time, estimated from the total number of rosette leaves, was significantly reduced only in the 21 DAG plants transferred to W + FR, the only group in which flower stalks were not visible before transfer (Figure 1B). In this group, the number of rosette branches (those that emerge directly from the axillary buds located at the base of each petiole) and cauline branches (those that emerge from the main flower stalk) were strongly affected compared to plants kept under W during the whole period (Figure 1B). The number of rosette branches reflects the phytochrome control of the bud outgrowth via the transcription factor BRANCHED1 by shade signals (Finlayson et al., 2010; Gonzalez-Grandio et al., 2013). The number of cauline branches, however, might reflect the strong apical dominance imposed by the early treatment with simulated shade. Transfer of W-grown plants that were already bolting (even if the flower stalk was small, e.g., 28 or 35 DAG) to simulated shade also reduced the number of rosette branches but did not significantly lower the number of rosette leaves (Figure 1B). These observations showed that exposure to simulated shade when flowering was already initiated resulted in minor changes in plant architecture, in contrast to exposure of younger plants (21 DAG, in which bolting was not visible). Therefore, to minimize the effect of simulated shade on plant architecture, for the rest of the experiments (unless otherwise stated), W-grown plants were transferred to W + FR when flowering was already induced, i.e., when plants that were growing under LD and W had the main flower stalk already visible of about 1–5 cm (usually around 20–30 DAG). In these conditions, W + FR treatments promoted the elongation of petioles, siliques, silique pedicels and the main flowering stem (used to measure plant height) (Figure S1A-D).

In *B. rapa* plants, low R:FR not only promoted silique elongation but reduced seed number per silique. Whereas silique elongation was not affected in phyB-deficient mutant (*ein194*) plants, seed number per silique was significantly reduced in W, a trait that was further attenuated under W + FR (Procko et al., 2014). We observed that, in Arabidopsis plants, W + FR similarly reduced silique seed number per silique (Figure 1C). In this species, siliques are significantly shorter in *phyB* mutant plants in W than in W + FR, suggesting that phyB activity did not appear to play a significant role in the determination of this trait (Figure 1C). This reduction in length could be an indirect effect caused by the reduced number of seeds per silique (silique length and seed number per silique usually positively correlate) (Barendse et al., 1986; Alonso-Blanco et al., 1999; Bac-Molenaar et al., 2015) and/or the enhanced elongation of the aerial part of this line. By contrast, seed number per silique was significantly reduced in *phyB* plants in W, suggesting that this character is determined by phyB. Two-way ANOVA analyses support that this phenotype was attenuated in *phyB*

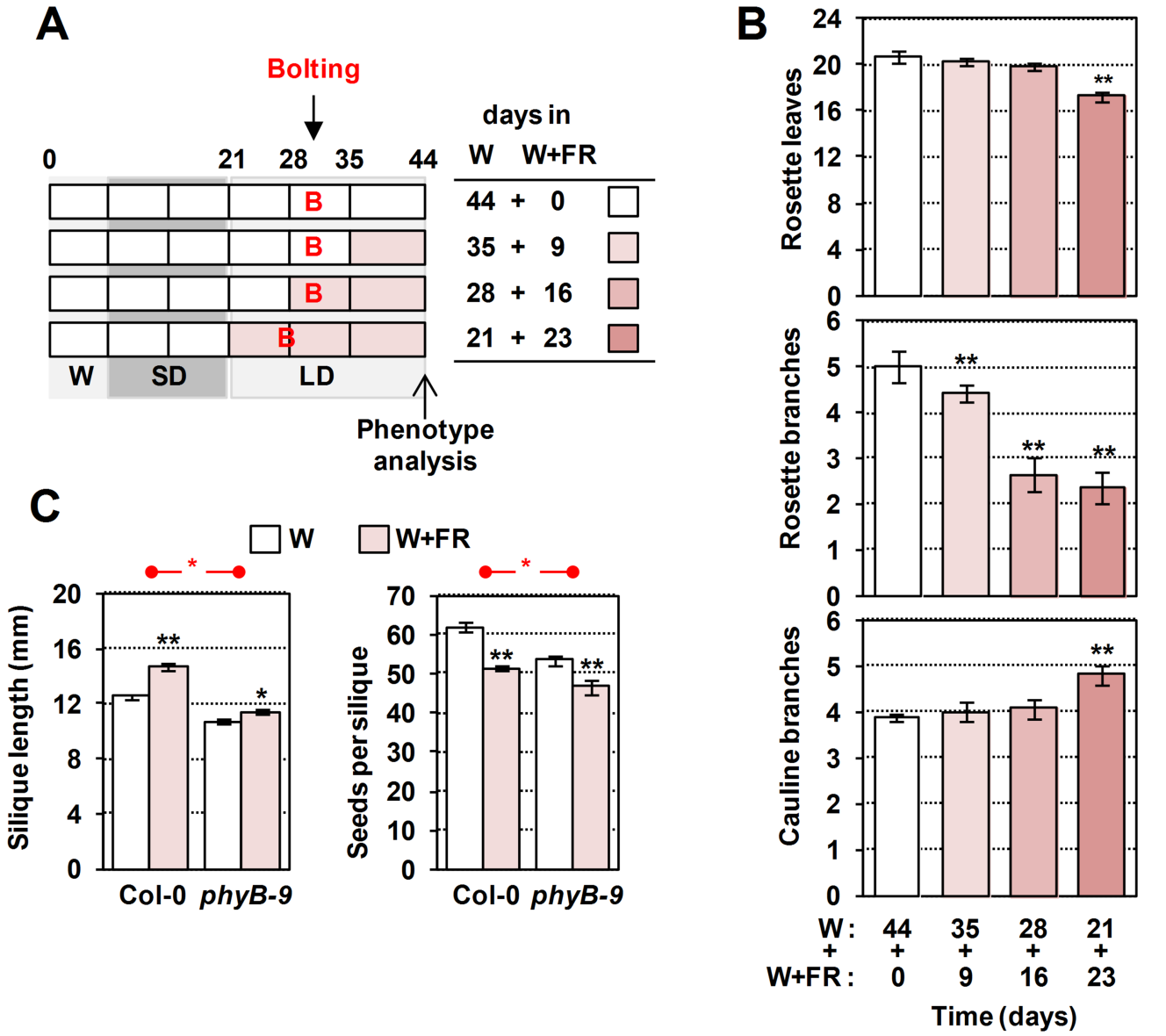

FIGURE 1 Simulated shade reduces the number of seeds per silique. (A) Cartoon representing the setting of the experiment. Col-0 plants were grown under W (white boxes) and later transferred to simulated shade (W + FR, pink boxes) for the indicated days. "B" in red indicates when flowering bolts were longer than 1 cm. After germinating and growing for 7 days under continuous W, well-grown seedlings were transferred into individual pots and grown under SD for the following two weeks. Plants were next transferred to LD for the indicated days. Transfer to W + FR is detailed in each case. (B) Flowering time is measured as the number of rosette leaves until bolting (top), rosette branches (middle) and cauline branches (bottom) in plants treated as indicated in A. (C) Silique length (left) and number of seeds per fruit (right) in Col-0 wild-type and *phyB-9* mutant plants treated with W or W + FR. Plants grown under W for 35 days, already bolting, were kept for 9 additional days either under W or W + FR (two top conditions in A). Graphs correspond to the average of at least 9 (B), 30 (silique length) or 15 (seeds per silique) (C) biological replicates (error bars, SE). Black asterisks indicate significant differences relative to control plants grown only under W (Student's *t*-test, $^{**}p < 0.01$, $^{*}p < 0.05$). Red asterisks indicate significant differences between the mutant and wild-type genotypes in response to W + FR (two-way ANOVA, $^{*}p < 0.05$).

mutant plants (Figure 1C), which indicates that this response is regulated by phyB and other phytochromes in these species. Simulated shade might reduce seed number per silique by impacting several key steps of ovule and seed development, such as (i) establishment of number of ovule primordia, (ii) correct development of ovules, (iii) fertilization, or/and (iv) correct and coordinated development of the embryo and endosperm inside the seeds (Schneitz et al., 1997; Sun et al., 2010; Hamamura et al., 2012; Cucinotta et al., 2014). To distinguish among these possibilities, we first analyzed floral buds of wild-type (Col-0) plants treated with W or W + FR (Figure 2A) by Differential Interference Contrast (DIC) microscopy. In these samples, young flowers have developed from the meristem either under W or W + FR (Figure 2A). We observed that treatment with W + FR significantly reduced (from 54.1 to 47.8, about 10%) the number of ovules

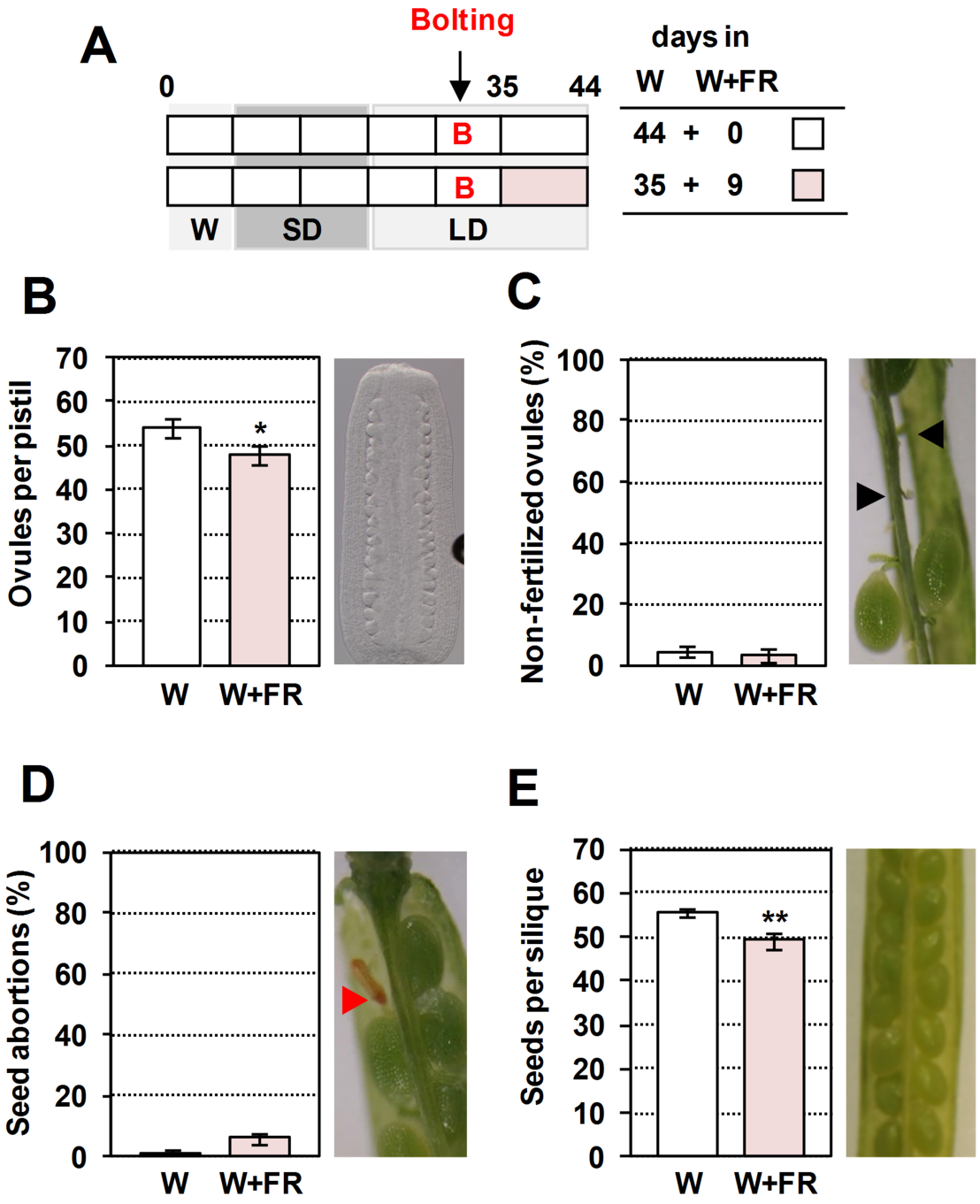

FIGURE 2 Simulated shade affects the number of ovule primordia produced per pistil. (A) Cartoon representing the setting of the experiment. After germinating and growing for 7 days under continuous white light (W), well-grown seedlings were transferred into individual pots and grown under SD for the following two weeks. Plants were next transferred to LD for the indicated days. Col-0 plants grown under W (white boxes) for 35 days, already committed to flowering, were kept for 9 additional days either under W or W + FR (pink boxes). Effect of simulated shade on the (B) number of ovules per pistil, (C) percentage of non-fertilized ovules and (D) percentage of seed abortions, and (E) number of seeds per silique in wild-type (Col-0) plants treated with W or W + FR, as indicated in A. Representative images of a pistil under Nomarski optical microscopy (B) and fruits under the stereomicroscope (C-E) are shown for visualization of the analyzed phenotypes. Black arrowheads point to non-fertilized ovules and red arrowheads to seed abortions. Graphs correspond to the average of at least 18 biological replicates (error bars, SE). Asterisks indicate significant differences relative to plants grown only under W (Student's *t*-test, **p < 0.01, *p < 0.05).

per pistil (Figure 2B). Simulated shade treatment had no effect on the percentage of non-fertilized ovules and seed abortions analyzed in mature siliques (Figure 2C-D) but still significantly reduced (from 55.7 to 49.2, about 10%) the number of seeds per silique (Figure 2E). Therefore, we concluded that the shade-induced reduction in seed number per fruit was caused by the reduction of the number of ovule primordia formed in young pistils. In addition, seeds produced by shade-exposed plants were slightly but significantly bigger than those

produced by W-grown plants (Figure S1E). Together, these results indicate that simulated shade impacted the reproductive development of Arabidopsis not only by promoting elongation of several organs and structures (e.g., fruit and pedicel length) but also by altering traits such as seed number per silique, which is reduced, and seed size, which is enhanced.

The shade treatment also reduced seed yield in the two accessions tested (Col-0 and L*er*), although the reduction was only significant in L*er* (Figure S2). As these plants were treated with simulated shade once they were already bolting, we deduced this reduction was not caused by alterations of the plant architecture. Altogether, our results suggest that whereas alteration of plant architecture by early treatments with W + FR has a strong but indirect impact on seed yield because of the reduction in branches produced per plant, the reduction in seed number per silique, which has a milder impact on seed yield, appeared to be caused by the exposure of flowering tissues to simulated shade.

In seedlings and adult leaves, a rapid (1–4 h) increase in the levels of IAA is essential for shade-induced hypocotyl and petiole elongation (Tao et al., 2008; Hornitschek et al., 2012; Bou-Torrent et al., 2014; de Wit et al., 2015; Pucciariello et al., 2018; Molina-Contreras et al., 2019). Using the DR5:GUS auxin reporter line, GUS activity was shown to take longer times (4–8 h) to increase in seedling cotyledons after shade treatment (Tao et al., 2008) or in adult leaves after 24 h (de Wit et al., 2015). We observed that 4–8 h of shade treatments also enhanced GUS activity in cauline leaves of adult DR5:GUS plants but not in their inflorescences, suggesting this treatment differently impacted auxin levels or activity in these two different tissues (Figure S3A). Next, we quantified auxin levels in floral buds before (0 h) and after 1 h of differential exposure to W or W + FR treatment. Simulated shade had no impact on IAA levels in the floral buds (Figure S3B). The distinct effect of 1 h of W + FR in auxin levels in floral buds, seedlings and leaves suggests that different molecular mechanisms may control the shade-induced reduction in seed number per silique and the promotion of hypocotyl and petiole length.

3.2 | Simulated shade is perceived in reproductive tissues

To learn whether reproductive tissues were perceiving simulated shade, we analyzed the expression levels of the shade marker gene *PIF3-LIKE 1* (*PIL1*) in Arabidopsis flowers, either in whole inflorescences or floral buds (which were enriched in young pistils with ovule primordia) (Figure 3). Expression of *PIL1* was higher in inflorescences from plants grown for several days (>8–10 days) under W + FR than W (Figure 3A, B). Importantly, *PIL1* expression was also rapidly induced (after 1 hour) by simulated shade in both whole inflorescences and floral buds (Figure 3C, D). Using a reporter *pPIL1:GUS* line (Hornitschek et al., 2012), GUS activity was detected in young pistils and siliques from W-grown plants (red arrows). After 6 h of W + FR treatment, GUS activity expanded to adjacent tissues (pedicels and petals, brown arrows) (Figure S4A). An equivalent W + FR treatment induced GUS activity much more strongly in seedlings (Figure S4B). Because *PIL1* is known to be a direct target gene of PIFs (Hornitschek et al., 2012; Li et al., 2012), our results indicate that reproductive tissues sense changes in vegetation proximity and rapidly respond by inducing the expression of shade marker genes.

Next, we checked if simulated shade could also regulate the expression of *ANT*, *CUC1* and *CUC2* genes known to control ovule primordia number (Figure 4). The expression of these genes was downregulated after long W + FR treatments compared with W treatments, in both whole inflorescences and floral buds (Figure 4A, B). However, their expression did not change under short simulated shade treatments (Figures 4C,D and S5), suggesting that those changes required longer times of shade exposure. Therefore, these results did not clarify whether the observed changes in *ANT*, *CUC1* and *CUC2* expression could be a cause or a consequence of the observed reduction in the ovule primordia number in the shade-exposed plants.

3.3 | Simulated shade reduces ovule number in *ant-4* mutant plants

To address whether the changes in the expression of these genes could be required for the shade-induced reduction in the number of ovule primordia observed, we took a genetic approach (Figure 5). We analyzed the response to shade in mutant plants deficient in *ANT*, *ant-4*. This mutant was chosen because *ANT* activity does not seem redundant, in clear contrast with *CUC* genes (Cucinotta et al., 2014; Cucinotta et al., 2020). First, we analyzed if the reproductive tissues of *ant-4* plants responded to shade by measuring the elongation of their siliques. Arabidopsis siliques of wild-type L*er* plants elongate when ovule fertilization occurs. The *ant-4* siliques present aberrant ovules due to defects during their development and thus lack of fertilization; therefore, these plants display shorter siliques already in W (Figure 5A, B). More importantly, *ant-4* siliques still elongated in response to shade, like wild-type L*er* siliques did (Figure 5B). Then, we analyzed the number of ovules per pistil of L*er* and *ant-4* plants in response to W + FR treatments. As expected, *ant-4* mutant plants produced fewer ovule primordia per silique than the wild type (Figure 5C-E). Regardless of that, we observed a reduction in ovule primordia number in *ant-4* in response to shade, similar to the wild type (Figure 5E). These results showed that shade exposure reduced the number of ovule primordia formed in both wild-type and *ant-4* plants. Importantly, the remarkable reduction of ovule number in the mutant line *ant-4* under simulated shade indicates that *ANT* is not a main factor regulating the shade response in reproductive tissues, i.e., the reduction of the ovule primordia formed.

3.4 | Simulated shade affects gene expression in reproductive tissues

In order to gain insights into the molecular responses to shade in the reproductive tissues, we performed a whole transcriptome analysis

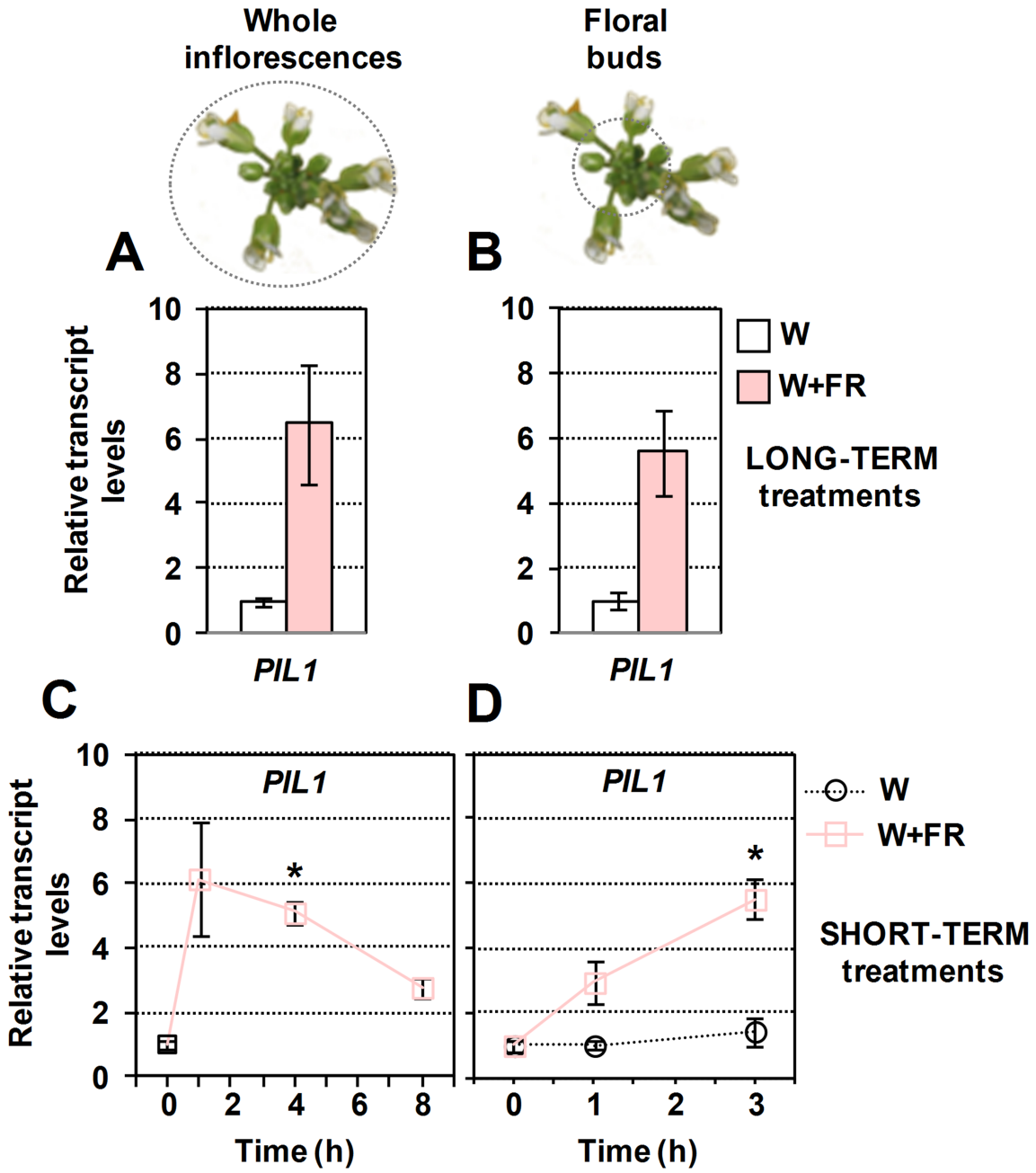

FIGURE 3 Simulated shade rapidly induces gene expression in the reproductive tissues. Relative expression of *PIL1* in whole inflorescences (A, C) and in floral buds (B, D) in wild-type (Col-0) plants differentially treated with W or W + FR since bolting (long-term treatment) (A, B) or during the indicated hours (short-term treatment) (C, D). Plants were grown as indicated in Figure 2. Graphs correspond to the average of three biological replicates (error bars, SE). Asterisks indicate significant differences relative to plants grown under W (B), or relative to plants grown at time 0 h (C) or at the same time point under W (D) (Student's *t*-test, *p < 0.05).

(RNA-sequencing) of Col-0 wild-type inflorescences. Plants were grown until they began to bolt (as shown in Figure 2A) and were then differentially treated with either W or W + FR for up to 24 h. In these plants, floral buds were harvested at the start of the differential treatment (W, 0 h), after 1, 3 and 24 h of W + FR, and after 24 h of W (Figure 6A). The central time points under W were not included in this study, although prior qPCR experiments (Figures 3 and 4) demonstrated minimal changes in gene expression of a few analyzed genes at these time points under W when compared to the 0 h time point.

To evaluate the overall variation in transcriptomic profiles, we performed Principal Component Analysis (PCA). The first two principal components (PC1 and PC2) explained 33.42% and 29.97% of the total variance, respectively, capturing the majority of the variability in the dataset (Figure S6). Samples grouped distinctly according to their respective light treatments and time points, with minimal overlap between conditions. The clear separation of groups and consistent clustering of biological replicates indicate robust data quality with minimal technical variation for most groups, supporting the reliability

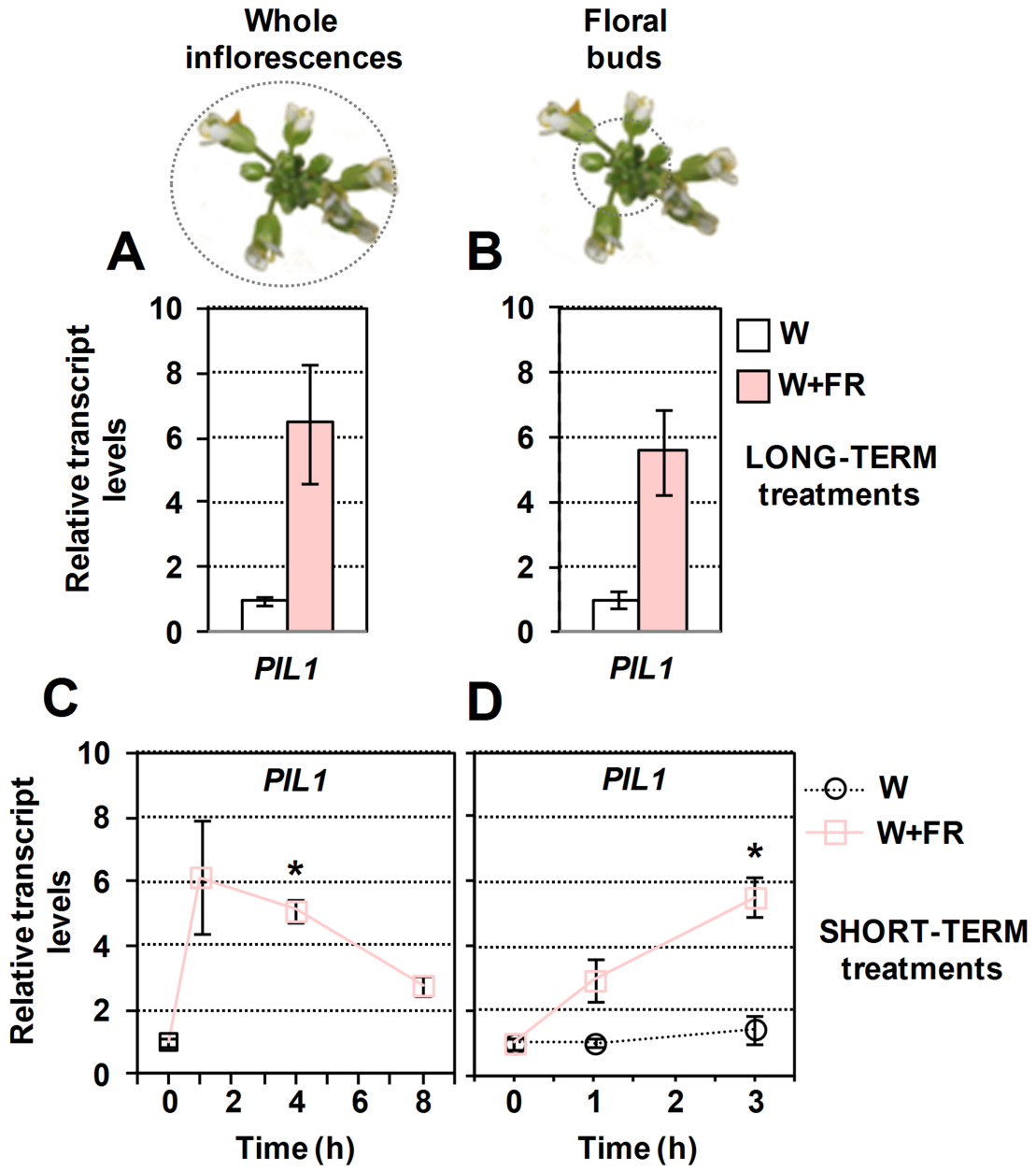

FIGURE 4 Simulated shade represses the expression of genes that determine ovule number. Relative expression of *ANT*, *CUC1* and *CUC2* in whole inflorescences (A, C) and in floral buds (B, D) in wild-type (Col-0) plants submitted to long- (A, B) or short-term (C, D) treatments as indicated in Figure 3. Graph corresponds to the average of three biological replicates (error bars, SE). In A and B, asterisks indicate significant differences relative to plants grown under W (Student's *t*-test, **p < 0.01, *p < 0.05).

of the dataset for identifying transcriptomic differences. Therefore, in these samples, several DEGs were identified as shade-regulated (FDR <0.05, fold change ≥ |1.5|) after 1 (258 DEGs), 3 (589 DEGs) and 24 (169 DEGs) hours of simulated shade treatment compared to their expression before the treatment (0 h) (Tables S2-S4). The well-known shade-marker genes *ATHB2* and *PIL1* were up-regulated at all three time points, while other established shade-marker genes were up-regulated only at specific time points (e.g., *YUC9* at 1 h; *PIL2* at 1 and 3 h; *HFR1* at 24 h). However, other well-established shade-induced genes, such as *IAA19* and *IAA29*, were not identified in these analyses (Figure 6B). The comparison of the transcriptome after 24 h of differential W and W + FR treatments identified 1511 and 169 DEGs after W and W + FR treatments, respectively (Figure 6C). Despite the differences in the number of DEGs after both treatments, this analysis also identified well-known shade-marker genes in the samples grown under W + FR compared to those in W (e.g., *ATHB2* and *HFR1*). In the case of *PIL1*, which was up-regulated in both treatments, the induction was higher in the simulated shade samples (Figure 6C).

Circadian regulation of transcription, which is well-known to occur in plants grown in day-night cycles, might have an impact on the 1–3 h DEGs identified in our analysis (no time points at 1 and 3 h

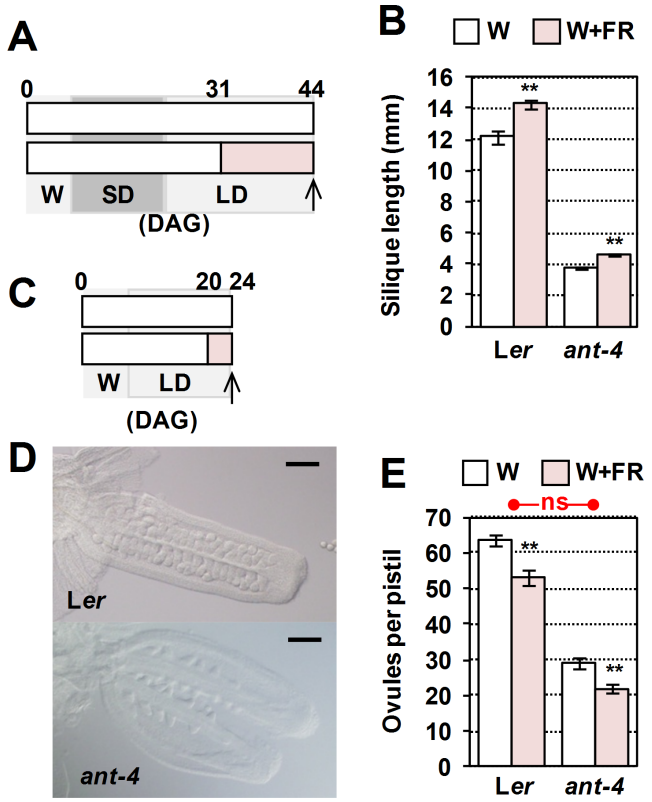

FIGURE 5 Simulated shade affects the number of ovule primordia produced per pistil in the *ant* mutant similarly to the wild type. (A, C) Cartoons representing the setting of the experiment. After germinating and growing for 7 days under continuous white light (W), well-grown seedlings were transferred into individual pots and either (A) grown under SD for the following two weeks and then transferred to LD for the indicated days, or (C) grown under LD for the following two weeks. Plants grown under W (white boxes) were kept for the indicated days either under W or W + FR (pink boxes) until committed to flowering. Arrows indicate the day in which the samples were harvested and analyzed. (B, E) Effect of simulated shade on the silique length (B) and ovules per pistil (E) in wild-type (*Ler*) and *ant-4* mutant plants treated with W or W + FR as indicated in A and C, respectively. (D) Representative images of a *Ler* wild-type (top) and an *ant-4* (bottom) pistils under Nomarski optical microscopy of plants grown in W as shown in C. Scale bars = 100 µm. Graphs correspond to the average of at least 10 (B) or 6 (E) biological replicates (error bars, SE). Black asterisks indicate significant differences relative to plants grown under W (Student's *t*-test, **p < 0.01). Red label indicates that the response to W + FR between the mutant and wild-type genotypes was not significant (ns, two-way ANOVA).

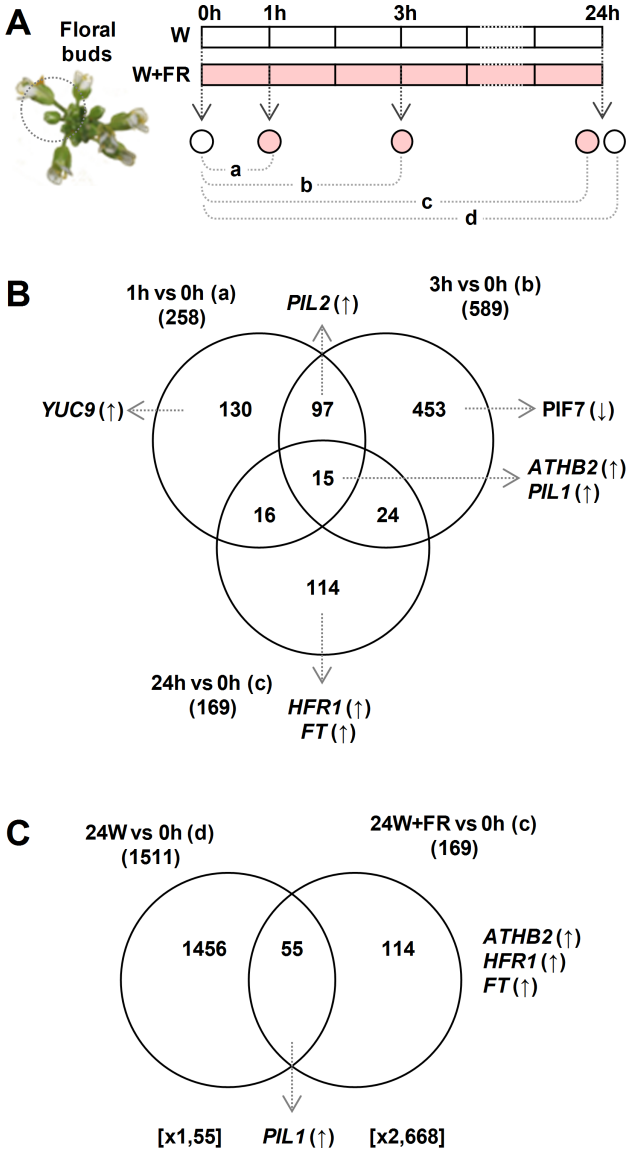

FIGURE 6 Simulated shade rapidly regulates gene expression in the reproductive tissues. (A) Cartoon representing the setting of the experiment. Plants were grown as indicated in Figure 2. Wild-type Col-0 adult plants grown under W were either kept in W (white boxes) or transferred to simulated shade (W + FR, pink boxes) for the indicated hours. Circles indicate the time in which the samples (floral buds) were harvested and the corresponding treatment. Letters "a" to "d" indicate the comparisons to identify the Differentially Expressed Genes (DEGs). (B) Venn diagram presenting DEGs ($p < 0.05$, fold change ≥ |1.5|) after 1, 3 and 24 hours of simulated shade treatment compared to their expression before the treatment (0 h). (C) Venn diagram presenting the DEGs ($p < 0.05$, fold change ≥ |1.5|) after 24 h of differential W or W + FR treatments compared to their expression before the treatment (0 h). In B and C, some of the DEGs found in the analyses are indicated.

of W-grown plants were included in our RNA-seq experiment) but not in the 24 h time point. To investigate potential circadian influences at these early time points, we consulted the publicly available database DIURNAL containing expression data subjected to standardized light and temperature cycles. This database includes regression-based analyses to identify genes exhibiting rhythmic expression patterns (Mockler et al., 2007). We searched for the expression of several genes identified in our RNA-seq in the "long day" condition of DIURNAL, taking into account that, in our experiment, the simulated shade treatment was initiated at Zeitgeber Time 3 (ZT3) (Table S5, Figure S7). These analyses indicated (1) that not all the shade-regulated DEGs (after 1–3 of W + FR exposure) appear to have a circadian expression (e.g., *YUC9*), (2) while some genes (e.g., *HFR1*, *ATHB2*, *PIL2*) show a potential link between circadian rhythm and shade-induced expression, their circadian expression peaks before (*HFR1*, *ATHB2*, at ZT0) or several hours after (e.g., *PIL2*, at ZT10) shade treatment was initiated, suggesting that a potential rapid (1–3 h) shade-induced

increase in their expression is not affected by their circadian regulation; (3) as the expression of well-established shade markers, such as *IAA19* and *IAA29*, is not under circadian control, we can conclude that their expression is not shade-induced in reproductive tissues; and (4) the shade-induced expression of *PIL2* and *IAA1* might also be affected by its clock regulation. Although our DIURNAL analyses only cover a small number of well-known shade-induced DEGs, they are consistent with our previous conclusion that a reduced number, but well-known, of shade-marker genes is induced in the floral bud samples grown under 1–3 h W + FR.

A separate analysis of these transcriptomic data based on the varying expression patterns over time classified the several hundred DEGs into 17 modules (Figures 7 and S8; Table S3). Notably, (1) 639 genes (Magenta module) showed increased expression after just 1 h of shade treatment, which rapidly decreased afterwards; (2) 1844 genes (Brown module) exhibited increased expression 3 h after shade treatment, followed by a subsequent decrease; (3) 2808 genes (Blue module) showed a sustained increase in expression over time in response to shade; (4) 224 genes (Tan module) displayed repressed expression after 1–3 h of shade treatment but later recovered to the same levels as in the W control after 24 h; and (5) 5756 genes (Turquoise module) were repressed by shade over time. Analyses to investigate potential circadian influences at these early time points also indicate that the regulation of several shade-induced genes identified appears to be largely independent of the circadian clock (Figure S7; Table S5).

The gene onthology (GO) analyses of the functional annotations of the DEGs lists corresponding to the above-mentioned modules in relation with the "Biological processes" (BP) and "Cellular component" (CC) categories were also obtained (Table S4). While Brown, Magenta and Tan modules were enriched with genes involved in metabolic processes related to photosynthesis, chloroplasts and abiotic (light) stimuli, the Blue and Turquoise modules were enriched with genes associated with other cellular processes (such as protein modification and transport) and development. Thus, we found that genes that rapidly and reversibly respond to shade (Brown, Magenta and Tan modules) are involved in light responses, whereas genes that show a sustained response to simulated shade (Blue and Turquoise modules) are primarily related to developmental changes. Consistently, shade-marker genes such as *ATHB2*, *ATHB4*, *HFR1*, *PAR1*, *PIL1* or *PIF5* were found in the Magenta and Brown modules, while hormone and development-related genes such as *TAA1*, *IAA1*, *BEE1/2*, *BIM1/2*, *ANT*, *STK* and *SHP2* were included in the Blue and Turquoise modules (Figure 7). Altogether, these results support our previous conclusion that reproductive tissues sense changes in vegetation proximity and rapidly respond by inducing the expression of shade marker genes. Moreover, vegetation proximity regulates the expression of development-related genes in a slower manner.

3.5 | Simulated shade has a stronger impact on gene expression in seedlings than in adult tissues

As mentioned above, several of the well-described shade marker genes previously described in seedlings are found among the shade-regulated DEGs in floral buds (e.g., *PIL1* or *ATHB2* in Magenta and Brown modules). Next, we wanted to explore the similarities and differences in the shade-induced transcriptomic changes between seedlings and adult (reproductive) tissues. To address this question, we compared our RNA-seq results with available microarray data of young W-grown seedlings treated with 0, 1, 3 and 24 h of W + FR (Leivar et al., 2012) (Table S6; Figure S9). In seedlings, hundreds of DEGs were identified as shade-regulated ($p < 0.05$, fold change ≥ |1.5|) after 1 (905 DEGs), 3 (2973 DEGs) and 24 (5633 DEGs) hours of simulated shade treatment compared to their expression before the treatment (0 h). The differences in the transcriptomic approaches used (microarrays *vs.* RNAseq) and the possible interference of their circadian regulation (that might increase the number of DEGs identified as false shade-regulate genes) complicate direct comparisons between these experiments. However, the substantially lower number of DEGs identified in floral buds after 1, 3 and 24 h of W + FR compared to seedlings is striking (Figure S9). For each time of exposure, the number of overlapping genes was also reduced, but *ATHB2*, *PIL2* and/or *HFR1* were consistently found (*PIL1* gene was absent in the microarrays). Altogether, these results suggest that floral buds are transcriptionally much less shade-responsive than seedlings.

3.6 | The shade-induced reduction in the number of ovule primordia in the placenta takes several hours

The promotion of hypocotyl elongation starts about 45 minutes after the exposure to low R:FR (Cole et al., 2011) and follows the rapid changes in gene expression observed as early as 15 minutes after the initial exposure to shade (Kohnen et al., 2016). The shade-induced hypocotyl elongation involves only changes in cell elongation (Pastor-Andreu et al., 2024). By contrast, the shade-reduction in seed number results from ovules that arise from a meristematic tissue within the carpel referred to as placenta (Cucinotta et al., 2014). As such, the formation of ovules from the placenta might take longer times. To explore the time it takes for the placenta to develop ovules in our growth conditions, we used a *pAP1:AP1-GR ap1 cal* (MB2) line. In the Arabidopsis double knockout mutant of *APETALA 1* (*AP1*) and *CAULIFLOWER* genes (*ap1 cal*), flower development is arrested, leading to an overproliferation of inflorescence meristems (IMs) (Kempin et al., 1995), which resembles a cauliflower (Figure 8A). In the MB2 plants, this double mutant is complemented with the construct *pAP1:AP1-GR*, where the *AP1* gene is fused to a glucocorticoid receptor (GR) under the control of its own *AP1* promoter (Lloyd et al., 1994). Treatment of MB2 plants with dexamethasone (DEX) induces the nuclear translocation of AP1-GR, synchronizing flower development. We applied DEX on day 20 of plants growing under LD conditions when, in our growth conditions, plants presented abundant IMs. Inflorescences were harvested after 3 (d23) and 4 (d24) days of mock or DEX treatment (±DEX) and analyzed at the microscope to record the progression of pistil development stages (Figure 8B). Although pistil development was not completely halted in the mock-treated plants (-DEX), hundreds of relatively synchronized floral buds

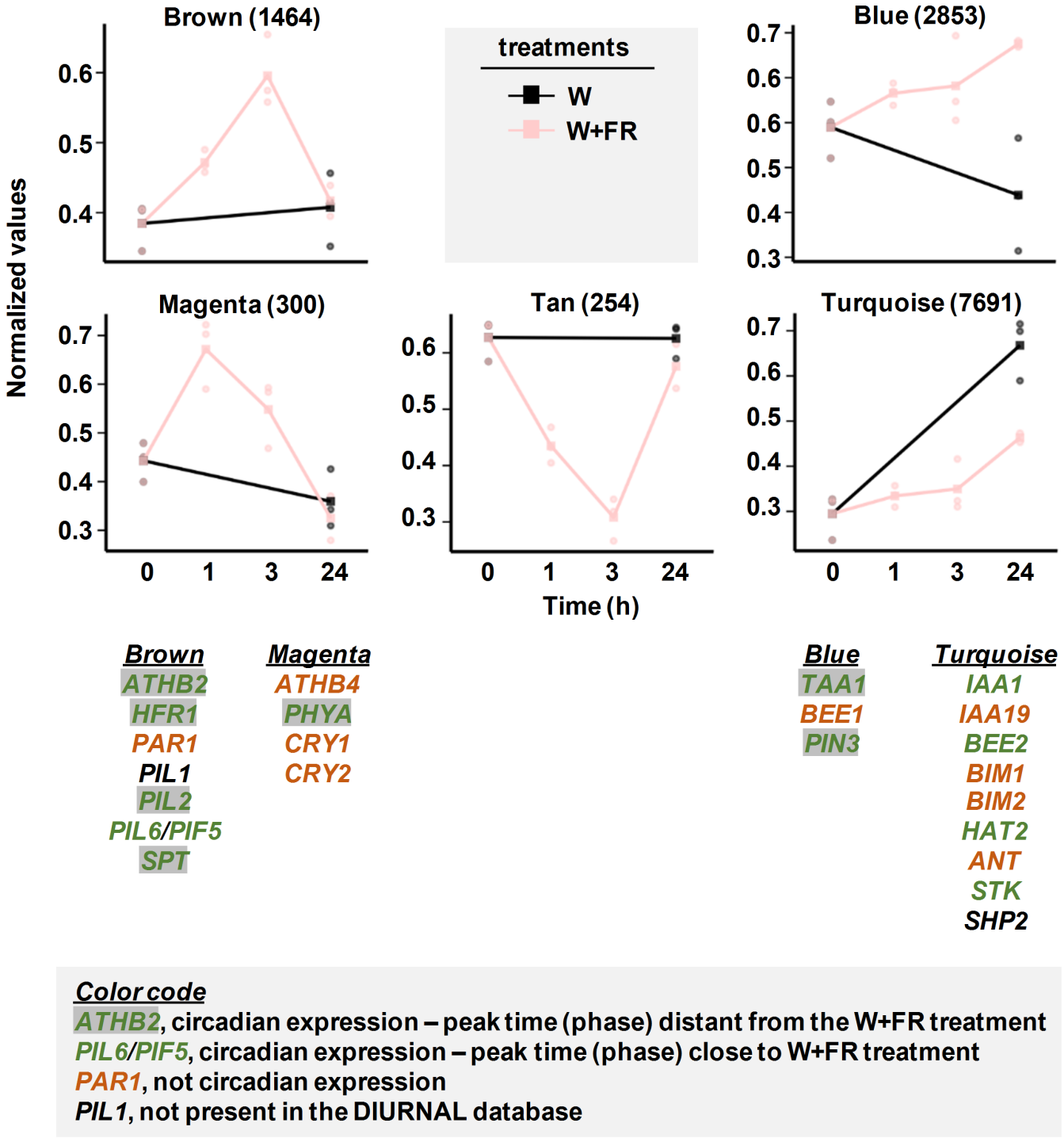

FIGURE 7 Genes rapidly regulated by shade in reproductive tissues are classified into modules according to their expression patterns. Temporal expression patterns under the simulated shade of the main five modules (named with a color). Each module contains a distinct set of Differentially Expressed Genes (DEGs). The simulated shade conditions are described in Figure 6. Numbers represent the total number of genes included in each module. At the bottom, a short list of representative shade-marker genes as well as hormone and development-related genes found in four of these modules is provided. Color code is explained at the bottom of the figure.

produced on each inflorescence of DEX-treated samples were observed at d23. Moreover, in contrast with the mock-treated plants (-DEX), the DEX-treated tissues were rich in pistils in stages 7 and 8 (Alvarez-Buylla et al., 2010) (Figure 8C), where ovule primordia initiate (i.e., indicating that ovule number is established). By d24, although the inflorescences still contained lots of pistils at stages 7 and 8, there were also pistils at more advanced stages compared to d23). Overall, these analyses indicated that the determination of ovule number requires at least 2–3 days in our experimental conditions, supporting our hypothesis that the shade-reduction in seed number appears as a developmental decision that requires days (rather than minutes) to be implemented.

4 | DISCUSSION

High planting density reduces the productivity of many crops as the increase in vegetation proximity might, eventually, affect radiation intercepted per plant (Villalobos et al., 1994). Hence, vegetation proximity associated with high plant density activates a series of SAS responses. In seedlings and young plants, vegetation proximity promotes hypocotyl and stem elongation, petiole length and flowering (Casal, 2012; Roig-Villanova and Martinez-Garcia, 2016; Martinez-Garcia and Rodriguez-Concepcion, 2023; Roig-Villanova and Martinez-Garcia, 2022). In adult plants, one of these responses is the reduction in seed yield (i.e., seed production per plant) (Villalobos et al., 1994; Libenson et al., 2002). The cellular and/or molecular causes of the reduction of seed yield appear to differ among species. In soybean, shade reduced seed yield, an effect attributed primarily to reductions in the number of branches and pods per plant as there was no effect in seed number per pod and seed weight (Green-Tracewicz et al., 2011). In Arabidopsis adult plants, two of the better-studied responses to plant proximity and shade are the induction of flowering and the suppression of axillary bud outgrowth. Shade-induced promotion of flowering takes place even when the plant is very young (i.e., after 1–2 weeks after germination) and might reduce the number of rosette leaves (Cerdan and Chory, 2003; Devlin et al., 2003; Halliday et al., 2003). Therefore, early treatments with W + FR have a strong impact on seed yield (Procko et al., 2014), presumably because of the reduction in rosette leaves and branches produced per plant (Figure 1B). However, shade-induced suppression of axillary bud outgrowth happens even when flowering has started and the numbers of rosette leaves and axilary buds are not distinct to control-treated plants (Finlayson et al., 2010; Gonzalez-Grandio et al., 2013) (Figure 1B). Similarly, when shade treatments were applied once the plant had entered the reproductive phase, simulated shade had a milder impact on plant architecture but still reduced the number of seeds per fruit (Figures 1 and 2) and eventually seed yield (Figure S2) by reducing the number of ovule primordia formed within the pistil. A 10% reduction in production, as observed in this study, is generally considered significant in both horticultural and extensive cropping systems (O'Connor et al., 2023). Given that the observed effect of plant proximity on seed number is likely preceded by additional impacts on plant architecture, the influence of plant proximity on overall production could be substantial. Furthermore, while not explored in the present study, the interplay between plant proximity and other environmental or biotic stresses, as well as the cumulative effects of these stimuli on plant performance, represents an interesting field for future research.

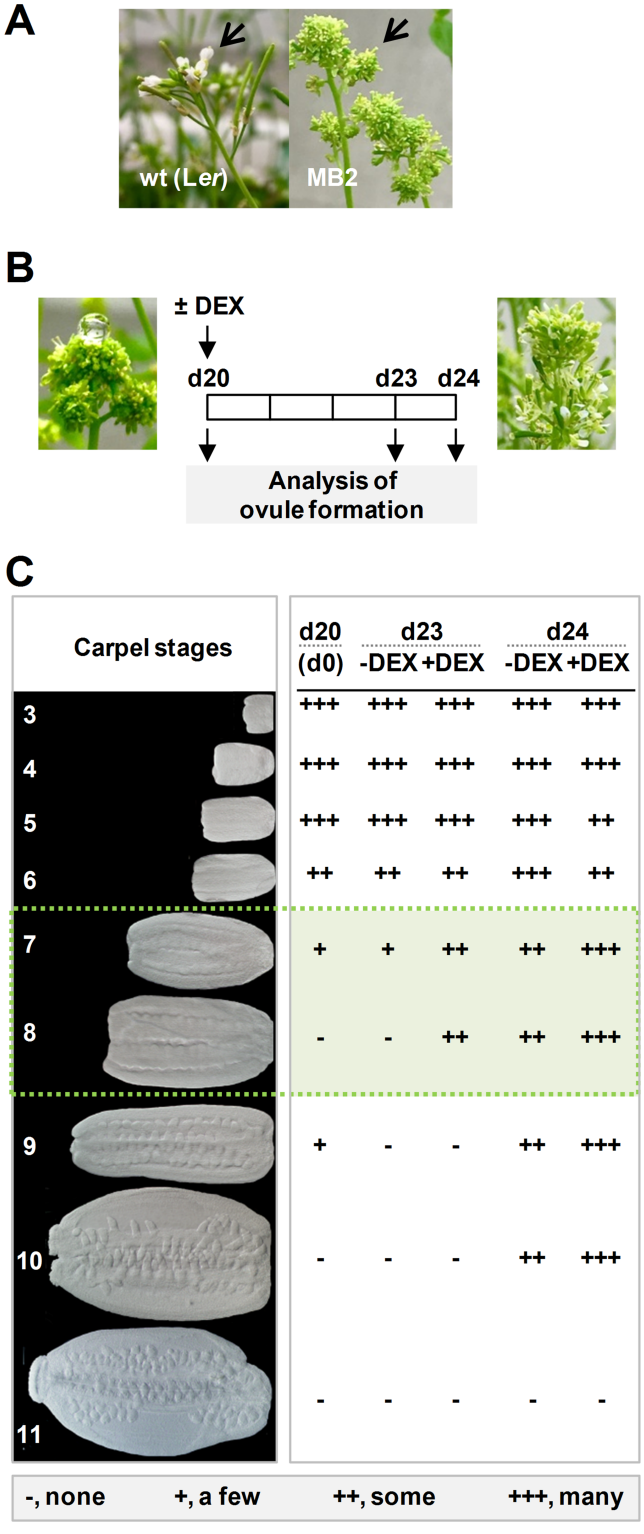

FIGURE 8 Phenotypical analyses of the *pAP1:AP1-GR ap1 cal* (MB2) plants indicate that formation of ovules from the placenta might take several hours. (A) Aspect of wild-type (*Ler*) and MB2 inflorescences. Arrows indicate the morphological differences between the inflorescences. Plants were growing as indicated in Figure 5C: after germinating and growing for 7 days under continuous white light (W), well-grown seedlings were transferred into individual pots and grown under LD for the following days. (B) Young inflorescences of MB2 plants grown for 20 days from germination (d20) under W were treated once with or without dexamethasone (DEX) to induce pistil development. At d20 and three and four days after the DEX treatment (d23 and d24), plant material was harvested and analyzed by Normaski microscopy. (C) Summary of the evolution of carpel development (stages 1 to 11) before (d20) and after (d23 and d24) the ± DEX application.

Shade exposure of inflorescences also promoted elongation of some floral organs, such as siliques and pedicels (Figures 1C and S1). A similar effect was reported in the crop species *B. rapa*, in which shade exposure poorly affected flowering but promoted silique elongation that produced fewer and smaller mature seeds (Procko et al., 2014). In pepper, shade also promoted the length of flower pedicels (Chen et al., 2024). Some of the molecular mechanisms and/or components involved in promoting the length of leaf petioles and stem-like structures (e.g., hypocotyls) in response to shade might also participate in promoting the elongation of pedicels and siliques. There are however some obvious differences, such as the role of phyB, that appear to promote rather than inhibit silique length (Figure 1C), in clear contrast with the role of this photoreceptor in inhibiting hypocotyl and petiole elongation (de Wit et al., 2015; Pantazopoulou et al., 2017; Molina-Contreras et al., 2019). It is important to note that both SAS responses, reduction in ovule number and promotion of elongation of pedicels and fruits, occur in separated organs within the inflorescence and even in different moments of the same: pistils start to differentiate from stage 6 to 8 of Arabidopsis flowers (Alvarez-Buylla et al., 2010), whereas pedicels and siliques elongate later in the flower development. The shade-induced reduction in seed number per silique likely ensures that the plant limited energy investment in reproduction results in fewer but larger, well-formed and viable seeds (Figures 1C, S1E, and S2B) (Procko et al., 2014).

In Arabidopsis, silique length and seed number per silique usually positively correlate (Barendse et al., 1986; Alonso-Blanco et al., 1999; Bac-Molenaar et al., 2015). This is also the case of siliques of *ant-4* (Figure 5), PAR1-RNAi (Roig-Villanova et al., 2007) and several other low-fertility mutant lines (Gomez et al., 2019). However, shade treatments led to the production of longer siliques that contained fewer and bigger seeds, a contrasting phenotype only reported in Arabidopsis plants submitted to a pruning treatment (Bennett et al., 2012). This phenotype has been proposed to be a consequence of the reduction in the sink strength caused by the removal of secondary inflorescences and lateral branches. Changes in the sink strength for assimilates between apices and flowers, which limit assimilate import into flowers, has also been proposed to contribute to the shade-induced increase in flower and fruit abortion in pepper plants (Chen et al., 2024). These results suggest that the low R:FR treatments might lower the sink strength balance between the different tissues in the inflorescences, indirectly causing a reduction in ovule primordia number but an increase in silique length. Alternatively, the low R:FR exposure might directly and independently alter both traits or responses (promote silique length and reduce seed number per silique) by unknown mechanisms, suggesting that simulated shade uncouples these two responses, which appear as associated in low-fertility mutant lines. As such, they might be regulated by different mechanisms or sets of genes. When comparing the results of our RNA-seq analysis of reproductive tissues with shade-responsive genes in seedlings, we found that shade regulates a common core of genes across these different stages of Arabidopsis development to perceive and rapidly respond to shade. Meanwhile, other genes respond to shade in a more tissue-specific manner. Therefore, it is tempting to speculate that the core of shade-regulated genes might have a role in controlling elongation responses in different organs and/or stages of development.

The reduction in rosette branches observed in plants exposed to simulated shade when flowering was already induced (e.g., grown for 28 d under W, Figure 1B) is consistent with previous conclusions that the main pathway of phytochrome control of branching is via its promotion of bud outgrowth (Finlayson et al., 2010; Gonzalez-Grandio et al., 2013). The inhibition of rosette branches is controlled by the perception of proximity vegetation signals likely sensed by more or less distant parts of the plant, such as the rosette leaves. The number of ovule primordia formed per pistil can also be adjusted by sensing the proximity vegetation signals via the action of phytochrome photoreceptors (Figure 1). This response could be a direct consequence of inflorescences being exposed to simulated shade. Since Arabidopsis inflorescences appear to both perceive and molecularly respond to simulated shade (Figures 3, 6, and S4), we can postulate that the observed changes in the number of ovule primordia in response to shade might be (at least in part) directly caused by this exposure. However, we cannot discard that these changes are triggered by signals arriving from nearby organs, as is known for flowering induction. Additional experiments are needed to distinguish between these possibilities.

We noticed that, in all the experiments, whether analyzed by qRT-PCR or RNA-seq, shade-induced expression of *PIL1* (as well as other well-known marker genes, such as *ATHB2*) was milder in floral buds tissues than in seedlings (Figures 3 and S9) (Molina-Contreras et al., 2019). Indeed, while GUS activity was very strong and widespread in seedlings, it was mild and restricted to parts of young pistils, siliques and/or pedicels in young inflorescences (Figure S4). These results suggest that the differences in levels and kinetics of the shade-induction of *PIL1* expression between seedlings and inflorescences are intrinsic to the tissue type, as the simulated shade applied to adult plants also worked in seedlings (Figure S4). The relative simplicity of the seedlings (comprising mostly roots, hypocotyls and cotyledons from a synchronized population of individuals) compared to floral buds (which are formed by a variety of tissues and organs - including sepals, petals, carpels, stamens and pedicels – at different developmental stages) might explain these differences. In this context, it is important to remember that the shade-induced responses of seedlings and floral buds occur over different time scales. In seedlings, responses mostly involve rapid changes in hypocotyl elongation, observable within 1 h of exposure to low R:FR (Cole et al., 2011). By contrast, the reduction in ovule primordia number is likely a cell fate decision that requires 2–3 days to be observed (Figure 8). While the molecular changes in seedlings result in the differential elongation of already formed (and differentiated) cells (Pastor-Andreu et al., 2024), the mechanisms altering ovule number are more complex and require more time, involving the formation of boundary regions to separate the ovule primordia once the placenta is formed (Cucinotta et al., 2014).

The repression of *CUC1*, *CUC2* and *ANT* after several days of shade exposure suggested that simulated shade might act via these genes to reduce ovule number. However, genetic analyses focused on the phenotypic characterization of individual mutants did not support

a role for these factors in controlling this trait. The use of transcriptomics did not provide obvious potential candidates to regulate the reduction in ovule number per silique. However, we acknowledge the potential for complex genetic interactions. Analyzing multiple mutant combinations, particularly those involving genes identified in our RNA-seq analysis, would be valuable for uncovering potential synergistic or epistatic effects. For instance, given the established role of *STK* and the *SHP* genes in ovule development (Pinyopich et al., 2003) and their observed downregulation in response to simulated shade (*STK* and *SHP2*, Figure 7), they might have a role underlying the decrease in seed number under shade. Future phenotypic analyses of the combination of their mutant combinations will be necessary to test this hypothesis.

Still, these analyses highlighted a first wave of DEGs related to light responses and photosynthesis (e.g., Magenta module) and a slower but sustained wave of DEGs more related to developmental changes, such as hormone-related genes (auxins and brassinosteroids), homeotic genes and regulators of flower development. If the regulatory genes of the shade-induced reduction in ovule number are transcriptionally regulated, their expression levels may be too low to be identified using this transcriptomic approach. In our RNA-seq analysis, we did not explicitly account for circadian regulation of transcription. This decision was based on prior knowledge that some genes involved in the studied processes are not circadian-regulated. However, we could not exclude the possibility that some of the identified DEGs in our analysis could be influenced by circadian rhythms. Additionally, it is possible that we may have missed other circadian-regulated genes relevant to the studied processes due to the experimental design or sampling times. The analysis of the expression of the main genes identified in our RNA-seq in the Diurnal database highlighted the complex interplay between shade responses and the circadian clock in regulating gene expression and plant development. Future studies with experimental designs specifically targeting circadian regulation would be valuable to fully elucidate the extent to which circadian rhythms influence the transcriptional changes observed here.

In summary, although simulated shade is believed to indirectly decrease plant yield by inducing early flowering and strongly reducing the amount of flowers per plant (i.e., it alters plant architecture), we have found that vegetation proximity has two opposite effects in *A. thaliana* reproductive tissues: (i) it promotes the elongation of the siliques and (ii) it reduces seed number per silique due to a reduction in the number of ovules formed from the placenta, a type of meristematic tissue within the carpel. These two responses can be uncoupled, suggesting that the underlying components and mechanisms are not the same. The shade reduction in seed number seems to be radically different from the shade promotion of hypocotyl elongation in seedlings, which involves just changes in cell elongation. We postulate that there are two major types of responses to simulated shade: (1) those that affect cells already differentiated (e.g., changes in elongation of hypocotyls, pedicels and siliques) and (2) those that involve fate decisions of meristematic cells (e.g., the transition from vegetative to floral meristem, or the establishment of a reduced number of ovule primordia in the placenta). We aim to test this hypothesis in future works.

AUTHOR CONTRIBUTIONS

I.R.-V. and J.F.M.-G. conceived the project. I.R.-V., E.L.-O., M.D.M., and A.S.-G. performed the experiments and harvested the samples for further analyses. A.G.-C. performed the IAA quantification. A.E.-C. perfomed RNA-seq and A.E.-C. and S.T.-M. analyzed these data. I.R.-V., S.T.-M., E.L.-O., M.D.M., A.S.-G. and J.F.M.-G. analyzed and discussed the data. I.R.-V. and J.F.M.-G. wrote the paper with contributions and/or comments from all authors. All authors reviewed the manuscript.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. The RNA-seq data presented in this article are accessible through GEO accession GSE230448 at the NCBI webpage.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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