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Arabidopsis HD-Zip II proteins regulate the exit from proliferation during leaf development in canopy shade

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Abstract

The shade avoidance response is mainly evident as increased plant elongation at the expense of leaf and root expansion. Despite the advances in understanding the mechanisms underlying shade-induced hypocotyl elongation, little is known about the responses to simulated shade in organs other than the hypocotyl. In Arabidopsis, there is evidence that shade rapidly and transiently reduces the frequency of cell division in young first and second leaf primordia through a non-cell-autonomous mechanism. However, the effects of canopy shade on leaf development are likely to be complex and need to be further investigated. Using combined methods of genetics, cell biology, and molecular biology, we uncovered an effect of prolonged canopy shade on leaf development. We show that persistent shade determines early exit from proliferation in the first and second leaves of Arabidopsis. Furthermore, we demonstrate that the early exit from proliferation in the first and second leaves under simulated shade depends at least in part on the action of the Homeodomain-leucine zipper II (HD-Zip II) transcription factors ARABIDOPSIS THALIANA HOMEBOX2 (ATHB2) and ATHB4. Finally, we provide evidence that the ATHB2 and ATHB4 proteins work in concert. Together the data contribute new insights on the mechanisms controlling leaf development under canopy shade.

Keywords: Arabidopsis, HD-Zip II transcription factors, HFR1/SICS1, leaf development, light quality changes, shade avoidance response.

Introduction

In dicots, plant growth is controlled by internal pathways that are strongly influenced by the environment. The effects of light on shoot growth are particularly interesting, because both light quantity and quality dynamically change during the day (Smith, 1982).

For instance, the reduction in the red/far red (R/FR) ratio of light acts as a signal that triggers the shade avoidance response, causing profound changes in stem and petiole elongation and leaf area growth in the angiosperms that have evolved the capacity to avoid shade. In contrast, shade-tolerant plants have adapted their photosynthesis to function optimally under low-light conditions. These plants are therefore capable of long-term survival under a canopy shade. In Arabidopsis, a typical shade-avoiding plant, the shade avoidance response consists of hypocotyl and petiole elongation and reduction of leaf lamina growth at the early stage of seedling development, and of elevation (hyponasty) and elongation of leaf petioles in older plants (Franklin, 2008; Casal, 2012).
The shade avoidance response occurs when plants grow close to each other, because the light environment is strongly enriched in FR by the light reflected by neighbour plants, and under foliar or canopy shade. In fact, light absorption by photosynthetic pigments occurs mainly in the blue (B) and R (400–500 nm and 600–700 nm, respectively) whereas most of the FR (700–750 nm) is reflected or transmitted by plant tissues. Hence, under canopy shade, both the amount of photosynthetically active radiation (PAR) and the R/FR ratio are significantly reduced (Smith, 1982; Martinez-Garcia et al., 2010; Casal, 2013; Roig-Villanova and Martinez-Garcia, 2016; Fiorucci and Fankhauser, 2017). Although the leading signal triggering the shade avoidance response is the low R/FR perceived through the phytochrome (phy) systems, the reduced R and B irradiance and the blue/green ratio are also important for the physiological and developmental responses (Casal, 2013; Pedmale et al., 2016; Roig-Villanova and Martinez-Garcia, 2016). Indeed, a decrease in the blue/green ratio causes stem elongation in Arabidopsis similar to a decrease in R/FR ratio (Sellaro et al., 2010), although operating through different mechanisms (Pedmale et al., 2016). The internode elongation seen in plants grown under a low R/FR ratio and/or low PAR appears to be mediated by changes in endogenous plant hormone levels and sensitivity (Morelli and Ruberti, 2000; Müller-Moulé et al., 2016; Ballaré and Pierik, 2017). It has been suggested that hypocotyl elongation caused by neighbour detection is promoted primarily by the production of auxin (Casal, 2013). In contrast, under canopy shade, the system operates with less auxin but with an increased sensitivity to the hormonal signal, probably through a differential activity of PHYTOCHROME INTERACTING FACTOR4 (PIF4), PIF5, and LONG HYPOCOTYL IN FAR RED 1/SLENDER IN CANOPY SHADE 1 (HFR1/SICS1) (Hersch et al., 2014). The PIF proteins are basic helix–loop–helix (bHLH) transcription factors that interact with phyB through the conserved N-terminal sequence, the active phyB-binding motif (Leivar and Quail, 2011). HFR1/SICS1 encodes an atypical bHLH protein, and acts as a HLH inhibitor (Hornitschek et al., 2009). PIF1, PIF3, and PIF7, like PIF4, PIF5, and HFR1/SICS1, have been implicated in shade avoidance (Sessa et al., 2005; Lorrain et al., 2008; Hornitschek et al., 2012; Leivar et al., 2012; Li et al., 2012; de Wit et al., 2015). Low R/FR-induced elongation response is attenuated in the pif4 pif5 double mutant and, even more, in pif1 pif3 pif4 pif5 quadruple and pif7 mutants (Lorrain et al., 2008; Leivar et al., 2012; Li et al., 2012). In contrast, transgenic seedlings expressing elevated levels of PIF4 and PIF5 display constitutively long hypocotyls and petioles (Lorrain et al., 2008). In addition, PIF4, PIF5, and PIF7 have been shown to bind directly the promoters of YUCCA8 and YUCCA9 (Hornitschek et al., 2012; Li et al., 2012), auxin biosynthetic genes essential for shade-induced elongation growth (Müller-Moulé et al., 2016).

In Arabidopsis, the shade avoidance response is regulated by a balance of positive and negative factors which, on one hand, ensures a rapid reshaping of the plant towards a light environment more favourable for growth, and, on the other hand, avoids an exaggerated reaction to low R/FR light (Sessa et al., 2005; Bou-Torrent et al., 2008; Crocco et al., 2010; Galstyan et al., 2011; Hao et al., 2012; Ciolfi et al., 2013). Very recently it has been shown that PIF proteins directly suppress the expression of miR156, a negative regulator of the SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) family of genes (Xie et al., 2017). As the SPL genes regulate several aspects of plant development, including leaf initiation rate, branching, vegetative phase change, and flowering time (Xu et al., 2016), the findings reported by Xie and co-authors imply that important aspects of the shade avoidance response are positively regulated through the PIF/miR156/SPL module (Xie et al., 2017).

Among the negative regulators of the shade avoidance response is HFR1/SICS1. HFR1/SICS1 is rapidly induced by low R/FR, and evidence exists that it is a direct target of PIF5 (Sessa et al., 2005; Hornitschek et al., 2009). Persistence of low R/FR results in HFR1/SICS1 accumulation and formation of non-functional heterodimers with PI4 and PIF5 (Sessa et al., 2005; Hornitschek et al., 2009). Accordingly, a number of genes rapidly and transiently induced by low R/FR, including ATHB2, are up-regulated in hfr1/sics1 mutants upon prolonged shade exposure (Sessa et al., 2005; Ruzza et al., 2014). ATHB2 induction by low R/FR does not require de novo protein synthesis (Roig-Villanova et al., 2006), and is reduced in loss-of-function pif mutants (pif1 pif3; pif4 pif5; and pif7; Lorrain et al., 2008; Hornitschek et al., 2009; Leivar et al., 2012; Li et al., 2012). Furthermore, it has been shown that ATHB2 is recognized in vivo by PIF5 (Hornitschek et al., 2012). Loss-of-function athb2 mutants display diminished hypocotyl elongation in low R/FR with respect to wild-type seedlings (Carabelli et al., 2013), whereas the phenotypes of plants overexpressing ATHB2 in high R/FR are reminiscent of those displayed by the wild type grown in low R/FR, indicating a role for this HD-Zip protein in the regulation of the shade avoidance response (Steindler et al., 1999).

ATHB2 is a member of the HD-Zip II family consisting of 10 genes, five of which [ATHB2, HOMEobox ARABIDOPSIS THALIANA4 (HAT4), HAT2, ATHB4, and HAT3] are regulated by changes in light quality that induce the shade avoidance response (Ciarelbi et al., 2008). Elevated levels of HAT1, HAT2, HAT3, and ATHB4 result in phenotypes analogous to those caused by ATHB2 overexpression (Sawa et al., 2002; Ciarelbi et al., 2008; Sorin et al., 2009; Ruberti et al., 2012; Ruzza et al., 2014), further indicating a redundant function of these transcription factors in shade avoidance. Interestingly, homologue genes have been found to be up-regulated in monocotyledonous and dicotyledonous plants exposed to low R/FR light, suggesting a conserved function of HD-Zip II transcription factors through evolution (Ueoka-Nakanishi et al., 2011; Chitwood et al., 2015; Wang et al., 2016).

Despite the significant advances over the last decade in understanding the mechanisms underlying shade-induced hypocotyl elongation (Casal, 2013), little is known about the cellular and molecular responses to low R/FR in the leaf. Previous studies in Arabidopsis have reported that light treatments that simulate neighbour detection or canopy shade promote petiole elongation and reduce leaf lamina...
growth (Kozuka et al., 2005; Carabelli et al., 2007; Kozuka et al., 2010; Sasidharan et al., 2010; de Wit et al., 2015; Roig-Villanova and Martínez-Garcia, 2016). Under simulated canopy shade, we provide evidence that low R/FR rapidly and transiently reduces the frequency of cell division in first leaf primordia through a non-cell-autonomous mechanism (Carabelli et al., 2007, 2008). However, the effects of canopy shade on leaf development are likely to be complex and need to be further investigated. Here, we show that prolonged low R/FR determines early exit from proliferation in the leaf and that this process requires the HD-Zip II transcription factors ATHB2 and ATHB4.

Materials and methods

Mutant, transgenic, and marker lines, and plant growth

The wild-type strain used was Arabidopsis thaliana (L.) Heynh. var. Columbia (Col-0). Other lines used were: athb2-1 (Khanne et al., 2006), athb2-2 (Turchi et al., 2013), athb3-2 (Turchi et al., 2013), athb4-1 (Sorin et al., 2009), athb4-3 (Turchi et al., 2013), athb6-1 athb4-1 (Turchi et al., 2013), hfr1-4/sics1-1 (Sessa et al., 2005), hfr1-1/sics1-2 (Sessa et al., 2005), ATHB2::ATHB2:GUS (Turchi et al., 2013), hfr1-4/sics1-1 ATHB2::ATHB2:GUS (Ruzza et al., 2014), ATHB2::GUS (Baima et al., 1995), CYCLINB1;1:GUS (CYCB1;1:GUS) (Cólón-Carmona et al., 1999; Kang and Dengler, 2002). Plants were grown as previously described (Sessa et al., 2005). Light outputs in High R/FRHigh PAR, Low R/FRLow PAR, and High R/FRLow PAR were as described by Cioffi et al. (2013).

Genetic analysis

To generate athb2-1 hfr1-4/sics1-1, athb4-1 hfr1-4/sics1-1, and athb2-2 hfr1-4/sics1-1, the following crosses were performed: athb2-1 × hfr1-4/sics1-1, athb4-1 × hfr1-4/sics1-1, and athb2-2 × hfr1-4/sics1-1. Double mutants were selected in F2 by phenotyping and PCR genotyping, using the primers previously described (Sessa et al., 2005; Turchi et al., 2013). The double mutants were reanalysed in F3 by phenotyping and genotyping. Homozygosity of the CYCB1;1:GUS (β-glucoronidase) reporter in athb2-2 and athb2-3 was determined by unanimous GUS staining of all seedlings tested (n ≥30).

Gene constructs and transformation

35S::ATHB4::GFP (green fluorescent protein) was constructed as follows: the coding sequence (cds) of ATHB4 (TAIR AT2G49410), excluding the stop codon, was amplified with Gateway™ ends and cloned into pDONR-201. Subsequently, the ATHB4 cds was transferred in pK7FWG2 (Curtis and Grossniklaus, 2003). 35S::ATHB4::GFP was then transformed to Agrobacterium tumefaciens GV3101RK and Col-0 plants were transformed as described (Steindler et al., 1999).

Phenotype analysis and microscopy

Leaves were cleared according to the protocol previously described (Weigel and Glazebrook, 2002). Cleared samples were excised under an MZ8 binocular microscope (Leica, Germany), and then analysed under dark-field optics or with differential interference contrast (DIC) optics, with an Axioskop 2 plus binocular microscope (Zeiss, Germany). Images were taken with a Coolpix 990 digital camera (Nikon Corp., Japan). To determine the mean leaf area, at least 10 samples were measured with the NIH Image Analysis Software [Research Services Branch (RSB) of the National Institute of Mental Health (NIMH), USA, http://rsb.info.nih.gov/ij/]. T-test statistical analysis was performed using QuickCalcs Online Calculators for Scientists (GraphPad Software, Inc. http://graphpad.com/quickcalcs/).

Adaxial subepidermal cells were analysed in cleared young leaves. Cells were viewed with an Axioskop 2 plus binocular microscope equipped with DIC optics, and photographed with the Coolpix 990 digital camera. In young leaves, the degree of cell differentiation and the mean cell area were determined in specific regions of the blade, as indicated. A distal location from the base of the leaf was recognized in the upper part of the leaf blade, inside the first loop of the secondary vein in the procambial or differentiated stage; a proximal region was determined close to the margin of the basal part of the leaf. To determine the mean cell area, 100 cells were measured in the indicated regions of 10 leaves (Horiguchi et al., 2005). Means were compared with t-test analysis (http://graphpad.com/quickcalcs/).

Histochemical detection of GUS activity

For histochemical detection of GUS activity, whole seedlings were treated as previously described (Scarpella et al., 2004; Carabelli et al., 2007). Incubation was 8, 3, and 5 h for ATHB2::ATHB2:GUS, ATHB8::GUS, and CYCB1;1:GUS, respectively. For microscopy, samples were cleared according to the protocol previously described (Weigel and Glazebrook, 2002) and viewed with an Axioskop 2 plus binocular microscope equipped with DIC optics; images were taken with the Coolpix 990. Expression pattern distributions of CYCB1;1:GUS in wild-type and mutant leaves were compared using a contingency table followed by Fisher’s exact test (http://www.physics.csbsju.edu/stats/contingency.html).

Cyclin index determination

In cleared leaves the total cell number and the number of cells with GUS activity in the adaxial subepidermal layer were counted at the proximal region along the proximo-distal axis of the blade by photographing leaves under DIC optics. A 0.01 mm² area was recognized in the proximal region close to the margin of the basal part of the leaf blade. The total cell number and the number of cells with GUS activity were counted, and expressed as the cyclin index (no. of cells with GUS activity/no. of total cells×100) (Donnelly et al., 1999). The data are presented in the form of box-and-whiskers plots, generated by means of the software available at http://plot.ly. A ‘boxplot’ function with default parameters was used (Carabelli et al., 2007).

Real-time PCR

For gene expression analysis, mRNA purification, cDNA synthesis, and quantitative real-time PCR (RT-qPCR) were performed as previously described (Carabelli et al., 2008). Primers and Universal Probe Library (UPL) probes for RT-qPCR analyses are described in Supplementary Table S1 at JXB online. Statistical analyses were performed on log-transformed relative expression ratio values as described by Rieu and Powers (2009). The relative transcript abundance of each gene was normalized to the Col-0 level in High R/FR. Subsequent to data standardization (Willems et al., 2008), one-way ANOVA followed by a Tukey’s post-hoc test was used to assess differences among means (Prism 5, GraphPad Software, CA, USA).

Results

Prolonged shade determines early exit from proliferation in the leaf

We have previously shown that leaves grown in simulated shade (Low R/FRLow PAR) are significantly smaller than those grown in simulated sun (High R/FRHigh PAR) and that cell number, not cell size, contributes to the reduction of leaf area under Low R/FRLow PAR. We have also provided evidence...
that Low \(R/FR_{\text{Low\,PAR}}\) rapidly and transiently reduces the frequency of cell division in first leaf primordia (Carabelli et al., 2007). Here, we reasoned that if the only effect of shade on leaf development is to induce a transient arrest of cell division in young leaf primordia, at later developmental stages leaves of equal area grown in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\) environments should show no significant morphological difference. To test this, first and second leaves of different ages but the same area in High \(R/FR_{\text{High\,PAR}}\) (7- and 8.5-day-old seedlings; Supplementary Fig. S1) and Low \(R/FR_{\text{Low\,PAR}}\) (8- and 10-day-old seedlings; Supplementary Fig. S1) were analysed by examining adaxial subepidermal cells that are good markers of cell differentiation and leaf expansion. No significant difference was observed in the adaxial subepidermal ground meristem cells of leaves with an area of ~0.1 mm\(^2\) (see legend to Fig. 1 for mean leaf area) in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\) (Fig. 1A, B, insets; mean cell area of distal leaf region 73.7 ± 1.8 \(\mu\)m\(^2\) and 69.5 ± 1.6 \(\mu\)m\(^2\) in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\), respectively). Consistent with this, the ground meristem cells showed the same morphological features: rectangular shape, mitotic activity, and absence of airspaces (Fig. 1A, B, insets and data not shown).

In contrast, adaxial subepidermal cells of leaves with an area of ~0.5 mm\(^2\) (see legend to Fig. 1 for mean leaf area) were larger throughout the organ in Low \(R/FR_{\text{Low\,PAR}}\) than in High \(R/FR_{\text{High\,PAR}}\) (Fig. 1C, D, insets; mean cell area of distal leaf region 187.9 ± 4.3 \(\mu\)m\(^2\) and 218.9 ± 6.7 \(\mu\)m\(^2\) in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\), respectively). A large percentage of leaves with an area of ~0.25 mm\(^2\) (see legend to Fig. 2 for mean leaf area) displayed an MP and P pattern in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\), suggesting that mesophyll cell differentiation is initiated earlier in Low \(R/FR_{\text{Low\,PAR}}\) than in High \(R/FR_{\text{High\,PAR}}\) (Fig. 1C, D, insets and data not shown).

To investigate further the effects of shade on leaf development, the complexity of the vascular system was analysed. At the 0.1 mm\(^2\) stage, first and second leaves in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\) have both formed the first-order vein (Fig. 1A, B). Moreover, histochemical localization of GUS activity in 0.1 mm\(^2\) first and second leaves of seedlings expressing the ATHB8::GUS marker (Baima et al., 1995) showed no difference in the number of GUS-stained procambial strands in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\) (Fig. 1E, F; mean number of GUS-stained strands 14.9 ± 0.7 and 13.9 ± 0.5 in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\), respectively, \(n=10\) leaves). At the 0.5 mm\(^2\) stage, first and second leaves in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\) have both formed the vascular loop of second-order veins (Fig. 1C, D). However, they have developed more procambial strands of higher order in High \(R/FR_{\text{High\,PAR}}\) than in Low \(R/FR_{\text{Low\,PAR}}\), as deduced by the expression pattern of the ATHB8::GUS marker (Fig. 1G, H; mean number of GUS-stained strands 26.5 ± 0.7 and 17.8 ± 0.9 in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\), respectively, \(n=10\) leaves, \(P<0.0001\)).

This finding further suggests that mesophyll cell differentiation is initiated earlier in Low \(R/FR_{\text{Low\,PAR}}\) than in High \(R/FR_{\text{High\,PAR}}\). In fact, there is evidence that the Arabidopsis vein pattern is not inherently determinate, but arises through reiterative initiation of new pre-procambial branches until this process becomes terminate by the differentiation of mesophyll (Scarpella et al., 2004).

Mesophyll cell differentiation is associated with cessation of cell cycling which occurs from the leaf apex to the base. In fact, while dividing cells are initially distributed uniformly and diffusely throughout the leaf, cells near the blade apex cease dividing first and the region of frequent cell divisions gradually becomes restricted to the leaf base, forming a strong longitudinal gradient (Pyke et al., 1991; Van Lijssebettens and Clarke, 1998; Donnelly et al., 1999; Carabelli et al., 2007; Kazama et al., 2010; Andriankaja et al., 2012). Thus, to investigate whether the differentiation process is indeed initiated earlier in shade, we analysed the changes in the spatial pattern of cell cycling during leaf expansion in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\) using the widely utilized CYCB1;1:GUS marker (Donnelly et al., 1999). To this end, CYCB1;1:GUS first and second leaves of different ages but the same area in High \(R/FR_{\text{High\,PAR}}\) (7.5- and 8.5-day-old seedlings) and Low \(R/FR_{\text{Low\,PAR}}\) (9- and 10-day-old seedlings) were analysed. The histochemical localization of GUS activity in the adaxial subepidermal cell layer revealed that CYCB1:1:GUS spatial patterns differ in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\), and indicated that cell cycling of mesophyll precursors is terminated earlier in Low \(R/FR_{\text{Low\,PAR}}\) than in High \(R/FR_{\text{High\,PAR}}\) (Fig. 2A–D). To perform a quantitative analysis, first and second leaves were grouped into four GUS expression patterns indicated as distal (D), distal–median (DM), median–proximal (MP), and proximal (P), and the percentage of leaves showing each specific GUS pattern was determined (Fig. 2E–G). A large percentage of leaves with an area of ~0.25 mm\(^2\) (see legend to Fig. 2 for mean leaf area) displayed an MP and P pattern in High \(R/FR_{\text{High\,PAR}}\) and Low \(R/FR_{\text{Low\,PAR}}\), respectively (no. of leaves High \(R/FR_{\text{High\,PAR}}=13/19\); no. of leaves Low \(R/FR_{\text{Low\,PAR}}=21/32\); Fig. 2A, B, E, F). Furthermore, the totality of leaves with an area of ~0.50 mm\(^2\) (see legend to Fig. 2 for mean leaf area) had a P pattern in High \(R/FR_{\text{High\,PAR}}\) and no GUS labelling in Low \(R/FR_{\text{Low\,PAR}}\) (no. of leaves High \(R/FR_{\text{High\,PAR}}=10/10\); no. of leaves Low \(R/FR_{\text{Low\,PAR}}=11/11\); Fig. 2C, D, E, G). Consistent with the CYCB1;1:GUS spatial patterns, precursors mesophyll cells are larger in Low \(R/FR_{\text{Low\,PAR}}\) than in High \(R/FR_{\text{High\,PAR}}\) (Supplementary Table S2).

Together, the data indicate that prolonged shade provokes early differentiation of mesophyll cells which correlates with a precocious termination of vein formation.

The HD-Zip transcription factor \(ATHB2\) is required for early exit from proliferation during leaf development in shade

Consistent with \(HFR1/SICS1\) acting as a negative master regulator of the shade avoidance response (Sessa et al., 2005; Hornitschek et al., 2009), the leaf cell phenotype is exaggerated in \(hfr1/sics1\) loss-of-function mutant seedlings in Low \(R/FR_{\text{Low\,PAR}}\) (Fig. 3). No significant difference was observed between wild-type and \(hfr1/sics1\) leaves with an area of
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Among the genes up-regulated in hfr1/sics1 mutants is ATHB2 (Sessa et al., 2005; Ruzza et al., 2014), which has been suggested to play a role, together with HAT1 and HAT2, in the control of cell proliferation during leaf development in a sun-simulated environment (Ciarbelli et al., 2008).

Under a sun-simulated environment, ATHB2 expression is mainly localized in provascular cells in either the embryo or leaf primordia (Turchi et al., 2013). Light quality changes rapidly, and transiently induces ATHB2::GUS expression in all cell layers of the elongating portion of the hypocotyl and cotyledon petioles of young seedlings (Carabelli et al., 2013; Ruzza et al., 2014). Consistent with HFR1/SICS1 function in shade avoidance, ATHB2::ATHB2::GUS is significantly up-regulated in the hypocotyl and cotyledon petioles of hfr1-4/sics1-1 loss-of-function mutant seedlings upon prolonged exposure to simulated shade (Ruzza et al., 2014). To determine whether ATHB2 is regulated by shade in leaves as well, ATHB2::ATHB2::GUS and hfr1-4/sics1-1 ATHB2::ATHB2::GUS seedlings were grown for 7 d in High R/FR_High PAR and then transferred to Low R/FR_Low PAR for different times. Interestingly, ATHB2::GUS expression is transiently induced by shade in the wild type at the leaf margin (Fig. 4A–C), which is known to play a significant role in leaf development (Reinhardt et al., 2007; Bilsborough...
In accordance with HFR1/SICS1 function in plant response to shade, the GUS signal in epidermal cells was stronger in hfr1-4/sics1-1 ATHB2::ATHB2:GUS than in ATHB2::ATHB2:GUS leaves exposed to prolonged Low R/FRLow PAR (Fig. 4D–F, insets).

To investigate whether ATHB2 has a role in the early exit from proliferation during leaf development in shade, the leaf cell phenotype of athb2 loss- and gain-of-function mutants was analysed. Both athb2-1 and athb2-3 loss-of-function mutants display precursor mesophyll cells smaller than the wild type in leaves with an area of ~0.35 mm² in Low R/FRLow PAR, whereas no significant difference was observed between mutants and the wild type in High R/FRHigh PAR (Fig. 3). In contrast, the athb2-2 gain-of-function mutant displays precursor mesophyll cells larger than the wild type in both High R/FRHigh PAR and Low R/FRLow PAR (Fig. 3).

To gain further insights into the role of ATHB2 in leaf development in shade, the CYCB1;1:GUS marker was introgressed into athb2-2 and athb2-3 mutant plants, and the cyclin index in the adaxial subepidermal cell layer of leaves with an area of ~0.35 mm² in High R/FRHigh PAR and Low R/FRLow PAR was calculated (Donnelly et al., 1999). As expected from leaf cell size analysis, the cyclin index value is higher in the leaf proximal region of athb2-3 upon exposure to Low R/FRLow PAR relative to the wild type, whereas it is lower in the athb2-2 gain-of-function mutant (Fig. 5).

Together, the data demonstrate a role for ATHB2 in the control of cell proliferation during leaf development in shade. This is further supported by the finding that at the end of vegetative development, fully expanded first leaves are significantly larger in the athb2-2 gain-of-function mutant (Fig. 5).

ATHB2 and ATHB4 act together in controlling early exit from proliferation during leaf development in shade

Adaxial subepidermal cell size analysis and cyclin index determination of leaves with an area of ~0.35 mm² (see Figs 3 and
functional redundancy has been reported for members of the HD-Zip II γ and δ subfamilies (Ciarbelli et al., 2008; Turchi et al., 2013), suggesting that one or more HD-Zip II protein may contribute, together with ATHB2, to the early exit from proliferation during leaf development in shade. As a first step to test this, we investigated how a hfr1-4/sics1-1 loss-of-function mutation affects the expression of ATHB4, HAT1, and HAT3 in Low R/FR

To explore whether ATHB4 has any role in the leaf cell response to prolonged shade, the leaf cell phenotype of athb4-3 and athb4-1 hfr1-4/sics1-1 double mutant relative to the wild type in Low R/FR Low PAR, whereas no significant functional redundancy has been reported for members of the HD-Zip II γ and δ subfamilies (Ciarbelli et al., 2008; Turchi et al., 2013), suggesting that one or more HD-Zip II protein may contribute, together with ATHB2, to the early exit from proliferation during leaf development in shade. As a first step to test this, we investigated how a hfr1-4/sics1-1 loss-of-function mutation affects the expression of ATHB4, HAT1, and HAT3 in Low R/FR

5) demonstrated that the leaf response to Low R/FR Low PAR is strongly reduced but not abolished in plants lacking ATHB2 function, thus indicating that other factor(s) are also involved in the early exit from proliferation provoked by canopy shade.

Functional redundancy has been reported for members of the HD-Zip II γ and δ subfamilies (Ciarbelli et al., 2008; Turchi et al., 2013), suggesting that one or more HD-Zip II protein may contribute, together with ATHB2, to the early exit from proliferation during leaf development in shade. As a first step to test this, we investigated how a hfr1-4/sics1-1 loss-of-function mutation affects the expression of ATHB4, HAT1, and HAT3 in Low R/FR

To explore whether ATHB4 has any role in the leaf cell response to prolonged shade, the leaf cell phenotype of athb4-3 and athb4-1 hfr1-4/sics1-1 double mutant relative to the wild type in Low R/FR Low PAR, whereas no significant
difference was observed between athb4 mutants and the wild type in High R/FR High PAR (Fig. 3). As observed in seedlings lacking ATHB2, however, the adaxial subepidermal cells of athb4 mutant leaves are larger in Low R/FR Low PAR than in High R/FR High PAR (Fig. 3), indicating that in the absence of ATHB4 the leaf response to prolonged shade is reduced but not abolished. In contrast, 35S::ATHB4:GFP leaves display precursor mesophyll cells larger than the wild type in both High R/FR High PAR and Low R/FR Low PAR (Fig. 3).

The up-regulation of ATHB2 and ATHB4 in hfr1-4/sics1-1 in Low R/FR Low PAR and the phenotype of athb2 and athb4 loss- and gain-of-function mutants strongly suggest that the exaggerated leaf cell phenotype of hfr1/sics1 loss-of-function mutants in shade may be attributable at least in part to the high levels of ATHB2 and ATHB4 expression in this mutant. To test this, we isolated and characterized the athb2-3 hfr1-4/sics1-1 and athb4-1 hfr1-4/sics1-1 double mutants. First, we analysed the expression levels of ATHB2 and ATHB4, respectively, in athb4-1 hfr1-4/sics1-1, and athb2-3 hfr1-4/sics1-1 upon exposure to Low R/FR Low PAR and found that the two genes are indeed up-regulated in the double mutants relative to the wild type (Supplementary Fig. S2). Next, we investigated the leaf cell phenotype of athb2-3 hfr1-4/sics1-1, and athb4-1 hfr1-4/sics1-1 upon prolonged exposure to simulated shade. Leaf adaxial subepidermal cells of athb2-3 hfr1-4/sics1-1 and athb4-1 hfr1-4/sics1-1 resemble, respectively, those of athb2-3 and athb4-1 single mutants in Low R/FR Low PAR (Fig. 3), implying that the exaggerated leaf cell phenotype of hfr1/sics1 in shade is indeed caused by elevated levels of ATHB2 and ATHB4. Interestingly, however, the leaf cell phenotype of the double mutants also indicates that the enhanced response of hfr1/sics1 leaves is lost in the absence of either ATHB2 or ATHB4, thus suggesting that the two proteins work in concert. To gain further insights on this, the leaf cell phenotype of the athb2-3 athb4-1 and athb2-2 athb4-1 double mutants was analysed. Consistent with the two proteins acting together, the size of the adaxial subepidermal cells of the athb2-3 athb4-1 double loss-of-function mutant exposed to prolonged Low R/FR Low PAR does not differ from that of the single mutants, whereas the leaf cell phenotype of athb2-2 athb4-1 resembles that of athb4-1 in both High R/FR High PAR and Low R/FR Low PAR (Fig. 3). Thus, this implies that the leaf cell phenotype caused by the athb2-2 gain-of-function mutation depends on the presence of ATHB4.

**Fig. 4.** ATHB2 expression is induced in the leaf margin by shade. (A–F) Histochemical localization of GUS activity in first and second leaves of ATHB2::ATHB2:GUS (A–C) and ATHB2::ATHB2:GUS hfr1-4/sics1-1 (D–F) seedlings grown for 7 d in a light/dark cycle (16/8 h) in High R/FR High PAR (A, D), and then exposed to Low R/FR Low PAR under the same regimen for 2 h (B, E) and 8 h (C, F). The insets show a paradermal view of adaxial epidermal cells. At least 30 leaves for each line and each time point were analysed. Scale bars: (A–F), 50 μm; insets, 10 μm.

Low PAR determines early exit from proliferation in the leaf through an ATHB2-independent pathway

Shade avoidance response is triggered by both changes in spectral composition and a reduction in the total amount of visible light. Recent work argues that there is more than one pathway leading to growth of the hypocotyl in response to changes in light quality [low R/FR or low blue light (LBL)] or quantity (PAR) (Keller et al., 2011; Keuskamp et al., 2011; Pedmale et al., 2016). Under our simulated shade conditions, R is reduced and FR is increased, maintaining total light quantity (400–800 nm) constant (Sessa et al., 2005; Carabelli et al., 2007; Ciarbelli...
leaf size and shape (Gonzales et al., 2012). These processes are dynamically regulated by environmental cues (Carabelli et al., 2007; Skirycz and Inzé, 2010; Skirycz et al., 2011; Kalve et al., 2014). A number of studies have indicated that low light intensity reduces the leaf area in several plant species (Wilson, 1966; Dengler, 1980; Yano and Terashima, 2004; Kim et al., 2005; Cookson and Granier, 2006). It has been suggested that a reduction in cell number, not in cell size, contributes to the reduced leaf size of plants grown under a simulated shade light regime consisting of low R/FR and low photosynthetic light radiation (Low R/FR<sub>Low PAR</sub>) (Carabelli et al., 2007). Moreover, evidence has been provided that Low R/FR<sub>Low PAR</sub> rapidly and transiently reduces the frequency of cell division in young leaf primordia (<0.008 mm<sup>2</sup>) through a non-cell-autonomous mechanism (Carabelli et al., 2007, 2008; Ruzza et al., 2014). Here we show that persistence of Low R/FR<sub>Low PAR</sub> later during leaf development (>0.25 mm<sup>2</sup>), determines early exit from proliferation, which in turn results in smaller leaves at the end of vegetative development. By combining adaxial subepidermal cell size determination and the expression pattern of the CYCB1;1:GUS marker in the first and second leaves of plants grown in High R/FR<sub>High PAR</sub> and Low R/FR<sub>Low PAR</sub>, we indeed gained evidence that prolonged exposure to simulated shade provokes early termination of cell cycling of mesophyll precursors (Figs 1, 2, Supplementary Table S2). Furthermore, taking advantage of the ATHB8::GUS marker (Baima et al., 1995), we demonstrated that the early differentiation of leaf mesophyll cells in plants grown in Low R/FR<sub>Low PAR</sub> relative to those in High R/FR<sub>High PAR</sub> correlates with a precocious termination of vein formation (Fig. 1). This finding is consistent with the evidence that Arabidopsis vein pattern is not inherently determinate, but arises through reiterated initiation of new pre-procambial branches until this process becomes terminate by the differentiation of mesophyll (Scarpella et al., 2004).

Very recently it has been shown that soybean plants grown in shade produced smaller leaves as a result of a lower mitotic activity. These results are also corroborated by a lower expression of key genes involved in the regulation of cell proliferation. In addition, shade significantly increased the auxin and gibberellin content, and significantly decreased the cytokinin (CK) content in soybean leaves (Wu et al., 2017). Several studies pointed to auxin as a major player in shade avoidance response and in neighbour detection in Arabidopsis and crop species (Casal, 2013; Iglesias et al., 2018, and reference therein). We have previously shown that shade light triggers a rapid arrest of leaf primordia growth by the breakdown of CKs through the action of the auxin-induced cytokinin oxidase 6 gene (AtCKX6) (Carabelli et al., 2007). Moreover, long exposure to shade light results in an up-regulation of AtCKX5 (Ciolli et al., 2013). Considering the positive role of CK in the regulation of cell proliferation in the shoot (Kieber and Schaller, 2018) and the negative effects of an increased CK degradation on leaf size (Holst et al., 2011), it is tempting to speculate that a reduced CK signalling could be responsible in part for the smaller leaves found in plants grown under shade light.

A rapid and transient arrest in cell cycle progression (pause) has also been observed in young Arabidopsis leaves subjected to simulated shade light (Holst et al., 2011). Varying R/FR while maintaining total light quantity constant implies a non-constant supply of PAR (400–700 nm). Consistent with the existence of more than one pathway promoting hypocotyl growth in response to light quality changes or low PAR, we have previously shown that there is no significant difference in the expression of several genes rapidly induced by shade such as ATHB2 (Carabelli et al., 1996), PIL1 (Salter et al., 2003), and HFR1/SICS1 (Sessa et al., 2005) between High R/FR<sub>High PAR</sub> and High R/FR<sub>Low PAR</sub>, thus demonstrating that these genes are specifically regulated at the transcriptional level by light quality changes under our simulated shade environment (Ciolli et al., 2013).

The wild type exposed to High R/FR<sub>Low PAR</sub> displays precursor mesophyll cells larger than those of seedlings grown in High R/FR<sub>High PAR</sub> (Supplementary Fig. S3). To investigate whether ATHB2 has any role in this process, the leaf cell phenotype of athb2-3, hfr1-4/sics1-1, and athb2-3 hfr1-4/sics1-1 was analysed. No significant difference was observed between mutants and the wild type in High R/FR<sub>Low PAR</sub>, thus indicating that low PAR affects leaf development through a pathway independent of ATHB2 (Supplementary Fig. S3).

**Discussion**

**Effects of shade on leaf morphogenesis**

Leaf growth is driven by two tightly controlled processes, cell proliferation and subsequent cell expansion which determine leaf size and shape (Gonzales et al., 2012). These processes are dynamically regulated by environmental cues (Carabelli et al., 2007; Skirycz and Inzé, 2010; Skirycz et al., 2011; Kalve et al., 2014). A number of studies have indicated that low light intensity reduces the leaf area in several plant species (Wilson, 1966; Dengler, 1980; Yano and Terashima, 2004; Kim et al., 2005; Cookson and Granier, 2006). It has been suggested that a reduction in cell number, not in cell size, contributes to the reduced leaf size of plants grown under a simulated shade light regime consisting of low R/FR and low photosynthetic light radiation (Low R/FR<sub>Low PAR</sub>) (Carabelli et al., 2007). Moreover, evidence has been provided that Low R/FR<sub>Low PAR</sub> rapidly and transiently reduces the frequency of cell division in young leaf primordia (<0.008 mm<sup>2</sup>) through a non-cell-autonomous mechanism (Carabelli et al., 2007, 2008; Ruzza et al., 2014). Here we show that persistence of Low R/FR<sub>Low PAR</sub> later during leaf development (>0.25 mm<sup>2</sup>), determines early exit from proliferation, which in turn results in smaller leaves at the end of vegetative development. By combining adaxial subepidermal cell size determination and the expression pattern of the CYCB1;1:GUS marker in the first and second leaves of plants grown in High R/FR<sub>High PAR</sub> and Low R/FR<sub>Low PAR</sub>, we indeed gained evidence that prolonged exposure to simulated shade provokes early termination of cell cycling of mesophyll precursors (Figs 1, 2, Supplementary Table S2). Furthermore, taking advantage of the ATHB8::GUS marker (Baima et al., 1995), we demonstrated that the early differentiation of leaf mesophyll cells in plants grown in Low R/FR<sub>Low PAR</sub> relative to those in High R/FR<sub>High PAR</sub> correlates with a precocious termination of vein formation (Fig. 1). This finding is consistent with the evidence that Arabidopsis vein pattern is not inherently determinate, but arises through reiterated initiation of new pre-procambial branches until this process becomes terminate by the differentiation of mesophyll (Scarpella et al., 2004).

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**Discussion**

**Effects of shade on leaf morphogenesis**

Leaf growth is driven by two tightly controlled processes, cell proliferation and subsequent cell expansion which determine
Interestingly, Skirycz and co-authors (2011) in addition found that when the osmotic stress persists, cells exit the mitotic cell cycle and initiate the differentiation process (stop) (Skirycz et al., 2011). These data together with those observed under simulated shade (Carabelli et al., 2007; this work) lead to speculation that ‘pause-and-stop’ may be a general mechanism by which plants respond to environmental conditions suboptimal for growth.

Role of HD-ZIP II transcription factors in the morphogenesis of shade leaves

In the attempt to identify the regulatory gene(s) involved in the early exit of leaf adaxial subepidermal cells from proliferation in plants grown under simulated shade, we searched for genes up-regulated in hfr1/sics1 loss-of-function mutant seedlings in Low R/FR\textsubscript{Low PAR}, which, in accordance with HFR1/SICS1 acting as a negative master regulator of the shade avoidance response (Sessa et al., 2005; Hornitschek et al., 2009), display an exaggerated leaf cell phenotype. Among them is ATHB2 (Sessa et al., 2005; Ruzza et al., 2014), a HD-Zip II transcription factor gene, which has been suggested to play a role, together with the HD-Zip II genes HAT1 and HAT2, in the control of cell proliferation during leaf development in a sun-simulated environment (Ciarbelli et al., 2008). It has indeed been observed that the mean area of leaf adaxial subepidermal cells of plants overexpressing ATHB2, HAT1, or HAT2 was significantly larger than that of the wild type in High R/FR\textsubscript{High PAR}. In contrast, overexpression of a dominant negative derivative of ATHB2 (ATHB2\textsubscript{N51A}), thought to sequester the endogenous ATHB2 protein and, probably, related HD-Zip II proteins in functionally inactive heterodimeric complexes, resulted in a decrease of the mean area of leaf adaxial subepidermal cells relative to the control (Ciarbelli et al., 2008). Remarkably, by combining adaxial subepidermal cell size determination and cyclin index calculation in the first and second leaves of plants grown in High R/FR\textsubscript{High PAR} and Low R/FR\textsubscript{Low PAR}, here we show that loss of function of ATHB2 delays exit from proliferation relative to the wild type under simulated shade, whereas gain of function of ATHB2 causes early termination of cell cycling of mesophyll precursors under both light regimes, thus implying a major role for ATHB2 in leaf development during shade avoidance (Figs 3, 5). Consistent with this, at the end of vegetative development, fully expanded first leaves were significantly larger in the athb2 loss-of function mutant than in the wild type in High R/FR\textsubscript{Low PAR} and Low R/FR\textsubscript{Low PAR}, whereas no significant difference was observed in mesophyll cell area between wild-type and mutant leaves in High R/FR\textsubscript{High PAR} and Low R/FR\textsubscript{Low PAR}. This is particularly significant considering the potential to recover the decreased cell number through the activity of meristemoids (Skirycz et al., 2011). Among the genes up-regulated in hfr1/sics1 loss-of-function mutants is also ATHB4, a gene encoding a HD-Zip II protein closely related to ATHB2 (Fig. 6). Interestingly, athb4 loss-of-function mutants in Low R/FR\textsubscript{Low PAR} display the same leaf cell phenotype observed in plants lacking ATHB2 grown under simulated shade (Fig. 3). In contrast, leaves of plants overexpressing ATHB4 display early exit from proliferation in...
both High R/FR<sub>High PAR</sub> and Low R/FR<sub>Low PAR</sub> with respect to the wild type (Fig. 3). Together, the data indicate that ATHB4 also contributes to leaf development during shade avoidance.

Intriguingly, athb2 hfr1/sics1 and athb4 hfr1/sics1 loss-of-function double mutants display a leaf cell phenotype in Low R/FR<sub>Low PAR</sub> analogous to that of athb2 and athb4 single mutants grown under simulated shade, respectively (Fig. 3). This further indicates a major role for ATHB2 and ATHB4 in leaf development during shade avoidance. Moreover, considering that ATHB2 and ATHB4 are up-regulated in athb4 hfr1/sics1 and athb2 hfr1/sics1 double mutants upon exposure to Low R/FR<sub>Low PAR</sub> relative to the wild type (Supplementary Fig. S2), respectively, the leaf cell phenotype of the double mutants under simulated shade indicates that the exaggerated response of hfr1/sics1 leaves is lost in the absence of either ATHB2 or ATHB4, thus suggesting that the two proteins work in concert. Consistent with this, the leaf cell phenotype of the athb2-3 athb4-1 double loss-of-function mutant grown in Low R/FR<sub>Low PAR</sub> resembles that of the single athb2-3 and athb4-1 mutants (Fig. 3). Furthermore, remarkably, the phenotype of the athb2 gain-of-function mutant is lost in plants lacking ATHB4 (Fig. 3), thus implying that the early termination of cell cycling of mesophyll precursors under both High R/FR<sub>High PAR</sub> and Low R/FR<sub>Low PAR</sub> caused by the athb2-2 mutation depends on the presence of ATHB4. These data further indicate that ATHB2 and ATHB4 proteins function as a complex in the regulation of leaf development during shade avoidance. They probably form heterodimers as suggested by yeast two-hybrid assays (Trigg et al., 2017).

A number of leaf growth regulators have been identified, and connections between these regulators have started to emerge (Gonzales et al., 2012; Bar and Ori, 2014); some of these factors are involved in the regulation of leaf polarity and cell proliferation (Husbands et al., 2015). Intriguingly, members of the HD-Zip II family, including ATHB2 and ATHB4, have been shown to regulate leaf polarity as well as shade avoidance response (Steindler et al., 1999; Sorin et al., 2009; Bou-Torrent et al., 2012; Turchi et al., 2013, 2015; Paz et al., 2016). Understanding how ATHB2 and ATHB4 interact with known regulators affecting leaf proliferation will be essential to unravel the mechanisms underlying leaf development under canopy shade.

Supplementary data
Supplementary data are available at JXB online.

Fig. S1. Kinetics of first and second leaf growth in High R/FR<sub>High PAR</sub> and Low R/FR<sub>Low PAR</sub>.

Fig. S2. ATHB2 and ATHB4 genes are up-regulated respectively in athb4-1 hfr1-4/sics1-1 and athb2-3 hfr1-4/sics1-1 double mutants in Low R/FR<sub>Low PAR</sub>.

Fig. S3. Leaf phenotype of Col-0, athb2-3, hfr1-4/sics1-1, and athb2-3 hfr1-4/sics1-1 seedlings in High R/FR<sub>High PAR</sub> and High R/FR<sub>Low PAR</sub>

Table S1. Primers and UPL probes for RT-qPCR analyses.

Table S2. Leaf adaxial subepidermal cells are larger in Low R/FR<sub>Low PAR</sub> than in High R/FR<sub>High</sub>.

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