A Gas-and-Brake Mechanism of bHLH Proteins Modulates Shade Avoidance\(^1\)[OPEN]\(^2\)

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Plants detect proximity of competitors through reduction in the ratio between red and far-red light that triggers the shade avoidance syndrome, inducing responses such as accelerated shoot elongation and early flowering. Shade avoidance is regulated by PHYTOCHROME INTERACTING FACTORS, a group of basic helix-loop-helix (bHLH) transcription factors. Another (b) HLH protein, KIDARI (KDR), which is non-DNA-binding, was identified in de-etiolation studies and proposed to interact with LONG HYPOCOTYL IN FAR-RED1 (HFR1), a (b)HLH protein that inhibits shade avoidance. Here, we established roles of KDR in regulating shade avoidance in Arabidopsis (Arabidopsis thaliana) and investigated how KDR regulates the shade avoidance network. We showed that KDR is a positive regulator of shade avoidance and interacts with several negative growth regulators. We identified KDR interactors using a combination of yeast two-hybrid screening and dedicated confirmations with bimolecular fluorescence complementation. We demonstrated that KDR is translocated primarily to the nucleus when coexpressed with these interactors. A genetic approach confirmed that several of these interactions play a functional role in shade avoidance; however, we propose that KDR does not interact with HFR1 to regulate shade avoidance. Based on these observations, we propose that shade avoidance is regulated by a three-layered gas-and-brake mechanism of bHLH protein interactions, adding a layer of complexity to what was previously known.

Plants harvest light energy during photosynthesis, especially blue (\(\sim 400–500\) nm wavelengths) and red (\(\sim 600–700\) nm wavelengths) light, while mostly reflecting far-red light (\(\sim 700–800\) nm wavelengths). As a consequence, the ratio of R to FR is reduced by light reflected or transmitted through plant leaves and neighbors use this to detect the presence of nearby plants. Shade intolerant plants, such as Arabidopsis (Arabidopsis thaliana), respond to this lowered R:FR ratio with the shade avoidance syndrome (SAS). The main shade avoidance characteristics in Arabidopsis are hypocotyl, internode, and petiole elongation; early flowering; and upward leaf movement called hyponasty (Ballaré et al., 1991; de Wit et al., 2015, 2016; Pantazopoulou et al., 2017; Galvão et al., 2019). SAS is typical of most plants, including crops, and although it improves individual plant fitness, it may compromise total crop yield (Robson et al., 1996; Boccalandro et al., 2003). By contrast, shade-tolerant species, such as those from forest understories, have developed alternative strategies to cope with shade conditions without investing in shade avoidance growth (Gommers et al., 2013, 2017; Molina-Contreras et al., 2019).

In an attempt to unravel the strategy of SAS suppression in some species, Gommers et al. (2017) previously described the contrasting shade-tolerant and -intolerant responses of two selected Geranium species when exposed to low R:FR. In a transcriptome approach between these species, putative regulators of these two different responses were identified. One of these regulators is a basic helix-loop-helix (bHLH) protein-encoding gene, called KIDARI (KDR)/PACLOBUTRAZOL RESISTANCE6 (PRE6; Gommers et al., 2017). The expression of KDR in Arabidopsis has been shown to rely on functional PHYTOCHROME-INTERACTING FACTOR4 (PIF4), PIF5, and PIF7 in both white light and low R:FR conditions (Gommers et al., 2017); however, the precise role of KDR in shade avoidance responses is poorly understood.

The main mechanism by which KDR has been previously shown to regulate growth in dark versus
monochromatic light is by acting as a cofactor. KDR does not directly regulate gene transcription because it lacks the capability to bind DNA, but it is able to interfere with the action of other proteins. KDR cannot bind DNA directly because it misses specific amino acids (Glu and Arg) in the basic domain, which are essential for DNA binding (Hyun and Lee, 2006). Its function is mainly determined by the HLH domain, and through this domain, KDR was proposed to interact with LONG HYPOCOTYL IN FAR-RED1 (HFR1; Hyun and Lee, 2006; Hong et al., 2013), another non-DNA-binding (b)HLH protein. HFR1 is an established regulator of shade avoidance and binds to PIF proteins (Hornitschek et al., 2009), preventing them from activating the transcription of genes associated with SAS. HFR1 and PIF4 are both members of the bHLH transcription factor (TF) family, but whereas PIF4 promotes SAS through the transcriptional activation of specific genes, HFR1 plays a negative role in SAS by suppressing PIF4 action through direct binding (Sessa et al., 2005; Hornitschek et al., 2009). It is proposed that the regulation of both positive and negative regulators upon shade exposure helps plants tune the intensity of their shade avoidance responses (Sessa et al., 2005; de Wit et al., 2016; Gommers et al., 2017). PIF4 and other members of the same subfamily, such as PIF1, PIF3, PIF5, and PIF7, are not transcriptionally upregulated in shade conditions, but their proteins are stabilized (Alsady et al., 2006; Shen et al., 2007; Leivar et al., 2008; Li et al., 2012; Lorrain and Fankhauser, 2012). In standard light, phytochromes are active and their interaction with PIFs leads to PIF inactivation and often degradation, whereas phytochromes are inactivated in shade, relieving the repression of PIF activity (Chen and Chory, 2011; Leivar and Quail, 2011). PIF proteins act as positive regulators of SAS primarily by promoting auxin synthesis, transport, and response (Hornitschek et al., 2012; Li et al., 2012). Simultaneously, PIFs also promote the expression of several negative regulators, such as HFR1, PHYTOCHROME RAPIDLY REGULATED1 (PAR1), and PAR2 (Sessa et al., 2005; Roig-Villanova et al., 2006), all non-DNA-binding (b)HLH proteins. Thus, there is a high redundancy as well as specification within the bHLH family in the regulation of SAS, allowing a highly flexible response that can integrate different environmental parameters.

In this study, we established the role of KDR in shade avoidance. Using established and novel Arabidopsis kdr mutant and overexpression lines, we demonstrated that KDR is a positive regulator of low R:FR-induced hypocotyl elongation. We showed that KDR overexpression, in addition to promoting hypocotyl elongation, also stimulates primary root elongation, bolting, and flowering. Using yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) approaches, we identified several interactors of KDR and showed that they colocalize with KDR in the nucleus. Experiments on mutants and transgenics to modulate the expression levels of these putative interactors, combined with published knowledge about these genes, suggest that all KDR-interacting proteins found here are themselves negative regulators of low R:FR-induced hypocotyl elongation. We propose that KDR interaction with these growth suppressors disables them from interacting with their downstream targets, alleviating the restraint on shade avoidance.

RESULTS
KDR Promotes Shade Avoidance Response

We confirmed the upregulation of KDR in seedlings of Arabidopsis exposed to a low R:FR treatment (R:FR = 0.2) in comparison to control conditions (R:FR = 2; Fig. 1A). To further investigate the role of KDR in SAS, we studied the response of kdr lines to low R:FR conditions. We measured the elongation of hypocotyls in seedlings and of petioles in rosette plants upon exposure to low R:FR. Hypocotyl elongation of a KDR knockout line kdr-1 was reduced in low R:FR conditions, whereas the activation-tagged line kdr-D, which results in four times the insertion of the CaMV 35S enhancer sequence in the promoter region of KDR, displayed an exaggerated elongation (Fig. 1B). When the same lines were tested for petiole elongation in rosette plants, a similar suppression of the response was found for kdr-1, but less severely. By contrast, the overexpressing line kdr-D showed no statistically significant difference compared to Col-0 wild type regarding low R:FR-induced petiole elongation. The petiole elongation responses between wild type and mutants were not dependent on day length (Fig. 1C).

Overexpression of KDR Stimulates Shade Avoidance

We created novel lines overexpressing KDR in Arabidopsis Col-0 background in order to have improved genetic material over the kdr-D activation tagging line that only mildly overexpresses KDR. We then used four homozygous independent lines to study their response to low R:FR treatment at the seedling stage. We found that most of the novel 35S:KDR transgenic lines showed an even more exaggerated response than that found for kdr-D and additionally displayed constitutive hypocotyl elongation in white light (Fig. 2A). Interestingly, the variation in hypocotyl length between the independent transgenic lines correlated with variation in KDR overexpression levels (Fig. 2, B and C). We selected the two independent lines with the highest KDR expression levels for further analyses. When looking more carefully at the phenotype of the selected lines, we observed that KDR overexpression increased elongation of most organs, including hypocotyl, petioles of cotyledons, petioles of true leaves, and primary root (Fig. 2D).

We also verified if petiole elongation in adult plants was affected in our strong overexpression lines. Interestingly, they did not show an increased petiole
leading to a more bent stems, which at a later life stage started to split open.KDR The lines accelerates the transition to reproduction early so also possible that the very high shade avoidance response (Halliday et al., 1994). It is

Supplemental Fig. S2A), which is another established flowering (Fig. 3; Supplemental Fig. S2A), which is another established shade avoidance response (Halliday et al., 1994). It is also possible that the very high KDR expression in these lines accelerates the transition to reproduction early so that fewer resources are invested in vegetative growth. The KDR overexpressors had very long flowering stems, which at a later life stage started to split open leading to a more bent flowering architecture, possibly another side effect of the ectopic expression of KDR (Supplemental Fig. S2B).

Overexpression of KDR Affects Regulation of PIF Targets

The main function of KDR described in the literature is its interaction with the negative growth regulator HFR1, which binds to PIFs and therefore interferes with the transcriptional activation of their target genes responsible for the induction of shade avoidance responses. We therefore verified if some of the well-known PIF targets were transcriptionally regulated in seedling of Arabidopsis overexpressing KDR in control white light conditions in comparison to Col-0. Interestingly, some of the PIF targets were significantly upregulated in the two independent overexpression lines, such as HFR1, PHYTOCHROME INTERACTING FACTOR 3-LIKE1 (PIL1), and XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 17 (XTH17), whereas several other targets, especially the auxin-related genes YUCCA8 (YUC8), INDOLE-3-ACETIC ACID INDUCIBLE29 (IAA29), and IAA19, were downregulated in the KDR overexpression lines compared to Col-0 (Supplemental Fig. S3). It was recently confirmed that KDR could be involved in auxin responses but in a rather contradictory way. Zheng et al. (2017) showed that in protoplasts of cells overexpressing KDR, the expression of the auxin response reporter DR5::GUS was reduced, whereas it was increased in protoplasts derived from the KDR knockout line. Since KDR was identified as an atypical TF, the repression of auxin response caused by KDR overexpression does not likely result from direct regulation of the expression of auxin responsive genes, but more likely from KDR interference with true TFs involved in a highly complex network of interactions around auxin response. This could be a partially PIF-independent network, since PIF activity would be increased, rather than inhibited, by KDR, and increased PIF activity would promote rather than suppress DR5::GUS expression (Sun et al., 2012).

Interactors of KDR from Y2H Screens

We performed a Y2H screen where the coding sequence (CDS) of KDR was cloned in frame with the GAL4 DNA-binding domain of the bait vector and used to screen a prey cDNA library of Arabidopsis cloned
with the GAL4 activation domain. The identity of the found interactors are displayed in Table 1, including the frequency with which the interactors were found and the strength of their interaction. Among the proteins identified, we focused on PAR1 and PAR2, two known PIF-interacting proteins. We confirmed the interactions by cloning the full-length CDSs of these proteins (rather than the truncated versions from the library) from Arabidopsis cDNA into the prey vector and using this to retransform yeast to perform a protein-protein interaction assay. In this direct Y2H assay, we also tested the previously published interaction of KDR with HFR1 (Hyun and Lee, 2006; Hong et al., 2013) but could not confirm this interaction (Fig. 4). Also, when swapping the bait-prey configuration, no interaction was found between HFR1 and KDR (Supplemental Fig. S4A). Finally, changing vectors to those used in Hyun and Lee (2006) and Hong et al. (2013; pGBK7 for the bait and pGADT7 for the prey) again did not confirm the interaction (Supplemental Fig. S4B). To rule out a putatively poor expression of HFR1 in yeast, we verified AD-HFR1 expression in an immunoblot experiment, and this was found to be comparable to AD-PAR1 (Supplemental Fig. S4C). Consistent with proper expression, we also found clear HFR1 homodimerization (Supplemental Fig. S4A), as previously published in Fairchild et al. (2000). As another positive control for the Y2H assays on HFR1, we did confirm interactions of HFR1 with PIF4 and PIF5 (previously published in Hornitschek et al., 2009) and found that HFR1 can also interact with PIF7 (Fig. 5A). We also found that KDR does not directly interact with PIFs in yeast, whereas HFR1 and PAR1 do (Fig. 5A). Lastly, we confirmed that PIF7 can interact with itself and other PIFs (Fig. 5B), which is consistent with the notion that PIFs form hetero- and homodimers to bind DNA regions and activate the expression of target genes (Leivar et al., 2008; Bu et al., 2011).

In order to maximize the number of relevant KDR interactors found, we performed a second Y2H screen using a completely different library consisting of only TFs of Arabidopsis cloned in full-length sequence (Pruneda-Paz et al., 2014). Ten putative interactors were discovered and their identity was verified by sequencing (Table 2). We narrowed the selection for
further studies to four (b)HLH candidates, ACTIVATION-TAGGED BR1 SUPPRESSOR1 (ATBS1)-INTERACTING FACTOR 2 (AIF2), AIF4, ILI1 BINDING BHLH 1 (IBH1), and IBH1-LIKE 1 (IBL1), since these proteins had previously been linked to growth regulation in association with some regulators of the SAS but had not been implemented in shade avoidance control before. The strength of interaction was verified by performing a Y2H direct interaction assay. All four candidates were able to grow at least up to the medium lacking His and supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT), meaning that the interactions were rather strong in yeast (Fig. 4).

KDR Is Localized Mainly in the Nucleus when Coexpressed with Strong Interactors

Next, we investigated the subcellular localization of KDR and its interactors identified with the Y2H screen. KDR was fused in frame to the N-terminal part of a cyan fluorescent protein (CFP), whereas the interactors were fused to the yellow fluorescent protein (YFP). Transient expression in Nicotiana benthamiana leaves was carried out and revealed that KDR was localized in both the cytoplasm and the nucleus, as previously published by Hong et al. (2013; Fig. 5C). However, the localization of the interactors appeared exclusively in subcellular compartments of the nucleus. Specifically, HFR1, PAR1, and PAR2 were detected in the nucleoplasm, whereas AIF2, AIF4, IBH1, and IBL1 appeared to be localized in the nucleus but with a very pronounced signal in the nucleolus (Fig. 5C). When KDR was transiently coexpressed with the putative strong interactors, KDR localized exclusively to the nucleus (Fig. 6), indicating that the interaction draws KDR to the nucleus. When coexpressing KDR with HFR1, there was still substantial KDR abundance in the cytoplasm (Supplemental Fig. S4, D and E), similar to when KDR was transiently expressed alone or coexpressed with free YFP, consistent with the lack of interaction described above.

In Planta BiFC Experiments Confirm Interactions in Nuclear Compartments

To further verify the interactions of KDR identified with the Y2H assays, we examined whether they were

| AT Code     | Gene Name | Frequency | Strength of Interaction |
|-------------|-----------|-----------|------------------------|
| AT3G58850   | PAR2      | 40        | Weak 1 –  –  –  38      |
| AT2G42870   | PAR1      | 13        | Mild 1 –  1  12         |
| AT3G11100   | VFP3      | 19        | Medium 19  2  2  –     |
| AT2G32900   | MIP1      | 4         | Strong –  2 –  –      |
| AT5G57760   | MRS2-1    | 2         | –  –  –  –           |
| AT1G16010   | –         | 1         | –  –  –  –           |
also occurring in planta. We performed a BiFC assay where the two parts of the split Venus fluorescent protein (YN or YC) were C or N terminally tagged to the proteins of interest and coexpressed in N. benthamiana leaves (Fig. 7). We detected the reconstituted YFP signal in the nucleus in all the different samples, apart from the interaction with HFR1, and some differences were noticed when KDR was found to interact with the different candidates. The reconstituted YFP signal was observed in different nuclear compartments, resembling the localization of the targets alone (Fig. 7). The interactions between KDR and PAR1 and PAR2 were observed in the nucleoplasm, whereas the interactions with AIF2, AIF4, IBH1, and IBL1 were found in the nucleus with the strongest signal in the nucleolus. Together, the Y2H and the BiFC data indicate that KDR can truly interact with all the identified targets and that these interactions seem to trigger its translocation primarily to subnuclear complexes, whereas no interaction with HFR1 could be confirmed. No signal was detected when a mutated version of PAR1, namely PAR1L66E, in which Leu-66 is mutated to Glu disabling protein-protein interaction (Galstyan et al., 2011), was used in combination with the proteins of interest.

Functional Involvement of KDR Interactors in Shade Avoidance

HFR1, PAR1, and PAR2 were already associated with shade responses and identified as negative regulators of SAS. Somewhat analogous to KDR, they are transcriptional cofactors which means they regulate transcription without physically binding DNA but by interacting with other proteins through the HLH domain (Roig-Villanova et al., 2007; Galstyan et al., 2012; Hornitschek et al., 2012). Also, AIF2, AIF4, IBH1, and IBL1 were described as non-DNA-binding (b)HLH proteins (Wang et al., 2009; Ikeda et al., 2012; Zhiponova et al., 2014); however, whereas their role is mainly related to elongation growth, nothing is known so far about shade avoidance in mutants for these genes. We first confirmed that in our conditions HFR1, PAR1, and PAR2 were also upregulated following a low R:FR treatment in seedlings of Col-0 (Fig. 8A). Since AIF2, AIF4, IBH1, and IBL1 were never associated with shade responses, we also verified if their expression level was differentially regulated upon exposure to low R:FR and we found that this was indeed the case (Fig. 8A).

Next, we studied the response to low R:FR conditions of different mutant and overexpression lines of these bHLH genes relative to Col-0 wild type (Fig. 8, B–D). Figure 8, B and C, show that low R:FR-induced hypocotyl elongation is increased in the hfr1-201, hfr1-5, and PAR RNAi line 9 mutants. Overexpressing any of these genes was sufficient to severely block the response to low R:FR. These phenotypes are entirely consistent with the roles of these proteins as negative SAS regulators. Instead, for the lesser-known genes AIF2, AIF4, IBH1, and IBL1, we studied the available T-DNA insertion lines, but unfortunately in the SALK lines for IBH1, we could not detect the T-DNA insertion and were therefore discarded. In low R:FR conditions, all the lines except for aif2-2 showed a moderately enhanced hypocotyl elongation response compared to Col-0 wild type (Fig. 8D). In the case of ibl1, a statistically significant difference, although minimal, was already seen in control conditions when compared to the wild type. The relatively mild phenotypes, albeit reproducible and statistically significant, may hint at genetic redundancy between the different KDR targets.
Higher order combinations of these mutants, as well as overexpression lines for these genes, would likely help understand the impact of these shade avoidance components in more detail.

Genetic Interaction between KDR and Downstream Shade Avoidance Regulators

We hypothesized that KDR would act to sequester negative regulators, such as PAR2, by direct interaction. We verified this hypothesis by crossing a PAR2 overexpression line with a KDR overexpression line. The high KDR abundance was expected to sequester PAR2 and restore elongation growth in the PAR2 over-expressor (Fig. 9). Indeed, we showed that KDR overexpression fully rescues hypocotyl elongation and response to low R:FR in the PAR2 overexpression line. Interestingly, KDR overexpression could not rescue low R:FR-induced hypocotyl elongation in an HFR1 overexpression line, but it could induce elongation in control conditions, similar to observations by Hong et al. (2013), where a combined KDR × HFR1 double overexpression line was exposed to monochromatic B light. Since PAR2 and HFR1 overexpression alone did not significantly affect hypocotyl lengths in control light, and the KDR × PAR2 and KDR × HFR1 overexpression lines were as elongated in control light as the KDR

Figure 5. Y2H protein–protein interaction studies and subcellular localization of KDR and its targets in planta. A, In the GAL4 Y2H assay, the GAL4 DNA-binding domain (BD) fused to KDR (left), HFR1 (middle), and PAR1 (right) was coexpressed with the GAL4-activation domain (AD) fused to PIF4, PIF5, and PIF7. The mating of the yeast was confirmed through the growth on nonselective medium (–LT). The assay showed that whereas the bait KDR does not interact with the preys PIF4, PIF5, and PIF7, the bait HFR1 does interact with PIF4, PIF5, and PIF7. PAR1 interacts most strongly with PIF4 and PIF5. B, PIF7 can interact with other PIFs in yeast. When used as bait, PIF7 interacts with the prey HFR1 but not with PAR1 and PAR2. C, KDR fused to CFP and the interactors HFR1, PAR1, PAR2, AIF2, AIF4, IBH1, and IBL1 fused to YFP were transiently expressed in epidermal leaf cells of N. benthamiana using A. tumefaciens. Images were taken 2 d after agroinfiltration. Scale bar = 20 μm. L, Leu; T, Trp; H, His; A, adenine.
overexpression line alone, we conclude that in control white light, PAR2 and HFR1 do not control hypocotyl length, nor does a putative interaction with KDR. It is possible that in control white light, KDR interactions with other proteins, such as AIF2, AIF4, IBL1, and IBH1 (Figs. 4 and 7) is causal to the elongated phenotype, whereas interaction with PAR2 plays a more prominent role under low R:FR light conditions. The observed phenotypes of the different overexpressor combinations are consistent with the observations that KDR interacts with PAR2, and with our observation that KDR does not seem to show interaction with HFR1 to functionally control plant development in low R:FR light conditions. Comparable results were also found when PAR2 and HFR1 overexpressor lines were crossed with the mild overexpression line kdr-D. Also, here the HFR1 overexpressor phenotype could not be rescued, whereas the elongation of the hypocotyl of PAR2 overexpressor was comparable to that of kdr-D. A closer look at this cross shows that the appearance of the cotyledons and their “petioles” are similar to those exhibited by 35S:PAR2 overexpression line (Supplemental Fig. S5).

Finally, we also generated transgenic lines overexpressing KDR in pif7, pif4 pif5, and pif4 pif5 pif7 backgrounds by floral dipping these mutants with a 35S:KDR construct using Agrobacterium-mediated transformation. We then studied their response when exposed to low R:FR using three independent lines for each background, after we verified their expression level (Fig. 10; Supplemental Fig. S6). As expected, pif4 pif5 shows a clear but reduced response to low R:FR, whereas pif7 and pif4 pif5 pif7 lost the hypocotyl response to low R:FR completely. Interestingly, in control light conditions, the overexpression of KDR is able to induce a strong elongation in pif7 and pif4 pif5 backgrounds, and more mildly when overexpressed in the triple knockout pif4 pif5 pif7. When these lines were exposed to low R:FR, pif4 pif5 35S:KDR had nearly the same hypocotyl phenotype as the same construct has in wild-type background, consistent with the relatively modest role of pif4 pif5 in low R:FR-induced hypocotyl elongation. By contrast, in the pif4 pif5 pif7 mutant, KDR overexpression could not rescue the hypocotyl elongation response to low R:FR, whereas it only slightly rescued the response in pif7.

We conclude that KDR interacts with PARs to regulate hypocotyl elongation in response to low R:FR, and this subsequently depends on PIFs, probably because PARs directly interact with PIFs to regulate their activity.

**DISCUSSION**

Significant discoveries have been made in the past decades to identify the molecular mechanisms through which plants perceive neighbors through light signals and activate the shade avoidance network. A previous transcriptome analysis on two wild Geranium species, one shade tolerant and the other shade avoiding, identified KDR as a molecular component whose expression was correlated with the ability to display elongation responses to shade (Gommers et al., 2017). Here, we attempted to unravel the role of KDR in SAS and found that overexpression of KDR in Arabidopsis resulted in an enhanced response to simulated shade conditions. Therefore, it was proposed that the interaction of KDR with HFR1 would release PIFs so that they could activate shade avoidance. If this would have been the only mode of action of KDR, then lines overexpressing KDR should have a similar phenotype to knockout lines of hfr1, which is not the case. Moreover, independent studies on nonmodel plants identified KDR orthologs as strong candidate regulators of contrasting elongation responses, and in these species no HFR1 orthologs could be found (van Veen et al., 2013; Gommers et al., 2017). Therefore, we speculated that other interacting partners might exist.

**KDR Is a Regulator of Established Shade Avoidance Components**

Results from one of the Y2H screens and further in planta confirmations identified PAR1 and its closest homolog PAR2 as interactors of KDR, proteins that were previously associated with SAS (Roig-Villanova et al., 2007; Bou-Torrent et al., 2008; Galstyan et al., 2011, 2012; Hao et al., 2012; Cifuentes-Esquível et al., 2013; Zhou et al., 2014). However, their interaction with KDR was not previously anticipated, and this sheds

| AT Code  | Type  | Gene Name | Involved In                                      |
|---------|-------|-----------|-------------------------------------------------|
| AT4G02590 | bHLH  | UNE12     | Fertilization                                   |
| AT2G31210 | bHLH  | bHLH091   | Developing Arabidopsis anther                   |
| AT2G28160 | bHLH  | FIT       | Regulation of iron uptake responses             |
| AT1G66425 | bHLH  | LHL3      | Regulation of early xylem development downstream of auxin |
| AT2G31280 | bHLH155-like protein | LHL2 | Interaction with Agrobacterium virulence protein VirF |
| AT3G11100 | trihelix | VFP3     | Interaction with agrobacterium virulence protein VirF |
| AT3G06590 | bHLH  | AIF2      | Negative regulation of cell elongation          |
| AT1G09250 | bHLH  | AIF4      |                                                   |
| AT2G43060 | bHLH  | IBH1      | Repressing the expression of PIF4               |
| AT4G30410 | bHLH  | IBL1      |                                                   |
new light on shade avoidance control. PAR1 and PAR2, as well as KDR and HFR1, are atypical non-DNA-binding (b)HLHs. HFR1 regulates shade avoidance by interacting with several PIFs (PIF1, PIF3, PIF4, and PIF5). This yields nonfunctional complexes unable to bind DNA and therefore blocks the activation of their targets (Hornitschek et al., 2009; Zhang et al., 2013). We show here that HFR1 can also interact with PIF7 (Fig. 5, A and B). Inactivation of PIF4 also occurs via interaction with PAR1 and PAR2, adding an extra level of regulation of cell elongation and plant development. Furthermore, PAR1 can also interact with BES1-INTERACTING-MYC-LIKE1 (BIM1) and with the BRASSINOSTEROID-ENHANCED EXPRESSION1 (BEE1), BEE2, and BEE3, which are positive regulators of SAS, by forming nonfunctional complexes also in this case (Cifuentes-Esquivel et al., 2013). Finally, overexpression of PRE1, another (b)HLH member of the same subgroup as KDR, can suppress the dwarf phenotype of PAR1 overexpression (Hao et al., 2012). In a comparable way, we found that overexpression of KDR can restore the growth defect of PAR2 overexpression (Fig. 9). This finding places KDR in a new third level of SAS regulation, above PAR1 and PAR2, which suppress PIF activity (Fig. 11). Our results cannot confirm the previously described suppressing role of KDR on HFR1, and our genetic data indicate that a putative interaction between HFR1 and KDR is unlikely involved in the regulation of low R:FR-induced hypocotyl elongation.

KDR Interacts with Several Negative Growth Regulators

In this study, we show that KDR physically interacts in yeast and in planta with a range of negative regulators of cell elongation, i.e. AIF2, AIF4, IBH1, and IBL1. None of these proteins have been previously associated with SAS, but they all share some similarities. For example, each of them has been identified already for their interaction with some of the PRE members (Wang et al., 2009; Zhang et al., 2009; Ikeda et al., 2013); they are atypical (b)HLH proteins, unable to bind DNA (Ikeda et al., 2012, 2013); and their overexpression results in a dwarf phenotype (Zhang et al., 2009; Ikeda et al., 2013). Nevertheless, the functions of these proteins remain poorly understood. They have been found to interact with growth-promoting TFs, possibly to suppress the elongation growth (Bai et al., 2012; Ikeda et al., 2012), similar to the mode of action of PARs and HFR1. Furthermore, the dwarf phenotype of IBL1 overexpression is restored when crossed with an overexpressing line of a PRE member (Zhang et al., 2009; Ikeda et al., 2013). Nevertheless, the functions of these proteins remain poorly understood. They have been found to interact with growth-promoting TFs, possibly to suppress the elongation growth (Bai et al., 2012; Ikeda et al., 2012), similar to the mode of action of PARs and HFR1. Furthermore, the dwarf phenotype of IBL1 overexpression is restored when crossed with an overexpressing line of a PRE member (Zhang et al., 2009; Ikeda et al., 2013). Nevertheless, the functions of these proteins remain poorly understood. They have been found to interact with growth-promoting TFs, possibly to suppress the elongation growth (Bai et al., 2012; Ikeda et al., 2012), similar to the mode of action of PARs and HFR1. Furthermore, the dwarf phenotype of IBL1 overexpression is restored when crossed with an overexpressing line of a PRE member (Zhang et al., 2009; Ikeda et al., 2013).
Figure 7. BiFC experiments confirm the interactions found with the Y2H assay. BiFC experiments performed by *A. tumefaciens* transient transformation of *N. benthamiana* leaf epidermis. The interaction of KDR with PAR1, PAR2, AIF2, AIF4, IBH1, and IBL1 was visualized as the reconstituted YFP signal in different nucleus compartments based on the type of interaction. No interaction was found between HFR1 and KDR and the negative controls using PAR1L66E. The autofluorescence of the chloroplasts is shown in red and the BiFC signal of Venus (YFP) in green. Images were taken 2 d after agroinfiltration. Scale bars = 10 μm.
elongation. Upon binding, KDR would then sequester PARs, AIFs, IBH1, and IBL1 so they are kept from binding their bHLH TF targets. This triantagonistic network of bHLHs fine-tunes general plant development as well as adaptation to environmental changes, i.e., changes in light quality. Although under natural conditions, each of these proteins has subtle impacts on development and plasticity, overexpression of these factors leads to a severe dwarfish phenotype and impaired cell elongation, in agreement with their inhibition of positive growth regulators (Zhang et al., 2009; Ikeda et al., 2013; Zhiponova et al., 2014). By contrast, ectopic expression of KDR leads to a strongly elongated phenotype. To test if the activity of these negative growth regulators is really inhibited by interaction with KDR, lines overexpressing KDR and the negative growth regulators at the same time could test this hypothesis, similar to our data here for combined PAR2 and KDR overexpression (Fig. 9). Alternatively, combinations of loss-of-function alleles for several of the KDR-interacting factors, combined with kdr loss of function, could further establish if these interactors act redundantly or if specific functions are associated with specific interaction pairs.

The Heterodimerization of KDR Leads to Its Nuclear Translocation

The interaction of KDR with the (b)HLH cofactors shown here is also reinforced by its translocation from the cytoplasm to the nucleus when coexpressed with the interacting bHLHs identified in the Y2H (Fig. 6). Since bHLH proteins are a family of TFs, they are thought to be localized mostly in the nucleus, where they can regulate the transcription of genes. On the other hand, literature presents evidence showing that the translocation from the cytoplasm to the nucleus is an important posttranslational regulatory mechanism in response to different stimuli and to different plant developmental stages (McGonigle et al., 1996; Nayar et al., 2014; Cui et al., 2016). A possible explanation for KDR translocation is that it could have a weak nuclear localization signal (NLS), whereas its interactors could have a strong NLS and therefore are localized only in the nucleus, even in absence of KDR. Consequently, the predominantly nuclear localization of KDR would rely on partners harboring strong NLS. Indeed, this is another possible mechanism of modulating SAS. In fact, all the strong interactors identified here are upregulated
following exposure to low R:FR, suggesting that KDR could change its localization during shade to form heterodimers with the negative growth regulators, inhibiting their function and having as final output the promotion of cell elongation through the release of the other positive growth regulators.

Several Triantagonistic (b)HLH/(b)HLH/bHLH Modules Control Cell Elongation in Response to Multiple Stimuli

Taken together, we propose that the response to shade is mediated by the coaction of several modules of heterodimers formed between bHLH proteins with opposite function in the regulation of elongation
(Fig. 11). Furthermore, we conclude that KDR can regulate a substantial number of shade avoidance regulators, certainly many more than previously anticipated, and this would explain the profound impact of variations in KDR expression levels on shade avoidance. Some of them are well-known negative regulators of shade avoidance responses, i.e. PAR1 and PAR2 (Sessa et al., 2005; Roig-Villanova et al., 2006, 2007), whereas the other interactors found, i.e. AIF2, AIF4, IBH1, and IBL1, had never been associated with shade avoidance. The expression levels of each of these KDR interactors were induced upon low R:FR treatment, consistent with their involvement in the regulation of shade avoidance (Fig. 8A).

The results described here uncovered further levels of shade avoidance regulation and also indicated that HFR1 and KDR might act independently to regulate low R:FR-induced hypocotyl elongation. The gas-and-brake mechanism of different layers of (b)HLH proteins described here gives tremendous opportunity to fine-tune shade avoidance expression. This could be instrumental to enhancing low R:FR response by simultaneous B-light deprivation (de Wit et al., 2016) or suppressing it during abiotic stress (Hayes et al., 2019). Unraveling the exact roles of these bHLH interactions through different life stages of the plant under different light conditions and in other stress response pathways would enable an integrative understanding of plant shade avoidance.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Measurements

Arabidopsis (Arabidopsis thaliana) lines are all in Col-0 background. The following published lines were used: the activation-tagged line kdr-D and the knockout kdr-1 (SALK_048383C; Hyun and Lee, 2006; Gommers et al., 2017), 35S:HFR1-GFP (also called G-BH (35; Galstyan et al., 2011), 35S:PAR1-GFP, hfr1-201 (Zhou et al., 2014), 35S:PAR2, par2-1, PAR RNAi #9 (Roig-Villanova et al., 2007), hfr1-5 (Sessa et al., 2005), pif7-1 (Leivar et al., 2008), pif4-101 pif5-1 (Lorrain et al., 2008), and pif4-101 pif5-1 (de Wit et al., 2015). In addition, seeds of aif2-1 (SALK_011076C), aif2-2 (SALK_061834), aif4 (GK-428G06), and ibh1 (SALK_119457C) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK) and genotyped using the primers listed in Supplemental Table S1. 35S:HFR1-GFP x kdr-D and 35S:PAR2 x kdr-D were created by crossing the respective genotypes described above, and experiments were performed using lines homozygous for both inserts.

The plants were cold stratified for 4 d on soil/perlite mix 1:2 (Primasta) supplemented with nutrients (2.6 mM KNO3, 2 mM Ca(NO3)2, 0.6 mM KH2PO4, 0.9 mM MgSO4, 6.6 mM MnSO4, 2.8 mM ZnSO4, 0.5 mM CuSO4, 66 mM H3BO3, 0.8 mM Na2MoO4, and 134 mM Fe-EDTA; pH 5.8) and then moved to a growth chamber under short-day conditions (8 h light, 16 h dark; 20°C; 70% humidity; PAR ~140 μmol m−2 s−1) for 11 d to allow germination. The plants were then transplanted and left to grow for 3 weeks until the beginning of the experiment. In the case of long-day conditions (16 h light, 8 h dark), the plants were grown for 7 d before being transplanted and then left to grow for an extra week before the light treatment started. Petiole length of the third youngest leaf was measured with a digital caliper before and after the treatment and the difference calculated. Pictures of the same plants were taken before (t = 0) and after the treatment (t + 8 h and t + 24 h), and the Δ in petiole angle was measured with ImageJ (https://imagej.net/Fiji/Downloads) to determine the hyponastic response. For bolting and flowering experiments, a single seed per pot was sown, cold stratified for 4 d, and then moved to the growth chamber under long-day conditions (16 h light, 8 h dark) for 4 d to allow germination before the light treatment started. The number of days to bolting and to flowering was calculated starting from the first day the pots were moved to the growth chamber until the flowering bolt appeared and the first flower opened, respectively. The number of rosette leaves and the total number of leaves (rosette leaves plus cauline leaves) were also measured at the moment of bolting and flowering.

For experiments on Arabidopsis seedlings, the seeds were gas sterilized with chlorine for 2 h, sown on sterile square petri dishes (120 × 120 × 17 mm, Greiner Bio One) containing one-half strength Murashige and Skoog (Duchefa Bio-chemistry), 1 g L−1 MES hydrate (Sigma-Aldrich), 8 g L−1 plant agar (Duchefa Biochemistry) at pH 5.8, and cold stratified for 4 d. The plates were placed in the light for 2 h followed by 1 d of darkness and then back to the light for 2 d, after which the light treatment started. The plates were finally scanned, and the hypocotyls were measured with ImageJ.

Nicotiana benthamiana seeds were germinated for 7 d (16 h light, 8 h dark; 20°C; 70% humidity; PAR ~140 μmol m−2 s−1) in 9 × 9 × 9.5-cm pots containing soil-perlite mix (1:2; Primasta). The seedlings were then transplanted to 7 × 7 × 8-cm pots and grown for 4 to 5 more weeks before agroinfiltration experiments were started.

Light Treatment

For simulated shade conditions, FR LEDs (730 nm, Philips GreenPower) were used to decrease the R:FR ratio from 2 (control conditions) to 0.2 (shade conditions) without changing the PAR (140 μmol m−2 s−1, Philips HPI 400 W). The light treatment lasted 5 d when using Arabidopsis seedlings, and 24 h for adult Arabidopsis plants. The light spectra of the different light conditions are shown in Supplemental Figure S7.

RNA Extraction and RT-qPCR

Whole seedling shoots of 4-d-old seedlings growing on plates (Figs. 1A and 8A; Supplemental Fig. S3) and whole seedling shoot of 8-d-old seedlings (Fig. 2C; Supplemental Fig. S6) were used to extract RNA using the RNeasy Plant Mini Kit (Qiagen). The RNA was quantified using a NanoDrop spectrophotometer and 1 μg of RNA was used for cDNA synthesis with the Synthascript cDNA Synthesis Kit (Epitexit) according to the manufacturer’s instructions.
mini kit (Qiagen) followed by DNase I (Qiagen) treatment. cDNA synthesis was performed using SuperScript III RNase H- reverse transcriptase (Thermo Fisher Scientific) together with random primers (Invitrogen). qPCR reactions were conducted in Viia7 real-time PCR (Thermo Fisher Scientific) using the SYBR green Supermix (Bio-Rad). Two technical replicates of three or four biological samples were used to calculate the average gene expression level normalized to the housekeeping genes AT4G26410 and AT5G25760 and relative to the expression level of wild-type Col-0 control condition. The primers used for RT-qPCR are listed in Supplemental Table S2.

Gene Cloning and Plant Transformation

The cDNA used to clone KDR CDS of Arabidopsis Col-0 was synthesized from RNA derived from leaf. The CDS was amplified using the Phusion DNA polymerase (Thermo Fisher Scientific) with the atfB primers listed in Supplemental Table S3 and cloned into the Gateway vector pDONR207 (Thermo Fisher Scientific) using the Gateway BP clonase II enzyme mix (Thermo Fisher Scientific). The reaction was used to transform competent cells of Escherichia coli DH5α. Colonies growing on the selective medium containing the antibiotic gentamycin (20 μg mL⁻¹) were checked by colony PCR and the plasmid DNA extracted using the QIAprep spin miniprep kit (Qiagen). A restriction reaction was performed on the extracted plasmid DNA, and the positive samples were sequenced. The entry vector with the right sequence was recombined into the Gateway destination vector pFAST-G02 (Shimada et al., 2010) using the Gateway LR clonase II enzyme mix (Thermo Fisher Scientific). The reaction was then used to transform E. coli DH5α competent cells. The colonies growing on the antibiotics streptomycin and spectinomycin (100 μg mL⁻¹ each) were checked by colony PCR. The plasmid DNA was extracted and double checked with restriction reaction. Less than 10 ng of construct was used to transform competent cells of Agrobacterium tumefaciens AGL-1 that were able to grow on the antibiotic rifampicin (20 μg mL⁻¹). Positive colonies growing on the selective antibiotics were picked, purified, and then used to transform flowering plants of Arabidopsis Col-0, pif6, pif4 pif5, and pif4 pif6 pif7 following the protocol of Zhang et al. (2006). Successfully transformed T1 seeds were selected through the GFP signal in dry seeds. T2 lines were selected for single insertion of the transgene using the selectable marker bar, which confers resistance to the herbicide Basta (25 mg mL⁻¹). DL-Phosphinothricin, Duchefa Biochemie). Finally, T3 seeds were screened for homozygosity using the GFP signal, and the insertion of the transgene was confirmed by PCR reaction performed on genomic DNA (gDNA) extracted from homozygous plants using the primers listed in Supplemental Table S4. Experiments were performed using T1 or T3 seeds.

Thermal Asymmetric Interlaced PCR

Leaf material was used to extract gDNA from transgenic lines 355:KDR lines 1, 3, 8, and 9, created using the pFAST-G02 vector, as described above. The gDNA was used to perform a thermal asymmetric interlaced PCR (TAIL-PCR) reaction as described in Liu et al. (1995) with minor modifications, using arbitrary degenerate, T-DNA left border end primers (Supplemental Table S5), and DreamTag DNA polymerase (Thermo Fisher Scientific). The cycle settings used for the TAIL-PCR reactions were adjusted based on the characteristics of the polymerase and primers used and listed in Supplemental Table S6. Purified fragments obtained in the second or third TAIL-PCR reactions were sequenced (Macrogen Europe) and analyzed through a BLAST search (NCBI, www.ncbi.nlm.nih.gov) to identify the flanking sequences. A schematic representation of the insertion sites is shown in Supplemental Figure S8.

Gene Cloning for Y2H Interactions

The procedure for cloning KDR CDS was as described before but with the use of the primers listed in Supplemental Table S7 and cloned into the Gateway vector pDONR221 (Thermo Fisher Scientific). The competent cells transformed with the entry vectors were selected for growth on the antibiotic kanamycin (50 μg mL⁻¹). The CDSs of the genes HFR1, PIF4, PIF5, and PIF7 cloned into the Gateway vector pENTR/D-TOPO and of PARI and PAR2 cloned into the Gateway vector pENTR223 were obtained from ARCG and sequence validated. The entry vectors containing the CDSs of KDR, HFR1, PARI, PAR2, PIF4, PIF5, and PIF7 were recombined into the Gateway destination vector pDEST32 (gentamycin 20 μg mL⁻¹), whereas KDR, HFR1, PARI, PAR2, PIF4, PIF5, and PIF7 were also recombined in the Gateway destination vector pDEST23 (carbenicillin 50 μg mL⁻¹). The pDEST22 vectors harboring the CDS of the genes AIF2, AIF3, IBH1, and IBL1 were obtained from the yeast TF library described by Pruneda-Paz et al. (2014). The yeast colonies were grown on 5-mL liquid synthetic complete (SC) medium (Formedium) lacking the selective amino acid Trp for 1 d at 30°C in shaking conditions. The plasmid DNA was then extracted using the QIAprep spin miniprep kit (Qiagen). Finally, the entry vectors containing KDR and HFR1 were both cloned also into the Gateway destination vectors pCBKT7 (kanamycin 50 μg mL⁻¹) and pCAD7 (carbenicillin 50 μg mL⁻¹).

Yeast Prey Plasmid cDNA Library of Arabidopsis

The yeast prey plasmid cDNA library of Arabidopsis was kindly provided by Dr. Guido van den Ackerveken (Utrecht University, the Netherlands) and created using In vitrigen Custom Services (Invitrogen). RNA was extracted from 15-d-old Arabidopsis seedlings subjected to five conditions: uninfected, infected with a compatible strain of Hyaloperonospora Arabidopsidis (Hpa), infected with incompatible strain of Hpa, sprayed with benzothiadiazole, or infiltrated with NIN-like proteins. This variety of optimal and stress conditions likely yielded a very broad library of different transcripts. Synthesis of cDNA was performed on the RNA extracted, cloned into the Gateway donor vector pENTR222, and then recombined into the Y2H Gateway destination vector pDEST22 to generate a GAL4 activation domain fused to the N-terminal part of Arabidopsis proteins. Competent cells of Saccharomyces cerevisiae strain Y8800 (genotype MATa try1–301 leu2–112 ura3–52 his3–200 Gal4α gal80Δ cph1–3 GAL1 HIS3 & GLY2 GAL2 ADE2 GAL2 LacZαMet2) were transformed with the expression vectors. At least one million colonies were harvested in yeast extract peptone dextrose (YPED) medium to ensure a good coverage of all the different proteins present in the library. Finally, aliquots of the library were made and stored in glycerol stocks.

Yeast Transformation

The bait constructs cloned into the pDEST32 or pCBKT7 were transformed into the yeast strain Y8930 (genotype MATa try1–301 leu2–112 ura3–52 his3–200 Gal4α gal80Δ cph1–3 GAL1 HIS3 & GLY2 GAL2 ADE2 GAL2 LacZαMet2), whereas the prey constructs cloned into the pDEST22 or pCAD7 were transformed into the strain Y8800 using the LiAc (Sigma-Aldrich) method (Schiestl and Gietz, 1989). Yeast transformed with the expression vectors pDEST32 and pCAD7 were plated on SC medium lacking the selective amino acid Leu. The same was done for the colonies transformed with the pDEST22 and pCBKT7, but in this case SC was used without the selective amino acid Trp. Four-day-old single colonies growing at 30°C were isolated and the insertion of the plasmids was confirmed with colony PCR using the primers listed in Supplemental Table S8. Positive transformed colonies were resuspended in YEPD containing 24% (v/v) glycerol and stored at −80°C. To test for auto-activation of the bait constructs, yeast strain Y8930 carrying the expression vectors pDEST32 or pCBKT7 were grown on a SC medium lacking His and adenine (2-adenine) in the following combinations: –Leu –His + 5 μg 3-AT; –Leu –His + 5 μg 3-AT and –Leu –Ade for pDEST32 constructs, whereas for colonies carrying the pCBKT7 the medium was lacking Trp instead of Leu. Colonies expressing the proteins PIF4 or PIF5 were able to activate the HIS3 and ADE2 reporter genes and for this reason they were not used in the experiments in the bait construction.

Y2H cDNA Library Screening and Individual Interactions

The Y2H library screening was performed using a mating-based approach described previously (Fromont-Racine et al., 2002). The yeast bait construct expressing KDR cloned in the pDEST32 was grown overnight in 10 mL YEPD medium under shaking conditions at room temperature. The day after a 1-mL aliquot of yeast prey cDNA library was thawed on ice, mixed with 100 mL YEPD, and incubated for 1 h. The library was then pelleted at 380g for 5 min and washed twice with sterile double distilled water, after which it was resuspended with 10 mL YEPD. Finally, the OD600 of the prey library and of the yeast containing the bait construct was measured, and they were mixed in equal amount of OD600 = 6. The yeast mix was spun down at 380g for 5 min, resuspended in 300 μL of sterile double distilled water, and plated on a 10-cm round plate containing YEPD supplemented with carbenicillin (100 μg mL⁻¹). The YEPD plate was incubated at 30°C for 4 h to allow the mating of the yeast. After the incubation, the YEPD plate was washed with 3 mL of sterile double distilled water.
water, the yeast suspension was collected, centrifuged at 380g for 5 min, and the pellet was resuspended in 600 μL of sterile double distilled water. Finally, the yeast was plated on round plates of 15-cm diameter containing SC medium – Leu – Trp – His supplemented with carbenicillin (100 μg mL⁻¹) and incubated at 30°C for 4 d. After the period of incubation, colonies growing on the selective medium were picked, resuspended in 25 μL of sterile double distilled water, and plated on two fresh SC – Leu – Trp – His plates for 2 d at 30°C. Hereafter, individual colonies of yeast from one plate were used for colony PCR using the primers listed in Supplemental Table S8. The resulting product reactions were purified using Agencourt AMPure XP beads (Beckman Coulter) according to the manufacturer’s protocol and sequenced to identify the prey proteins interacting with the bait of interest. The second – Leu – Trp – His plate was replica plated on SC – Leu – Trp – His + 2 μM 3-AT and on SC – Leu – Trp – His + 5 μM 3-AT, which were subsequently incubated for 2 to 3 d at 30°C, and also plated on SC – Leu – Trp – Ade for 5 d at 20°C. Yeast colonies expressing at least one of the two reporter genes were considered positive. A selection of the proteins found in the screening were full-length cloned in the prey vector, as described previously, and the yeast strain Y8800 was transformed. All the baits and the preys used for individual interactions were cloned for 2 d at 30°C on SC – Leu or – Trp, based on the type of vectors in which they were expressed. A small dot of a single yeast colony containing the bait or the prey was resuspended in 400 μL of sterile double distilled water. Then 10 μL of prey yeast was mixed with other 10 μL of the bait yeast. Five microliters were spotted on YEPD plate and incubated for 24 h at 30°C to allow the mating and the growth of the yeast. Finally, a small dot of individual mated colony was resuspended in 1 mL of sterile double distilled water and 10 μL was spotted on the following SC selective plates: – Leu – Trp, as confirmation of the mating; – Leu – Trp – His, as confirmation of the interaction; and – Leu – Trp – His + 2 μM 3-AT; – Leu – Trp – His + 5 μM 3-AT; – Leu – Trp – Ade to determine the strength of interaction. The plate lacking Ade was incubated at 20°C for 5 d while all the other plates were incubated at 30°C for 2 to 3 d. The yeast transformed with the empty vector pDEST22 (bait) in combination with the studied preys and the empty vector pDEST22 (prey) in combination with the different baits of interest were used as negative control.

Protein Extraction from Yeast and Detection Using Western Blot

Total protein lysates from yeast harboring the different constructs of pDEST22 were obtained according to the Yeast Protocols Handbook (http://www.takara.co.kr/www/index.php?m=manual&d=PT0024-1.pdf; Clontech), following the Urea/SDS method. Before loading, the lysates were boiled for 1 min at 96°C and loaded on a 4% to 15% mini-PROTEAN TGX stain-free protein gels (Bio-Rad). The gels were imaged with a Chemidoc station (Bio-Rad). The separated proteins were subsequently transferred onto a polyvinylidene difluoride membrane using a Bio-Rad transblot turbo. Blots were blocked with 5% (w/v) milk powder (Elk) in Tris-buffered saline (TBS) buffer. Immunodetection of the proteins was performed using the monoclonal antibody directed against the GAL4 activation domain (Clontech no. 630402, 1:1000 in 0.5% [w/v] Elk TBS). Goat anti-mouse IgG conjugated with horseradish peroxidase was used as secondary antibody (Cell Signaling Technology no. 7076, 1:5000 in 0.5% [w/v] Goat anti-mouse IgG conjugated with horseradish peroxidase was used as secondary antibody (Cell Signaling Technology no. 7076, 1:5000 in 0.5% [w/v] Elk TBS). The proteins of this study were cloned without the stop codon

Yeast Prey Plasmid TF Library of Arabidopsis

The construction of the prey TF library of Arabidopsis was made as described by Pruneda-Paz et al. (2014). In brief, the library consists of 1,956 TFs cloned in pDEST32 (bait) in combination with the studied preys. The plates were incubated for 3 d at 30°C and then moved to room temperature for 3 extra d. The growth on the yeast was checked after 2, 3, 4, and 6 d to score for interactors. The identity of the positive colonies was confirmed by repeating the result of a colony PCR used to amplify the Gateway cassette of the pDEST22 carried by the yeast. The primers listed in the Supplemental Table S8 were used to perform the colony PCR.

Gene Cloning for Localization, Colocalization, and BiFC Experiments

For in planta localization and colocalization experiments, cDNA deriving from Arabidopsis was used to amplify the CDSs of KDR, HFR1, PAR1, and PAR2, whereas AIF2, AIF4, IBH1, and IBL1 were amplified from the respective clones in pDEST22 (previously described) using the primers listed in the Supplemental Table S9, which were designed in a way that the CDSs were in frame with a C-terminal tag and without the stop codon. The PCR products containing the attB sequences were cloned into the Gateway donor vector pDONR207 (gentamycin 20 μg mL⁻¹). The resulting entry vector containing KDR was recombined into the Gateway destination vector pEarleyGate102 (Earley et al., 2006; kanamycin 50 μg mL⁻¹), whereas HFR1, PAR1, PAR2, AIF2, AIF4, IBH1, and IBL1 were recombined into the Gateway destination vector pEarleyGate101 (kanamycin 50 μg mL⁻¹). The empty Gateway destination vector pB7WG2 was used to visualize the free YFP (Karimi et al., 2005).

For BiFC experiments, the mutated version of PAR1 (called PAR1mut) was amplified from the vector P535/PAR1mut-G obtained from Galstyan et al. (2011) and cloned into the Gateway donor vector pDONR207. The following set of Gateway destination vectors were used in order to reconstitute the Venus fluorescent protein (YFP); pDEST-VYNEGW (N-terminal part of Venus, residues 1 to 173, referred to as VN in the “Results” section and cloned in frame to the C-terminal part of the protein of interest), pDEST-VYNECYC (C-terminal part of Venus, residues 156 to 239, referred to as YC and cloned in frame to the C-terminal part of the protein of interest), pDEST-VYNEDV (N-terminal part of Venus, residues 1 to 156, referred to as VN and cloned in frame to the N-terminal part of the protein of interest), pDEST-VYNEDV (C-terminal part of Venus, residues 156 to 239, referred to as YC in the “Results” section and cloned in frame to the N-terminal part of the protein of interest; Gehl et al., 2009). Transformed competent cells were all selected based on growth on kanamycin (50 μg mL⁻¹). The proteins of this study were cloned without the stop codon when the N- or C-terminal part of the Venus protein was fused to their C-terminal part. They were cloned with the stop codon when the N- or C-terminal part of the Venus protein was fused to their N-terminal part. For each BiFC experiment, we used as positive control the combination of the two interacting proteins published with this BiFC set of vectors (Gehl et al., 2009), and as negative controls the two combinations of empty vectors with the proteins of interest. The combinations of each protein studied here together with the mutated PAR1mut (Galstyan et al., 2011) were used as additional negative controls.

Transient Expression in N. benthamiana

Competent cells of A. tumefaciens AGL1 were transformed with the Gateway expression vectors described in the previous paragraph made for localization, colocalization, or BiFC experiments. Transformed colonies were selected using the antibiotic resistance of the different vectors and with rifampicin (20 μg mL⁻¹) carried by AGL1 cells. Single colonies were grown for 2 d at 28°C in 20 mL L-broth shaking medium under shaking conditions. After the OD595 was measured, the cells were pelleted and resuspended to a final OD595 of 0.5 with a one-half strength Murashige and Skoog medium (Duchefa Biochemie) supplemented with 10 mM MES hydrate (Sigma-Aldrich), 20 g L⁻¹ Suc (Sigma-Aldrich), and 200 μM acetosyringone (Sigma-Aldrich) at pH 5.6 and incubated

0.5, 10, 15, 20, or 40 mM 3-AT. No auto-activation was found. From the glycerol stock, the bait yeast was grown on SC – Leu at 30°C for 2 d. At the same time, also the yeast of the TFs library was grown, the 96-well plates were thawed on ice, and 5 μL were taken from each single well and spotted on a plate containing SC – Trp and left to grow at 30°C for 2 d. Then, the bait strain was resuspended in 11 mL of sterile double distilled water and 3 μL were spotted on YEPD plates. A small dot of each colony of the library was taken with a pipette tip, resuspended in 200 μL of sterile double distilled water, and 3 μL were spotted on top of the bait. The plates were grown for 3 d at 30°C to allow mating and growth, after which each colony spot was resuspended in 200 μL of sterile double distilled water, and 3 μL were plated on SC – Leu – Trp – His supplemented with 10 μM of 3-AT. The plates were incubated for 3 d at 30°C and then moved to room temperature for 3 extra d. The growth on the yeast was checked after 2, 3, 4, and 6 d to score for interactors. The identity of the positive colonies was confirmed by sequencing the result of a colony PCR used to amplify the Gateway cassette of the pDEST22 carried by the yeast. The primers listed in the Supplemental Table S8 were used to perform the colony PCR.

The Role of KIDARI in Shade Avoidance

Y2H TF Library Screening

The bait vector pDEST32 harboring the gene KDR was used to transform the yeast strain Pj69-4a. The auto-activation of the bait construct was tested by taking a small dot of yeast colony, resuspended in 50 μL of sterile double distilled water, and spotting 5 μL on the selective plates SC – Leu – His containing

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in darkness for at least 1 h. The solutions were used to agroinfiltrate the abaxial side of 4- to 5-week-old *N. benthamiana* leaves using a 1-ML syringe without the needle. In the case of colocalization or BiFC experiments, the cells of *A. tumefaciens* carrying the two different expression vectors were mixed before performing agroinfiltration. The plants were left to grow in normal light conditions and after 2 d leaf sections were taken from the agroinfiltrated regions and visualized through confocal microscopy.

Confocal Microscopy

Microscopy was performed using a Zeiss LSM 780 (Zeiss) confocal laser-scanning microscope using the 20× water immersion objective (Plan-Apochromat 20×/0.8 M27). Fresh leaf material was prepared on a glass slide with a cover slip. Excitation of YFP, CFP, and autofluorescence of chlorophyll was done at 488 nm, 405 nm, and 488 nm, respectively. Light emission of YFP was detected at 493 to 550 nm, CFP at 300 to 483 nm, and chlorophyll autofluorescence at 644 to 800 nm. Pinhole, gain, laser power, and detector offset were always set the same within experiments. Analyses of the images were performed with ZEN lite (blue edition).

Statistical Analysis

Growth data were analyzed by two-way ANOVA followed by post-hoc Tukey test, whereas RT-qPCR data were analyzed by Student’s *t* test or one-way ANOVA followed by post-hoc Tukey test. All the analyses were done using GraphPad Prism.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: AT1G26945 (KDR), AT5G25760, AT4G26410, AT1G02340 (HFR1), AT2G42870 (PAR1), AT1G58850 (PAR2), AT3G06390, AT1G09250 (AF2), AT2G43860 (BII1), AT1G9410 (BII1), AT2G43100 (PIF4), AT3G59960 (PIF5), AT5G61270 (PIF7), AT2G49670 (PIF3), AT2G41390 (XTH15), AT2G6310 (XTH17), AT3G38860 (PRE1), AT2G8720 (YUC8), AT3G15540 (IAP1), and AT4G32280 (IAP2).

Supplemental Data

- **Supplemental Table S1.** Primers used for genotyping.
- **Supplemental Table S2.** Primers for RT-qPCR.
- **Supplemental Table S3.** Primers with attB cloning sites for full-length KDR CDS amplification.
- **Supplemental Table S4.** Primers used for genotyping transgenic lines overexpressing KDR made using the vector pFAST-C02.
- **Supplemental Table S5.** Primers used for TAIL-PCR.
- **Supplemental Table S6.** Cycle settings used for TAIL-PCR.
- **Supplemental Table S7.** Primers with attB cloning sites for full-length KDR CDS amplification.
- **Supplemental Table S8.** Primers used for amplification of Arabidopsis gene fragments in Y2H vectors.
- **Supplemental Table S9.** Primers with attB cloning sites for CDS amplification without the stop codon.

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LITERATURE CITED

Al-Sady B, Ni W, Kircher S, Schäfer E, Quail PH (2006) Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. Mol Cell 23: 439–446

Bai M-Y, Fan M, Oh E, Wang Z-Y (2012) A triple helix-loop-helix/basic helix-loop-helix cascade controls cell elongation downstream of multiple hormonal and environmental signaling pathways in Arabidopsis. Plant Cell 24: 4917–4929

Ballaré CL, Scopek AL, Sánchez RA (1991) Photocycle of stem elongation in plant neighbourhoods: Effects of photon flux rate under natural conditions of radiation. Plant Cell Environ 14: 57–65

Bocalanandro HE, Ploschuk EL, Yanovsky MJ, Sánchez RA, Gatz C, Casal JJ (2003) Increased phytochrome B alleviates density effects on tuber yield of field potato crops. Plant Physiol 133: 1539–1546

Bou-Torrent J, Roig-Villanova I, Galstyan A, Martínez-García JF (2008) PAR1 and PAR2 integrate shade and hormone transcriptional networks. Plant Signal Behav 3: 453–454

Bu Q, Castillo A, Chen F, Zhu L, Huq E (2011) Dimerization and blue light regulation of PIF1 interacting bHLH proteins in Arabidopsis. Plant Mol Biol 77: 501–511

Chen M, Chory J (2011) Phytochrome signaling mechanisms and the control of plant development. Trends Cell Biol 21: 664–671

Cifuentes-Esquível N, Bou-Torrent J, Galstyan A, Gallemi M, Sessa G, Salla Martret M, Roig-Villanova I, Ruberti I, Martínez-García JF (2013) The bHLH proteins BEE and BIM positively modulate the shade avoidance syndrome in Arabidopsis seedlings. Plant J 75: 989–1002

Cui J, You C, Zhu E, Huang Q, Ma H, Chang F (2016) Feedback regulation of DYT1 by interactions with downstream bHLH factors promotes DYT1 nuclear localization and anther development. Plant Cell 28: 1078–1093

de Wit M, Keuskamp DM, Bongers FJ, Hornitscheik P, Gommers CMM, Reinen E, Martínez-Cerón C, Fankhauser C, Pierik R (2016) Integration of phytochrome and cryptochrome signals determines plant growth during competition for light. Curr Biol 26: 3320–3326

de Wit M, Ljung K, Fankhauser C (2015) Contrasting growth responses in lamina and petiole during neighbor detection depend on differential auxin responsiveness rather than different auxin levels. New Phytol 208: 398–409

Earley KW, Haag JR, Pontes O, Oppen K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J 45: 616–629

Fairchild CD, Schumaker MA, Quail PH (2000) HFR1 encodes an atypical bHLH protein that acts in phytochrome A signal transduction. Genes Dev 14: 2377–2389

Fromont-Racine M, Rain J-C, Legrain P (2002) Building protein-protein interaction networks by two-hybrid mating strategy. Mol Biol 350: 513–524

Galstyan A, Bou-Torrent J, Roig-Villanova I, Martínez-García JF (2012) A dual mechanism controls nuclear localization in the atypical basic-helix-loop-helix protein PAR1 of Arabidopsis thaliana. Mol Plant 5: 669–677
Galvano FCN, Bou-Torrent J, Martinez-Garcia JF (2011) The shade avoidance syndrome in Arabidopsis: A fundamental role for atypical basic helix-loop-helix proteins as transcriptional co-factors. Plant Cell 23: 256–267

Galvão VC, Fiorucci A-S, Trevisan M, Franco-Zorrilla JM, Goyal A, Schmid-Siegert E, Solano R, Fankhauser C (2019) PIF transcription factors link a neighbor cue to accelerated reproduction in Arabidopsis. Nat Commun 10: 4054–4067

Gehr C, Waadt R, Kudla J, Mendel R-R, Hänsch R (2009) New GATEWAY vectors for high throughput analyses of protein-protein interactions by bimolecular fluorescence complementation. Mol Plant 2: 1051–1058

Gommers CMM, Keuskamp DH, Buti S, van Veen H, Koornneef M, Whitelam GC (2012) Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. Plant Cell 24: 337–352

Gommers CMM, Visser EJW, St Onge KR, Voesenek LACJ, Pierik R (2013) Shade tolerance: When growing tall is not an option. Trends Plant Sci 18: 65–71

Halliday S, Fankhauser C (2009) Modular cloning in plant cells. Plant Cell 21: 379–386

Halliday KJ, Koomen M, Whitemall GC (1994) Phytochrome B and at least one other phytochrome mediate the accelerated flowering response of Arabidopsis thaliana L. to low red/far-red ratio. Plant Physiol 104: 1311–1315

Hao Y, Oh E, Choi G, Liang Z, Wang ZY (2012) Interactions between HLH and bHLH factors modulate light-regulated plant development. Mol Plant 5: 688–697

Hayes S, Pantazopoulou CK, van Gelderen K, Rein en E, Tween AL, Sharma A, de Vries M, Prat S, Schuurink RC, Testerink C, et al (2019) Soil salinity limits plant shade avoidance. Curr Biol 29: 1669–1676.e4

Heng S-Y, Yang Y, See PJ, Ryu JY, Cho S-HH, Won J-CC, Park C-KM (2013) A competitive peptide inhibitor kidneyed KIDARI negatively regulates HY1 by forming nonfunctional heterodimers in Arabidopsis photomorphogenesis. Mol Cells 35: 25–31

Hornitschek P, Kohnen MV, Lorrain S, Rougemont J, Ljung K, Lorrain S, Michelini O, Fankhauser C (2009) Inhibition of the shade avoidance response by formation of non-DNA binding bHLH heterodimers. EMBO J 28: 3895–3902

Hyun Y, Lee I (2006) KIDARI encoding a non-DNA binding bHLH protein, represses light signal transduction in Arabidopsis thaliana. Plant Mol Biol 61: 283–296

Ikeda M, Fujiwara S, Mitsuda N, Ohme-Takagi M (2012) A triantagonistic basic helix-loop-helix system regulates cell elongation in Arabidopsis. Plant Cell 24: 4483–4497

Ikeda M, Mitsuda N, Ohme-Takagi M (2013) ATB51 INTERACTING FACTORS negatively regulate Arabidopsis cell elongation in the triantagonistic bHLH system. Plant Signal Behav 8: e22448

Kimata M, De Meyer B, Hillson P (2005) Modular cloning in plant cells. Trends Plant Sci 10: 103–105

Leivar P, Monte E, Al-Sady B, Carle C, Storer A, Alonso JM, Ecker JR, Quail PH (2008) The Arabidopsis phytochrome-interacting factor PIF7, together with PIF3 and PIF4, regulates responses to prolonged red light by modulating phyB levels. Plant Cell 20: 337–352

Leivar P, Quail PH (2011) PIFS: Pivotal components in a cellular signaling hub. Trends Plant Sci 16: 19–28

Li L, Ljung K, Breton G, Schmitz RJ, Pruneda-Paz J, Jenkins H, Hallab A, Gan X, et al (2019) Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. Plant J 93: 312–323

Lorrain S, Fankhauser C (2012) Plant development: Should I stop or should I go on? Curr Opin Plant Biol 15: 475–483

McConigle B, Bouhidel K, Irish VF (1996) Nuclear localization of the Arabidopsis APETALA3 and PISTILLATA homeotic gene products depends on their simultaneous expression. Genes Dev 10: 1812–1821

Molina-Contreras MJ, Pauliščiūtė S, Then C, Moreno-Romero J, Pastora-Andreu P, Morelli L, Roig-Villanova J, Jenkins H, Hallab A, Gan X, et al (2019) Phytochrome activity contributes to contrasting responses to shade in cardamine and Arabidopsis seedlings. Plant Cell 31: 2649–2663

Nayar S, Kapoor M, Kapoor S (2014) Post-translational regulation of rice MADS29 function: homodimerization or binary interactions with other seed-expressed MADS proteins modulate its translocation into the nucleus. J Exp Bot 65: 5339–5350

Pantazopoulou CK, Bongers JF, Kupers JJ, Reinen E, Das D, Evers JB, Autin P, DPR, Prick D (2017) Neighbor detection at the leaf tip adaptively regulates upward leaf movement. Plant Physiol 174: 1051–1056

Pruneda-Paz J, Breton G, Nagel DH, Kang SE, Bonaldi K, Doherty CJ, Ravelo S, Galli M, Ecker JR, Kay SA (2014) A genome-scale resource for the functional characterization of Arabidopsis transcription factors. Cell Rep 8: 622–632

Robson PRH, McCormac AC, Irvine AS, Smith H (1996) Genetic engineering of harvest index in tobacco through overexpression of a phytochrome gene. Nat Biotechnol 14: 995–998

Roig-Villanova I, Bou J, Sorin C, Devlin PF, Martinez-Garcia JF (2006) Identification of primary target genes of phytochrome signaling: Early transcriptional control during shade avoidance responses in Arabidopsis. Plant Physiol 141: 85–96

Roig-Villanova I, Bou-Torrent J, Galvano FCN, Carretero-Paulet L, Portoles S, Rodriguez-Concepcion M, Martinez-Garcia JF (2007) Interaction of shade avoidance and auxin responses: A role for two novel atypical bHLH proteins. EMBO J 26: 4756–4767

Schiessel RH, Gietz RD (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet 16: 339–346

Sessa G, Carabeli M, Sassi M, Ciolfi A, Possenti M, Mittermperger F, Becker J, Morelli G, Ruberti I (2005) A dynamic balance between gene activation and repression regulates the shade avoidance response in Arabidopsis. Genes Dev 19: 2811–2815

Shen Y, Khanna R, Carle CM, Quail PH (2007) Phytochrome induces rapid PIF5 phosphorylation and degradation in response to red-light activation. Plant Physiol 145: 1043–1051

Shimada TL, Shimada T, Hara-Nishimura I (2010) A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of Arabidopsis thaliana. Plant J 61: 519–528

Sun J, Qi L, Li Y, Chu J, Li C (2012) PIF4-mediated activation of YUC2/8 expression integrates temperature into the auxin pathway in regulating Arabidopsis hypocotyl growth. PLoS Genet 8: e1002594

van Veen H, Mustroph A, Barding GA, Vergeer-van Eijk M, Welschen-Evertman RAM, Pedersen O, Visser EJW, Larive CK, Pierik R, Bailey-Serres J, et al (2013) Two Rumex species from contrasting hydrological niches regulate flooding tolerance through distinct mechanisms. Plant Cell 25: 4691–4707

Wang H, Zhu Y, Fujioka S, Asami T, Li J, Li J (2009) Regulation of Arabidopsis brassinosteroid signaling by atypical basic-helix-helix proteins. Plant Cell 21: 3781–3791

Zhang L-Y, Bai M-Y, Wu J, Zhu J-Y, Wang H, Zhang Z, Wang W, Sun Y, Zhao J, Sun X, et al (2009) Antagonistic PH/HLH transcription factors mediate brassinosteroid regulation of cell elongation and plant development in rice and Arabidopsis. Plant Cell 21: 3767–3780

Zhang X, Henrichues R, Lin S-S, Niu Q-W, Chua N-H (2006) Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nat Protoc 1: 641–646

Zhang Y, Mayba O, Pfeiffer A, Shi H, Pepperman JM, Speed TP, Quail PH (2015) A quartet of PIF bHLH factors provides a transcriptionally centered signaling hub that regulates seedling morphogenesis through differential expression-pattern of shared target genes in Arabidopsis. PLoS Genet 11: e1003244

Zheng K, Wang Y, Zhang N, Jia Q, Wang X, Hou C, Chen J-G, Wang S (2017) Involvement of PACLOBUTRAZOL RESISTANCE/5/KIDARI, an atypical bHLH transcription factor, in auxin responses in Arabidopsis. Front Plant Sci 8: 1513

Zhiponova MK, Morohashi K, Vanhoutte I, Machemer-Noonan K, Revakova M, Van Montagu M, Grotewold E, Russinova E (2014) Helix-loop-helix/basic helix-loop-helix movement factor network represses cell elongation in Arabidopsis through an apparent incoherent feed-forward loop. Proc Natl Acad Sci USA 111: 2824–2829

Zhou P, Song M, Yang Q, Su L, Hou P, Guo L, Zheng X, Xi Y, Meng F, Xiao Y, et al (2014) Both PHYTOCHROME RAPIDLY REGULATED1 (PARK1) and PARK2 promote seedling morphogenesis in multiple light signaling pathways. Plant Physiol 164: 841–852