Arabidopsis FHY3 and FAR1 integrate light and strigolactone signaling to regulate branching

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Branching/tillering is an important parameter of plant architecture and is tightly regulated by both internal factors (such as plant hormones) and external factors (such as light conditions). How the various signaling pathways converge to coordinately regulate branching is not well understood. Here, we report that in Arabidopsis, FHY3 and FAR1, two homologous transcription factors essential for phytochrome A-mediated light signaling, and SMXL6/SMXL7/SMXL8, three key repressors of the strigolactone (SL) signaling pathway, directly interact with SPL9 and SPL15 and suppress their transcriptional activation of BRC1, a key repressor of branching, thus promoting branching. In addition, FHY3 and FAR1 also directly up-regulate the expression of SMXL6 and SMXL7 to promote branching. Simulated shade treatment reduces the accumulation of FHY3 protein, leading to increased expression of BRC1 and reduced branching. Our results establish an integrated model of light and SL coordinately regulating BRC1 expression and branching through converging at the BRC1 promoter.
Branching (tillering in cereal crops) is a major component of plant architecture and critical determinant of crop productivity. A critical stage of branch development is bud outgrowth after the formation of axillary buds, and this process is intricately regulated by endogenous developmental cues and various environmental signals. Recent molecular genetic studies have revealed a conserved TBI/FC1/BRC1 pathway repressing axillary bud outgrowth in both monocots and dicots. TEOSINTE BRANCHED 1 (TBI), which encodes a TCP-family transcription factor, was initially identified as a key repressor of lateral branching in maize. Later studies showed that loss-of-function mutants of the genes homologous to TBI in rice and Arabidopsis (FINE CULM1 or FC1 in rice, Branched1 or BRC1 in Arabidopsis) display a similar excess tillering/branching phenotype. Moreover, recent studies showed that strigolactone (SL), a newly identified plant hormone, represses bud outgrowth in both monocots and dicots. Further, it was shown that SLs repress tiller outgrowth in rice through promoting the degradation of a central repressor protein, D53. Similarly, it has also been shown that in Arabidopsis, SL-induced degradation of three D53-like SUPPRESSOR OF AXILLARY GROWTH2-LIKE proteins SMXLs (SMXL6, SMXL7, and SMXL8) leads to activation of BRC1 to inhibit branching.

Substantial evidence suggests that the MIR156-SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE) module plays an important role in regulating diverse aspects of plant growth and development, ranging from vegetative to reproductive phase transition, branching, leaf development, flowering time, panicle/tassel architecture, fruit ripening, fertility, lateral root development, and abiotic stress responses. SPLs encode a family of plant-specific transcription factors that share a highly conserved DNA-binding domain, the SBP domain. It has been shown that Arabidopsis SPL9 and SPL15 negatively regulate branching by suppressing axillary meristem initiation and that their loss-of-function mutants display an enhanced branching phenotype. Similarly, a single-nucleotide polymorphism that escapes miR156 targeting or increase OsSPL14 expression via epigenetic regulation confer an ideal plant architecture to rice, including fewer unproductive tillers, stronger culm, enlarged panicle and ultimately, enhanced grain yield. Thus OsSPL14 was also named Ideal Plant Architecture1 (IPAI) or WEALTHY FARMER’S PANICLE (WFP). IPAI was used hereafter. Further analysis revealed that IPAI could directly bind to the promoter of OsTBI (FC1), to regulate branching/tillering. Recent studies further showed that IPAI can directly bind to the promoter of D53 and that D53 protein can inhibit the transcriptional activation activity of IPAI by direct physical interaction, thus forming a feedback loop to repress the expression of D53. Such a mechanism has also been shown to be conserved in wheat. These results clearly indicate that the SL signaling pathway and the MIR156/SPL regulatory module converge at the promoter of TBI/FC1/BRC1 to coordinateably regulate branching in plants.

Light is a major environmental factor that regulates branching pattern in plants. When plants sense a reduction in red to far-red light ratio (R/FR) due to competition for light from the neighbor plants (vegetation proximity) or grown under canopy shade, they initiate a set of adaptive responses collectively termed shade avoidance syndrome (SAS), including rapid shoot elongation, more erect leaves, accelerated leaf senescence, early flowering, reduced branching, and ultimately decreased biomass and seed yield if the shading is persistent. It has been shown that under simulated shade conditions (low R/FR ratios), the expression of BRC1 is elevated, leading to suppression of branching activity. We previously showed that under simulated shade conditions, accumulation of the phytochrome-interacting factors (PIFs) proteins increases, and they directly bind to the promoters of several MIR156 genes and repress their expression, thus releasing the downstream SPL transcription factors to regulate a wide range of physiological responses, including branching. In addition, earlier studies reported that mutations in Arabidopsis FHY3 and FAR1, which encode two homologous transcription factors essential for phytochrome A (phyA)-mediated far-red light signaling in Arabidopsis, caused reduced branching number. Despite the progress made in this area, however, how the light signaling pathway integrates with the SL and MIR156/SPL regulatory module to coordinately regulate branching remains poorly understood.

In this study, we report that FHY3 and FAR1 directly interact with SPL9 and SPL15 and inhibit the binding of SPL9 and SPL15 to the BRC1 promoter, whereas the D53-like proteins SMXL6/7/8 directly interact with SPL9 and SPL15 and suppress their trans-activation activity on BRC1, thus promoting lateral bud outgrowth and branching. In response to simulated shade conditions, FHY3 and FAR1 protein levels reduce, which on one hand leads to the release of SPL9 and SPL15 to bind to BRC1 promoter and activate its expression, and on the other hand, leads to reduced expression of SMXL6 and SMXL7, which is directly upregulated by FHY3 and FAR1, resulting in elevated expression of BRC1 and suppression of branching. Our results establish a complex regulatory network regulating branching under shade conditions through integrating the light and SL signaling pathways with the MIR156/SPL regulatory module.

Results

FHY3 and FAR1 regulate branching in response to shade. Previous studies reported that fhy3 loss-of-function has reduced lateral branching, indicating that FHY3 is a positive regulator of branching. To confirm this, we firstly compared the number of rosette branches (longer than 2 mm) in wild type (WT), fhy3-11 and far1-4 single mutants, the fhy3 far1 double mutant, and the FHY3 overexpressor (FHY3-OE) under normal high R/FR (white light, WL) conditions. Our observation showed that the FHY3-OE plants had much more rosette branches (27.3% more than that of WT), while the mutant plants all had less branches compared with the WT (only 16.4% and 13.4% of WT for fhy3-11 and fhy3 far1, respectively) (Fig. 1a, b). Further RT-qPCR analysis showed that the transcript level of BRC1 was significantly higher in both the fhy3-11 and fhy3 far1 plants but obviously lower in the FHY3-OE plants compared with the WT plants (Supplementary Fig. 1).

To test whether FHY3 and FAR1 play a role in shade-induced repression of branching, we compared the number of rosette branches of WT, fhy3 far1 double mutant and FHY3-OE plants. We treated the plants with far-red light (15 μmol m−2 s−1) for 30 min at the end of each light period before returning to darkness (EOD-FR) to simulate the shade conditions. Seven-day-old seedlings of WT, fhy3 far1 double mutant and FHY3-OE were treated with EOD-FR for 30 min every day before returning to darkness. After 4 week’s treatment, the number of rosette branches (>2 mm in length) was counted. The results showed that compared with their counterparts grown under normal WL conditions, all EOD-FR treated plants had significantly reduced rosette branches (Fig. 1a, b), especially for the WT and FHY3-OE plants (~51% and ~62% reduction for WT and FHY3-OE, respectively), while the fhy3-11 and fhy3 far1 mutants had less decline (~31% and ~27% of reduction for fhy3-11 and fhy3 far1, respectively) (Fig. 1a, b). These observations suggest that the FHY3-OE plants are more sensitive, while the fhy3-11 and fhy3 far1 mutants are less sensitive to the simulated shade treatment than the WT plants. Consistent with this, RT-qPCR analysis revealed that the transcript level of BRC1 was significantly upregulated in all shade-treated materials compared with their
counterparts grown under normal WL conditions, with the highest relative increase of expression being detected in the \textit{FHY3-OE} plants subjected to simulated shade treatment (Supplementary Fig. 1).

We next examined whether the transcript levels of \textit{FHY3} and \textit{FAR1} are regulated by simulated shade treatment. RT-qPCR analysis revealed that, compared with the control plants, the expression of both \textit{FHY3} and \textit{FAR1} were significantly downregulated in plants treated with EOD-FR, with ~38.5% and 35.4% reduction for \textit{FHY3} and \textit{FAR1}, respectively (Fig. 1c). To assess the effect of simulated shade treatment on the protein accumulation of \textit{FHY3}, 7-day-old \textit{FHY3}-HA transgenic seedlings were treated with 15 μmol m$^{-2}$ s$^{-1}$ FR light for 30 min at the end of the light period. The seedlings were harvested at 1, 4, and 6 h post treatment for western blotting analysis. The results showed that accumulation of \textit{FHY3} protein rapidly decreased after the EOD-FR treatment (Fig. 1d). Consistent with this, the accumulation of the YFP-FHY3 fusion protein in the \textit{pFHY3::YFP-FHY3} transgenic seedlings also declined in the EOD-FR treated samples (Fig. 1e). We further used proteasome inhibitor MG132 to test whether the decline of \textit{FHY3} protein accumulation under simulated shade is regulated by 26S proteasome. Treatment with MG132 significantly slowed down the degradation of \textit{FHY3} in the samples treated with simulated shade (Supplementary Fig. 2a, b), indicating that \textit{FHY3} protein stability is regulated by the 26S proteasome pathway.

\textbf{SPL9/15 inhibit branching by repressing \textit{BRC1} expression.} Previous studies have shown that \textit{Arabidopsis} SPL9 and SPL15 negatively regulate branching and that their loss-of-function mutants display an enhanced branching phenotype$^{14,15}$. To confirm their roles in repressing branching, we generated \textit{sp9} \textit{sp15} and \textit{brc1 brc2} double knockout mutants, transgenic overexpressors of \textit{BRC1} (\textit{BRC1-OE}) and \textit{rSPL9} (\textit{rSPL9-OE}, which expresses a miR156-resistant form of SPL9) (Supplementary Fig. 3). As expected, the \textit{sp9} \textit{sp15} double mutant and the \textit{brc1 brc2} double mutant had more rosette branches than the WT (~39% and ~74.4% more than WT for \textit{sp9} \textit{sp15} and \textit{brc1 brc2}, respectively), whereas the \textit{rSPL9-OE} and \textit{BRC1-OE} plants had less
rosette branches than the WT (~72.4% and ~44.2% of WT for rSPL9-OE and BRC1-OE, respectively) (Fig. 2a and Supplementary Fig. 4). RT-qPCR assay showed that spl9 spl15 had significantly lower BRC1 expression level than WT, while rSPL9-OE had significantly higher BRC1 expression level than WT (Supplementary Fig. 5). To test whether SPL9 and SPL15 repress branching through BRC1 and BRC2, we generated the rSPL9-OE/brc1 brc2 and BRC1-OE/spl9 spl15 high-order mutants via genetic crosses. Phenotypic analysis showed that rSPL9-OE/brc1 brc2 had similar number of branches as the brc1 brc2 double mutant, while BRC1-OE/spl9 spl15 had similar branch number as the BRC1-OE plants (Fig. 2a and Supplementary Fig. 4).

RT-qPCR assay showed that brc1 brc2/rSPL9-OE had similar BRC1 expression level as brc1 brc2, whereas BRC1-OE/spl9 spl15 had similar BRC1 expression level as BRC1-OE (Supplementary Fig. 5). These observations support the notion that SPL9 and SPL15 repress branching through regulating the expression of BRC1.

As previous studies have shown that rice IPA1 can directly bind to the promoter of FC1 and activate its expression, and that bioinformatics analysis revealed the presence of GTAC motifs for SPL protein binding in the BRC1 and BRC2 promoters (Supplementary Fig. 6), we speculated that Arabidopsis SPL9 and SPL15, which are most closely related to IPA1 based on a
phylogenetic analysis\textsuperscript{13}, may also directly bind to the BRC1 and BRC2 promoters and regulate their expression. Yeast one-hybrid assay showed that both SPL9 and SPL15 could bind to the S1 fragment (which contains two GTAC motifs) of the BRC1 promoter, but not the BRC2 promoter. Mutation of the GTAC core motifs in the S1 fragment of the BRC1 promoter into TTTT abolished the binding, suggesting that the binding is specific (Fig. 2b and Supplementary Fig. 6). To confirm the binding, we performed electrophoresis mobility shift assay (EMSA) using purified recombinant proteins of the SBP domain of SPL9 and SPL15 fused with the glutathione S-transferase (GST) tag (Supplementary Fig. 7), and the results showed that both SPL9 and SPL15 could bind to the biotin-labeled probe of BRC1 (Fig. 2c). Further, we performed chromatin immunoprecipitation (ChIP)-qPCR assay using transgenic plants that constitutively express miR156-resistant forms of SPL9 and SPL15 fused with HA tag at their C terminal (35S::rSPL9-HA and 35S::rSPL15-HA, respectively). As shown in Fig. 2d, the S1 fragment of the BRC1 promoter was significantly enriched in the ChIP samples. Together, these observations indicate that SPL9 and SPL15 can directly bind to the BRC1 promoter.

Next, we carried out a transient expression assay in N. benthamiana leaves to examine the effect of SPL9 and SPL15 on BRC1 transcription. The results showed that the pBRC1::LUC reporter activity was significantly elevated when co-transfected with either the 35S::rSPL9 or the 35S::rSPL15 construct, compared with the control vector (pSPYCE) (Fig. 2e). To verify the physiological relevance of SPL9/15 activation of BRC1 expression in plants, we generated transgenic plants expressing BRC1 driven by its endogenous WT promoter (pBRC1wt::gBRC1) or BRC1 promoter mutated in the BRC-binding site (pBRC1m::gBRC1) in the spl9 spl15/brc1 brc2 and rSPL9-LE/brc1 brc2 backgrounds. We found that the branch number in the brc1 brc2 plants harboring the pBRC1wt::gBRC1 transgene was restored to WT level, whereas the brc1 brc2 plants harboring the pBRC1m::gBRC1 transgene had significantly more branches than the WT plants (similar to brc1 brc2). We also found that the spl9 spl15/brc1 brc2 plants carrying the pBRC1wt::gBRC1 or pBRC1m::gBRC1 transgenes had similar branch number as the spl9 spl15/brc1 brc2 plants (Supplementary Fig. 8). Moreover, we found that rSPL9-LE/brc1 brc2 plants harboring pBRC1wt::gBRC1 transgene had significantly fewer branches than brc1 brc2 and WT plants. However, no significant difference was detected between rSPL9-LE/brc1 brc2 plants harboring the pBRC1m::gBRC1 transgene and the brc1 brc2 mutant plants (Supplementary Fig. 8). Collectively, these results strongly support the notion that SPL9 and SPL15 act to regulate branch number through regulating BRC1 expression via binding to the SBP core motif of the BRC1 promoter.

**FHY3 and FAR1 directly interact with SPL9 and SPL15.** As FHY3 and FAR1 play an antagonistic role with SPL9 and SPL15 in regulating branching, we wondered whether these proteins physically interact with each other to coordinately regulate BRC1 expression. Yeast two-hybrid assay showed that both FHY3 and FAR1 interacted with both SPL9 and SPL15 (Fig. 3a). Domain deletion analysis revealed that both the FHY3-I (N-terminal domain containing the DNA-binding zinc finger motif) and FHY3-II domains, but not the FHY3-III domain, could interact with SPL9 and SPL15 (Fig. 3a). The interaction between FHY3 and FAR1 with SPL9 and SPL15 was further confirmed using bimolecular fluorescence complementation (BiFC) assay and luciferase complementation imaging (LCI) assay in N. benthamiana leaf epidermis, in vitro pull-down assay and in vivo co-immunoprecipitation (Co-IP) assay (Fig. 3b-d and Supplementary Fig. 9). Domain mapping analysis also revealed that the SBP-box domain of SPL9 and SPL15 is responsible for interacting with FHY3 and FAR1 (Supplementary Fig. 10).

**FHY3 and FAR1 inhibit SPL9/15 binding to the BRC1 promoter.** We next tested how the interaction between FHY3 and FAR1 with SPL9 and SPL15 may affect the transcriptional regulation of BRC1. Yeast one-hybrid assay showed that inclusion of an FHY3-expressing construct in the yeast obviously inhibited the activation of SPL9 and SPL15 on the BRC1 promoter-driven reporter gene (Fig. 4a), suggesting that the interaction between FHY3 and SPL9/15 may inhibit the binding of SPL9 and SPL15 to the BRC1 promoter. To confirm this, we carried out EMSA by adding the FHY3 protein (FHY3-I domain) to the mixture of SPL-SBP and biotin-labeled BRC1 probe. The results showed that FHY3 protein itself could not bind to the BRC1 probe, but addition of FHY3 protein (but not the MBP control) significantly reduced the amounts of shifted probes caused by binding of SPL9 and SPL15 (Fig. 2b). To further verify the inhibitory role of FHY3 on the DNA-binding activity of SPL9 and SPL15 in vivo, we performed ChIP-qPCR assay using WT, FHY3-OE and fhy3 far1 double mutants carrying the same 35S::rSPL9-HA transgene grown under normal white light conditions or subjected to EOD-FR treatment. Compared with the WT background, the enrichment of BRC1 DNA fragment declined drastically in the FHY3-OE background, but increased in the fhy3 far1 double mutant background (Fig. 4c). In addition, the enrichment of BRC1 DNA fragment was significantly higher in the EOD-FR treated WT and FHY3-OE plants compared with their counterparts grown under normal white light conditions (Fig. 4c and Supplementary Fig. 11). Together, these results strongly support the notion that FHY3 can inhibit the DNA-binding activity of both SPL9 and SPL15 to the BRC1 promoter and that SPL9 (and probably SPL15 as well) has stronger binding activity to the BRC1 promoter under simulated shade conditions.

Next, we performed a transient expression assay to test the effect of FHY3 on the transcriptional activation activity of SPL9 and SPL15 on BRC1 by co-infiltrating the 35S::rSPL9 and pBRC1::LUC reporter with or without 35S::FHY3 into N. benthamiana leaves. As shown in Fig. 4d, expression of the reporter gene declined significantly in the samples co-injected with 35S::FHY3, suggesting that FHY3 indeed represses the transcriptional activation activity of SPL9 and SPL15 on BRC1.

To provide genetic evidence supporting the above notion, we generated fhy3 far1/spl9 spl15 and FHY3-OE/spl9 spl15 mutant combinations via genetic crosses and compared their rosette branch numbers. The results showed that both fhy3 far1/spl9 spl15 and FHY3-OE/spl9 spl15 had similar number of rosette branches as the spl9 spl15 double mutant (Fig. 4e, f). RT-qPCR assay showed that fhy3 far1/spl9 spl15 and FHY3-OE/spl9 spl15 had similar BRC1 expression levels as spl9 spl15 (Supplementary Fig. 12). These results together support the notion that FHY3 and FAR1 indeed act upstream of SPL9 and SPL15 to promote rosette branching.

**SMXL6/7/8 interact with SPL9/15.** Previous studies also showed that in rice, D35 (homolog of SMXL6/7/8) can physically interact with IPA1 (homolog of SPL9 and SPL15) and suppress the transcriptional activity of IPA1\textsuperscript{19}. We thus tested whether SMXL6/7/8 can interact with SPL9 and SPL15. As expected, our yeast two-hybrid assay showed that all these three SMXL proteins could interact with both SPL9 and SPL15, with stronger interaction being observed for SPL9 (Fig. 5a). The interaction between SMXL6/7/8 and SPL9/15 was further verified by LCI assay and BiFC assay (Fig. 5b and Supplementary Fig. 13). We further selected SMXL6 as a test case and confirmed its interaction with
SPL9 and SPL15 using in vitro pull-down assay and in vivo Co-IP assay (Fig. 5c, d).

We next investigated whether SMXL proteins could affect the DNA-binding activity of SPL9 and SPL15 to the BRC1 promoter. Both yeast one-hybrid assay and EMSA assay showed that no obvious effect was observed when SMXL6 was added into these assays (Fig. 5e and Supplementary Fig. 14), suggesting that SMXL proteins are unlikely to affect the DNA-binding activity of SPL9 and SPL15. Next, we performed transient expression assay in N. benthamiana leaves to test the effect of SMXL proteins on SPL9 and SPL15 using in vitro pull-down assay and in vivo Co-IP assay (Fig. 5c, d).

Fig. 3 FHY3 and FAR1 directly interact with SPL9 and SPL15. a Yeast two-hybrid assay shows that both SPL9 and SPL15 interact with FHY3 and FAR1. LexA and JG serve as the negative controls. b BiFC assay shows that both SPL9 and SPL15 interact with FHY3 and FAR1 in N. benthamiana leaf epidermal cells. Both FHY3 and FAR1 were fused to the N-terminal fragment of YFP (nYFP) and both SPL9 and SPL15 were fused to the C-terminal fragment of YFP (cYFP). The AT-HOOK-RFP marker was used to indicate the nuclei. The interaction between nYFP and SPL9-cYFP or SPL15-cYFP serves as the negative controls. Scale bar = 50 μm. c Pull-down assay shows that FHY3 directly interacts with both SPL9 and SPL15 in vitro. GST-SPL9SBP or GST-SPL15SBP proteins were incubated with protein extracts containing His-FHY3-I and further immobilized with glutathione sepharose beads. d Co-IP analysis shows the interaction between FHY3/FAR1 and SLP9/15 using a transient expression system in tobacco leaves. Immunoprecipitation was performed using anti-HA matrix and the co-immunoprecipitated proteins were detected with anti-MYC antibodies. This assay was repeated for three times and similar results were obtained.
transcriptional activation of BRC1 expression. The results showed that addition of SMXL6 protein significantly reduced the activation activity of SPL9 and SPL15 on the reporter gene driven by the BRC1 promoter, compared with the addition of the pSPYNE empty vector. Similar results were also observed for the SMXL7 and SMXL8 proteins (Fig. 5f). These results demonstrate that SMXL6/7/8 inhibit the transcriptional activation activities of both SPL9 and SPL15 on BRC1.

To provide genetic evidence for the above notion, we generated smxl6/7/8 triple mutant and SMXL6D (a mutant form of SMXL6 resistant to degradation) overexpressors (SMXL6D-OE) as described[1] (Supplementary Fig. 15). As expected, the smxl6/7/8 triple mutant had fewer, while the SMXL6D overexpressors had more branches than the WT (Fig. 6a, b). We next generated smxl6/7/8/spl9/15 quintuple mutant and SMXL6D-OE/ rSPL9-OE combinations. Phenotypic assay showed that the smxl6/7/8/spl9/15 quintuple mutant had similar number of rosette branches as spl9/spl15, while SMXL6D-OE/ rSPL9-OE had slightly more (~15.7%) rosette branches than rSPL9-OE (Fig. 6a, b). RT-qPCR assay showed that smxl6/7/8/spl9/15 had similar BRC1 transcript level as spl9/spl15, while SMXL6D-OE/ rSPL9-OE had slightly more (~15.7%) BRC1 transcript level as rSPL9-OE (Fig. 6a, b). Together, these results suggest that SMXLs act to promote rosette branching through suppressing the transcriptional activation activities of both SPL9 and SPL15 on BRC1.

**Fig. 4 FHY3 inhibits the binding and transactivation function of SPL9 and SPL15.** a Yeast one-hybrid assay shows that FHY3 inhibits the binding of SPL9 and SPL15 to the BRC1 promoter. b EMSA assay shows that FHY3 inhibits the direct binding of SPL9SBP and SPL15SBP to the BRC1 promoter. MBP protein is used as the negative control. This assay was repeated for three times and similar results were obtained. c ChIP assay of BRC1 in axillary buds from 4-week-old 35S::rSPL9-HA transgenic plants in the WT, fhy3 far1, or FHY3-OE backgrounds. Values given are mean ± SD (n = 3). Letters indicate significant differences by two-sided LSD test (p < 0.05). d Transient expression assay and quantitative analysis of luminescence intensity show that FHY3 inhibits the transactivation activity of both SPL9 and SPL15 on the LUC reporter gene driven by the BRC1 promoter. Representative images of N. benthamiana leaves 72 h after infiltration were shown. Five independent determinations were assessed. e Comparison of the rosette branch number of 4-week-old fhy3 far1, spl9 spl15, FHY3-OE, rsPL9-OE, and their higher order mutants grown under normal white light conditions. Arrow indicates the short rosette branches. f Quantification of the number of rosette branches of fhy3 far1, spl9 spl15, FHY3-OE, rsPL9-OE, and their higher order mutants grown under normal white light conditions. Values shown are mean ± SD (n = 12). Letters indicate significant differences by two-sided LSD test (p < 0.05).
yeast two-hybrid assay showed that no direct interaction could be detected between FHY3/FAR1 and SMXL6/7/8 (Fig. 5a). Notably, bioinformatics analysis identified at least two putative FHY3/FAR1-binding sites (EBS, CACGCGC) in the first exon of both SMXL6 and SMXL7 (Supplementary Fig. 17). Thus, we tested whether SMXL6 and SMXL7 are direct target genes of FHY3. Yeast one-hybrid assay showed that FHY3 could directly bind to the first exon of SMXL6 and SMXL7 (Fig. 5c). Moreover, transient expression assay in N. benthamiana leaves showed that co-expression of FHY3 significantly enhanced the transcription of LUC reporter genes driven by the 35S minimal promoter fused with the 5’ UTR together with the first exon of SMXL6 and SMXL7 (Fig. 5d and Supplementary Fig. 18). Further, RT-qPCR assay showed that the transcript levels of SMXL6/7 were all downregulated in the fhy3 far1 double mutant, but upregulated in the FHY3-OE plants (Fig. 6c). In addition, the expression levels of SMXL6 and SMXL7 significantly declined in the WT and FHY3-OE plants grown under simulated shade conditions, compared with their counterparts under normal white light conditions (Fig. 6e). These results suggest that FHY3 (and possibly FAR1) can directly upregulate the expression of SMXL6 and SMXL7, and thus likely play a role in SL signaling.

To test whether FHY3/FAR1 and SMXL6/7 could directly regulate BRC1 expression, we first examined whether FHY3/FAR1 and SMXL6/7 can directly bind to the BRC1 promoter. Yeast one-hybrid assay revealed that there was no direct binding of FHY3/FAR1 or SMXL6/7 to the BRC1 promoter (Supplementary Fig. 19). Secondly, we tested whether FHY3/FAR1 and SMXL6/7 can regulate BRC1 activity through protein–protein interaction. Our yeast two-hybrid assay showed that no direct interaction was detected between BRC1 and FHY3/FAR1 or SMXL6/7 proteins (Supplementary Fig. 20). These results suggest that it is unlikely FHY3/FAR1 and SMXL6/7 directly regulate BRC1 expression or BRC1 activity.
Fig. 6 FHY3 and FAR1 directly upregulate the expression of SMXL6 and SMXL7. a. Comparison of the rosette branch number of smxl6/7/8, spl9, spl9, FHY3, FAR1, and their higher order mutants grown under normal conditions. Eight-day-old seedlings were moved into the soil and grown under normal conditions for 4 weeks before phenotyping. Arrow indicates the short rosette branches. b. Quantification of the number of rosette branches of fhy3, spl9, spl9, FHY3, FAR1, and their higher order mutants. Values shown are mean ± SD (n = 12). Letters indicate significant differences by two-sided LSD test (p < 0.05). c. Yeast one-hybrid assay shows that FHY3 and FAR1 bind to the promoters of SMXL6 and SMXL7. pLacZi2 and JG served as the negative controls. d. Transient expression assay shows that FHY3 and FAR1 promote the expression of SMXL6 and SMXL7. Representative images of N. benthamiana leaves 72 h after infiltration were shown. e. Comparison of the expression levels of SMXL6 and SMXL7 in 7-day-old wild type, fhy3, and FHY3-OE seedlings grown under normal white light or simulated shade (EOD-FR) conditions. Values given are mean ± SD (n = 3). Different letters indicate significant differences by two-sided LSD test (p < 0.05). f. A simplified schematic model depicting FHY3 and FAR1 integrate light and strigolactone signaling to regulate branching. FHY3/FAR1 and SMXL6/SMXL7/SMXL8 directly interact with SPL9 and SPL15 and suppress their transcriptional activation of BRC1, meanwhile FHY3 and FAR1 upregulate the expression levels of SMXL6 and SMXL7 and thus promote branching. Simulated shade treatment reduces the accumulation of FHY3 protein, leading to increased expression of BRC1 and reduced branching. Arrow: activate; Bar: repress.
Discussion

Previous studies have shown that light and SLs co-regulate many developmental and physiological processes in plants. For example, it has been shown that SL-mediated inhibition of hypocotyl elongation is dependent on phytochrome and cryptochrome signaling pathways. In addition, earlier studies showed that MAX2, which encodes a subunit of an SFR35 ligase, acts as a positive regulator of the SL signaling pathway and inhibits shoot branching, also functions positively in regulating seedling photomorphogenesis and seed germination. Moreover, it has been reported that high R/FR conditions promote the biosynthesis of SLs, while low R/FR conditions inhibit the biosynthesis of SLs in tomato and Lotus japonicus. These results collectively suggest that light and SLs coordinate the development of monocots and dicots, the results described in this report should have important implications for future design of crops with improved plant architecture and thus increased yields, particularly under high-density planting conditions.

Methods

Planta material and growth conditions. The Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used in this study. The mutants spl9-1 (SAIL_150_B05), spl15-1 (SALK_074426), fhy3-11 (SALK_002711), far1-4 (SALK_031652), brc1-2 (SALK_091920), and brc2-1 (SALK_023116) were obtained from the Nottingham Arabidopsis Stock Centre. The T-DNA insertion sites of these mutant alleles were shown in Supplementary Fig. 21. After surface sterilization, seeds were sown on half strength Murashige and Skoog media plates supplemented with 1% sucrose. Under a 3-day stratification period in the dark at 4 °C, the plates were then placed in a growth chamber (Percival, USA) at 23 °C with a 16-h-light/8-h-dark photoperiod to allow seed germination and seedling growth.

Plant growth and development. For phenotypic investigation, axillary bud and branches were developed as described in Aguilar-Martinez et al. and Stirnberg et al. At least 12 individuals for each genotype were grown under normal conditions or simulated shade conditions for 4 weeks after germination and then the number of rosette branches (longer than 2 mm) was counted.

Plasmid construction. For yeast one-hybrid assay, the full-length coding sequence of SPL9 or SPL15 was PCR amplified from WT cDNA and ligated to the pG4-5 vector (Clontech, USA) to produce the AD-SPL9 and AD-SPL15 constructs. Promoter fragments (~3 kb in length) of BRC1 or BRC2 were amplified from WT genomic DNA and ligated to the pLaZiZ vector to generate the 2 µ-BRC1 or 2 µ-BRC2 constructs. For yeast two-hybrid assay, the coding regions of FHY3, FAR1, or SMXLs were amplified and cloned into the pG202 vector (Clontech, USA) to generate the EcoR restriction site to produce the corresponding LexA fusion construct.

For BiFC, the coding sequences of SPL9 or SPL15 (without stop codon) were cloned into the pSPYCE vector to generate the 35S:SPL-SPYCE constructs. The coding regions of FHY3, FAR1, and SMXLs (without stop codon) were cloned into the pSPYNE vector to produce the corresponding SPYNE fusion constructs. For LCI assay, the coding sequences of SPL9 or SPL15 were cloned into the pCAMBA1300−LUC vector and the coding sequences of FHY3, FAR1, or SMXLs were cloned into the pCAMBA1300−N-LUC vector to generate the corresponding constructs. For luciferase assay, the promoter of BRC1 was cloned into the promoter−0800-LUC vector (Biovector, USA) at the SalI restriction site to produce the BRC1−LUC construct. All these constructs were introduced into the A. tumefaciens strain EHA105.

To generate the FHY3 and BRC1 overexpression constructs, the full-length coding sequences of FHY3 and BRC1 were cloned into the pCAMBA1301 vector (Biovector) to produce the FHY3-OE and BRC1-OE constructs, respectively. For rSPL9 overexpression, a mir156-resistant form of SPL9 was cloned into the pSPYCE vector at the SalI restriction site to generate the rSPL9-OE constructs.

The continuous increase in world population and reduced arable land, accompanied by continuously deteriorated climate conditions pose significant challenges to securing global food supply in the coming decades. Increasing planting density has been an effective means of increasing crop yield per unit land area. However, shade avoidance response, which is triggered in response to vegetative proximity or canopy shade, is detrimental to crop productivity. Emerging evidence supports the notion that phytochrome signaling pathway has been targeted for selection during crop domestication and genetic improvement. In addition, it has been shown that in response to shade, inactivation of phytochrome B leads to increased TBI expression and suppression of axillary bud outgrowth in sorghum and maize. Moreover, earlier studies reported that FHY3 promotes branching through an AXR1-dependent pathway. However, the detailed mechanism still remains unknown and will be worthy of further investigation.

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construct. For SMXL6D (a nondegradable mutant form of SMXL6) overexpression, SMXL6D was constructed into the pSPFY vector.

To test the H2O2 resistance of wild-type Arabidopsis, the GSTC1 promoter and the coding region was amplified from wild-type genomic DNA and ligated into the pCAMBIA1300 vector (Biovector) at the EcoRI/HindIII restriction sites. The pBRC1wt::gBRC1 construct was produced by replacing the WT BRC1 promoter with the mutated promoter which is amplified from pBRC1wt::gBRC1 construct in which the two SBP-binding sites GTAC were mutated into TTTT via site-directed mutagenesis.

All sequence confirmed transforming vectors were introduced into A. tumefaciens strain GV3101 and further transformed into WT Arabidopsis or specified backgrounds using the floral dip method. For overexpression vectors, more than 20 independent transgenic lines for each transformation were obtained and 3–5 representative lines (T, generation, homozygous plants) of each vector were selected for gene expression and phenotyping assays. For the smxl6/7/8 CRISPR knockout mutant, 27 independent transgenic lines were obtained and three homologous lines with mutated SMXL6/7/8 (T1 generation) were selected and used for phenotyping assays. For pBRC1wt::gBRC1 and pBRC1m::gBRC1 constructs, more than 10 independent transgenic lines of T1 generation were used for both phenotypic and gene expression assays.

All the primers used for plasmid construction are shown in Supplementary Table 1.

RNA extraction and gene expression analysis. For gene expression analysis, tissues were frozen in liquid nitrogen and total RNA was extracted using the Trizol reagent (Invitrogen, USA). For total RNA analysis, axillary buds were harvested for BRC1 and leaves from 3-week-old N. benthamiana plants for BRC1 and SPLs-SPYCE overexpression, and leaves from 3-week-old plants for BRC1 CRISPR knockout mutant, 27 independent transgenic lines were obtained and 5 representative lines (T3 generation, homozygous plants) of each construct were selected and used for gene expression analysis.

ChIP-qPCR assay. Eight-day-old transgenic seedlings of 35S::SPL9-HA or 35S::SPL15-MYC were cross-linked with 1% formaldehyde and ground in liquid nitrogen. The chromatin complex was prepared following the method of Xie et al.24. In brief, the supernatant was pre-cleared with 40 μl Protein-A-Agarose (16-157, EMD Millipore Corp.) and incubated at 4 °C for 1 h. Then the supernatant was moved into a microtube and 5 μl HA tag monoclonal antibodies (CB100005M, Cali-Bio) were added. After incubation at 4 °C for overnight with gentle agitation, 40 μl Protein-A-Agarose was added and incubated for 2 h. After washing, the immuno complex was eluted from the agarose beads. The precipitated DNA was then retrieved and quantified using quantitative PCR with their individual primer pairs. The values were standardized to the input DNA to obtain the enrichment fold. PP2A was used as the internal control.

Western blotting analysis. For western blotting, 8-day-old seedlings were ground in liquid nitrogen and homogenized in extraction buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1% Tween-20, 1 mM DTT, 1 mM PMSF, and phe-nylmethylsulphonyl fluoride, and complete protease inhibitor cocktail). After spinning, the supernatant was collected and the total protein quantified using the Bradford method. The purified anti-FLAG antibodies (M185-7, BML, Japan) with 1:5000 dilution were used to detect FHY3 or FAR1 proteins. For the actin internal control, the plant actin monoclonal antibodies (BE7008, EASYBIO, China) at a dilution of 1:5000 were used. The western blotting was imaged with the Chemi S 420 Chemiluminescence Imaging System (Biopest, German).

Pull-down and Co-IP assay. For pull-down assay, the purified GST-SPL9SBP or GST-SPL15SBP fusion protein was added to the total protein extract of His-FHY3-I or His-SMXL6, and then incubated for 1 h at 4 °C under rotation. After washing with the protein extract buffer for three times, the samples were boiled with loading buffer and run on 10% SDS-PAGE gels for separation. For the anti-GST antibodies (PM013-7, BML, Japan) and anti-His antibodies (D291-7, BML, Japan) with 1:5000 dilution were used to detect the protein level of SPL-SPB and HY3-I/SMXL6, respectively.

For Co-IP, N. benthamiana leaves infiltrated with Agrobacterium harboring the constructs SPL9/15-MYC and FHY3/SMXL6-HA were collected and homogenized in 2 mL of co-IP buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2 mM DTT, 0.1% Tween-20, 1 mM PMSF, and complete protease inhibitor cocktail). The supernatant was centrifugation at 12,000 g for 2 times (10 min each, the supernatant was collected and incubated for 2 h at 4 °C with anti-HA magnetic agarose beads (M180-10, BML, Japan). After washing with three times with 1 mL of co-IP buffer, the beads were boiled with loading buffer and run on 10% SDS-PAGE gels for separation. For GST antibodies (the PPA2 Antibody, anti-GST, and anti-HA antibodies (61S-7, BML, Japan) with 1:5000 dilution were used to detect the level of SPL9/15 or FHY3/SMXL6 proteins, respectively.
Measurement of relative fluorescence. The average fluorescence pixel intensity of each root was determined using Image J software (http://rsbweb.nih.gov/ij/; version 1.38) as described by Ye et al.5,25. In brief, draw a close freestyle line using the “straight” tool in the software along the outline of individual root and then do “subtract background” and calculate the average fluorescence using the “measure” tool.

Statistical analysis. All real-time PCR and other quantitative analysis were repeated at least three times. To evaluate the significant difference among genotypes treated with or without EOD-FR, the method of two-way analysis of variance with interaction was adopted by using SPSS software (version IBM SPSS Statistics 22.0). The least significance difference (LSD) test was adopted to analyze the significant difference among multiple groups. The Student t test was used to analyze the significant difference between two groups. The graphs with black dots and bars were produced using GraphPad Prism software (version 7.04) by entering the raw data.

Data availability
All data supporting the findings of this study are included within the article and its Supplementary Information files or available upon request from the authors. The source data for Figs. 1b–e, 2b–e, 3d–f, 4b–d, 5b–e, 6b and Supplementary Figs. 1–5, 7–9, 11 and 12, 14, 16, 18, 23 are provided in the Source Data file.

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Author contributions
H.Wang and Y.X. designed the research; Y.X., Y.L., M.M., Q.Z., Y.Z., B.Z., B.W. and H.Wei performed the research and analyzed the data; H.Wang and Y.X. wrote the paper.

Competing interests
The authors declare no competing interests.

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