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Research Article

bHLH transcription factors LP1 and LP2 regulate longitudinal cell elongation

Rui Lu ,[1] Jiao Zhang,[1] Yu-Wei Wu,[1] Yao Wang ,[1] Jie Zhang,[1] Yong Zheng ,[1] Yang Li [1] and Xue-Bao Li [1,*,†]

1 Hubei Key Laboratory of Genetic Regulation and Integrative Biology, School of Life Sciences, Central China Normal University, Wuhan 430079, China

*Author for communication: xbli@mail.ccnu.edu.cn
†Senior author

X.B.L. and R.L. conceived and designed the research; R.L., J.Z., Y.W.W., J.Z., Y.W., Y.Z. and Y.L. performed the experiments; R.L., Y.L. and X.B.L. analyzed data; R.L. and X.B.L. wrote the paper.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Xue-Bao Li (xbli@mail.ccnu.edu.cn)

Abstract

Basic helix–loop–helix/helix–loop–helix (bHLH/HLH) transcription factors play substantial roles in plant cell elongation. In this study, two bHLH/HLH homologous proteins leaf related protein 1 and leaf-related protein 2 (AtLP1 and AtLP2) were identified in *Arabidopsis thaliana*. LP1 and LP2 play similar positive roles in longitudinal cell elongation. Both *LP1* and *LP2* overexpression plants exhibited long hypocotyls, elongated cotyledons, and particularly long leaf blades. The elongated leaves resulted from increased longitudinal cell elongation. *lp1 and lp2* loss-of-function single mutants did not display distinct phenotypes, but the *lp1lp2* double mutant showed decreased leaf length associated with less longitudinal polar cell elongation. Furthermore, the phenotype of *lp1lp2* could be rescued by the expression of *LP1* or *LP2*. Expression of genes related to cell elongation was upregulated in *LP1* and *LP2* overexpression plants but downregulated in *lp1lp2* double mutant plants compared with that of wild type. LP1 and LP2 proteins could directly bind to the promoters of *Longifolia1 (LNG1)* and *LNG2* to activate the expression of these cell elongation related genes. Both LP1 and LP2 could interact with two other bHLH/HLH proteins, IBH1 (ILI1 binding BHLH Protein1) and IBL1 (IBH1-like1), thereby suppressing the transcriptional activation of LP1 and LP2 to the target genes *LNG1* and *LNG2*. Thus, our data suggested that LP1 and LP2 act as positive regulators to promote longitudinal cell elongation by activating the expression of *LNG1* and *LNG2* genes in Arabidopsis. Moreover, homodimerization of LP1 and LP2 may be essential for their function, and interaction between LP1/LP2 and other bHLH/HLH proteins may obstruct transcriptional regulation of target genes by LP1 and LP2.

Introduction

The plant leaf is an essential component of whole plant architecture, serving as the basis of primary productivity. Understanding how plants determine leaf shape is a priority issue in plant biology. Leaf morphogenesis is composed of leaf initiation, establishment of polarity, and leaf expansion (Dkhar and Pareek 2014). The leaf shape is initially built up by the leaf petiole and leaf blade. In *Arabidopsis thaliana*, leaf blades expand in two dimensions which are identified as leaf-length (longitudinal) and leaf-width (lateral) axes. The ratio of the two directions is used as the criterion to determine leaf blade shape (Bowman et al., 2002). *Angustifolia* (*AN*) regulated polar cell elongation in the leaf-width direction, and the loss function of AN results in narrower leaves

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(Kim et al., 2002). *Rotundifolia 3* (*ROT3*) is involved in polar elongation in the leaf-length direction. *ROT3* overexpression transgenic plants exhibited longer leaves in the longitudinal direction, but leaf shape is not altered in leaf width direction (Kim et al., 1999). Another two leaf-length regulation genes *Longifolia1* (*LNG1*) and *LNG2* were reported to function independently of *ROT3*. A dominant mutant *lng1-1D* exhibited a long leaf blade phenotype and other elongated organs, due to increased polar cell elongation rather than increased cell proliferation. Loss-of-function mutants for *lng1* and *lng2* exhibited a shortened length of leaf blades, associated with less longitudinal polar cell elongation (Lee et al., 2006b; Lee and Kim, 2018). LNG3 and LNG4 are two homologs of LNG1, and LNG1/2/3/4 function redundantly in regulating polar cell elongation in Arabidopsis leaves. Single loss-of-function mutants, *lng3* and *lng4*, do not show any distinctly altered phenotypes, but mutants of *lng quadruple* (*lngq*), and *lng1/2/3* and *lng1/2/4* triples, display decreased leaf length, compared with wild type (Lee et al., 2018). An Arabidopsis cold shock domain protein was shown to positively regulate leaf cell elongation by affecting LNG1 accumulation during leaf development (Yang et al., 2012).

Basic helix–loop–helix/helix-loop-helix (bHLH/HLH) proteins are a superfamily of transcription factors containing the HLH domain that is composed of 50–60 amino acids (Heim et al., 2003; Toledo-Ortiz., 2003). bHLH proteins consist of the N-terminal basic region and C-terminal helix–loop–helix region, which have distinct functions in transcriptional regulation process. The basic region, comprising about 15 amino acids with major basic residues, has the functions of identifying and binding to DNA. Meanwhile, the HLH region, containing two amphipathic α-helices separated by a loop region, usually forms homodimers or heterodimers with other HLH domains (Murre et al., 1989; Ferré-D'Amaré et al., 1994; Nair and Burley, 2000).

Previous studies indicated that bHLH/HLH proteins participate in plant hormone signaling, organ development, and so on in Arabidopsis (Heisler et al., 2001; Serna and Martin, 2006; Zhao et al., 2008, Josse et al., 2011; Leivar et al., 2011; Zhao et al., 2012; Dai et al., 2016). In the past decade, researchers suggested that bHLH/HLH proteins, including Paclobutrazol resistant1 (PRE1), Activator for cell elongation1 (ACE1), Homolog of bee2 interacting with IBH1 (HBI1), ILI1 binding BHLH protein1 (IBH1), IBH1-like1 (IBL1), ATBS1-interacting factors (AIFs), and other unidentified bHLH/HLH proteins, play vital roles in the control of cell elongation by a triantagonistic bHLH/HLH system in Arabidopsis. PRE1, encoding an HLH protein, and its homologous have redundant functions to activate gibberellin-dependent development (Lee et al., 2006a). Overexpression of *IBH1*, a negative regulator of brassinosteroid (BR) response, suppresses cell elongation and then causes dwarfism in Arabidopsis. PRE1 forms heterodimers with and inactivates IBH1 to repress the phenotype of *IBH1* overexpression transgenic Arabidopsis (Zhang et al., 2009). IBH1 plays negative roles by heterodimerzing with and inhibiting other DNA binding bHLH proteins, such as HBI1 and ACEs (Bai et al., 2012; Ikeda et al., 2012; Fan et al., 2014). *HBI1* overexpression Arabidopsis exhibited stimulated hypocotyl and petiole elongation, indicating that HBI1 is a positive regulator of cell elongation. HBI1 acts as a transcriptional activator and can directly bind to the promoters to activate two *Expansin* genes. IBH1 could inhibit HBI1's DNA binding and transcriptional activities, but the inhibitory effects of IBH1 were eliminated by PRE1 (Bai et al., 2012; Fan et al., 2014). ACE1/2/3 directly activates the expression of the enzyme genes for cell elongation. Overexpression of *ACE1/2/3* exhibited longer hypocotyls and cotyledons, larger rosette plants, flowers, and petals, due to increased cell length. IBH1 negatively regulates cell elongation by forming heterodimers with ACEs and thus disturbing their DNA binding ability. PRE1 interacts with IBH1 and neutralizes the ability of IBH1 to influence ACEs (Ikeda et al., 2012). Furthermore, IBL1, a close homolog of IBH1, also antagonized BR responses and cell elongation. Although defined as non-DNA binding, IBH1 represses the transcription of IBL1. IBH1 and IBL1 acted in tandem to restrain the expression of downstream *bHLH/HLH* genes, thus forming an incoherent feed-forward loop (Zhiponova et al., 2014). Besides, AIF1/2/3/4 interact with PRE1 and ACE1, similar to IBH1, and also negatively regulate cell elongation in the triantagonistic bHLH/HLH system (Wang et al., 2009; Ikeda et al., 2013; Kim et al., 2017). Additionally, many other bHLH/HLH proteins were reported to regulate cell elongation, including positive regulators (phytochrome interacting factor4 [PIF4]/5, PIL5, and BEE2) and negative regulators (PAR1) (Friedrichsen et al., 2002; Hornitschek et al., 2009; Leivar et al., 2011; Malinovsky et al., 2014; Oh et al., 2014). Here, we demonstrated two bHLH proteins, LP1 and LP2, promote longitudinal cell elongation in Arabidopsis. Our data suggested that LP1 and LP2 function as transcriptional activators to regulate expressions of *LNGs*, thus regulating longitudinal cell elongation redundantly. Both IBH1 and IBL1 interacted with LP1 and LP2 proteins and thus interfered with their transcriptional activities. Our findings provide a comprehensive insight into understanding LP1 and LP2 regulated cell elongation in plants.

Results

Characterization of *LP1* and *LP2* and their expression patterns

Our previous study identified a cotton bHLH protein (GhFP1) and clarified its positive roles in regulating fiber cell elongation of cotton (Liu et al., 2020). To identify the homologues of GhFP1 in Arabidopsis, GhFP1 was employed as a query to perform homologous blast searches against the Araport11 protein sequences (https://www.arabidopsis.org/cgi-bin/Blast/TAIRblast.pl). We found that two proteins leaf-related protein1 (LP1, At2g42300) and leaf-related protein2 (LP2, At3g57800) share 52% and 55% sequence similarities with a cotton bHLH protein GhFP1, respectively (Supplemental Figure S1). Organizational expression patterns of *LP1* and *LP2* were highly consistent, and they both were found to be expressed extensively in different tissues, and at relatively high levels in petals, stamens, carpels and rosette leaves (Figure 1A). In

addition, a 1,802-bp proximal part of *LP1* promoter and a 1,979-bp proximal part of *LP2* promoter were isolated from Arabidopsis genome and fused with the *GUS* reporter gene in pBI101 vector (LP1p:GUS and LP2p:GUS). The recombinant plasmids were introduced into Arabidopsis by *Agrobacterium*-mediated DNA transfer. The transgenic plants expressing *LP1p:GUS* or *LP2p:GUS* fusion genes were obtained for analyzing the expression of *GUS* under the control of *LP1* or *LP2* promoter. At the seedling developmental stage, GUS activity in *LP1p:GUS* transgenic plants was highly detected in the cotyledons, leaf primordia, roots of light-grown seedlings (Figure 1, B–D). At 4 weeks after sowing, GUS signals were mostly observed in leaf blades (Figure 1E). As further developed, the GUS activity was detected in floral organs, including petals, sepals, and pistil stigmas, but no or weak GUS staining was found in young fruits (Figure 1, F–H). Similarly, GUS activity in *LP2p:GUS* transgenic plants was highly detected in the cotyledons, leaf primordia, and roots in light-grown conditions after 6 d of sowing (Figure 1, I–K). Expression of GUS expanded to leaf blade along the leaf mid-veins of the *LP2p:GUS* transgenic plants after 4 weeks sowing (Figure 1L). Besides, the GUS activity was observed in floral organs of *LP2p:GUS* transgenic plants, but no or very weak signals were found in other tissues (Figure 1, M–O). To visualize the expression patterns of *LP1* and *LP2* in leaf primordia, 1,802-bp fragment of *LP1* promoter or 1,979-bp fragment of *LP2* promoter were fused to reporter *H2B-GFP*, generating *LP1p:H2B-GFP* or *LP2p:H2B-GFP* recombinant plasmids. The transgenic plants expressing *LP1p:H2B-GFP* or *LP2p:H2B-GFP* fusion genes were obtained. GFP signals were observed widely throughout leaf primordia of 6-d-old *LP1p:H2B-GFP* or *LP2p:H2B-GFP* transgenic seedlings (Supplemental Figure S2). These data suggested that *LP1* and *LP2* were widely expressed in the leaf blade at different growth stages of Arabidopsis and display overlapping expression patterns, indicating that *LP1* and *LP2* may be functionally overlapping genes.

LP1 and LP2 regulate longitudinal cell elongation redundantly in Arabidopsis

To investigate the roles of LP1 and LP2 in Arabidopsis, *LP1* and *LP2* under the control of Caulifower mosaic virus (CaMV) 35S promoter were introduced into the wild-type Arabidopsis to get heritable overexpression transgenic plants, respectively.

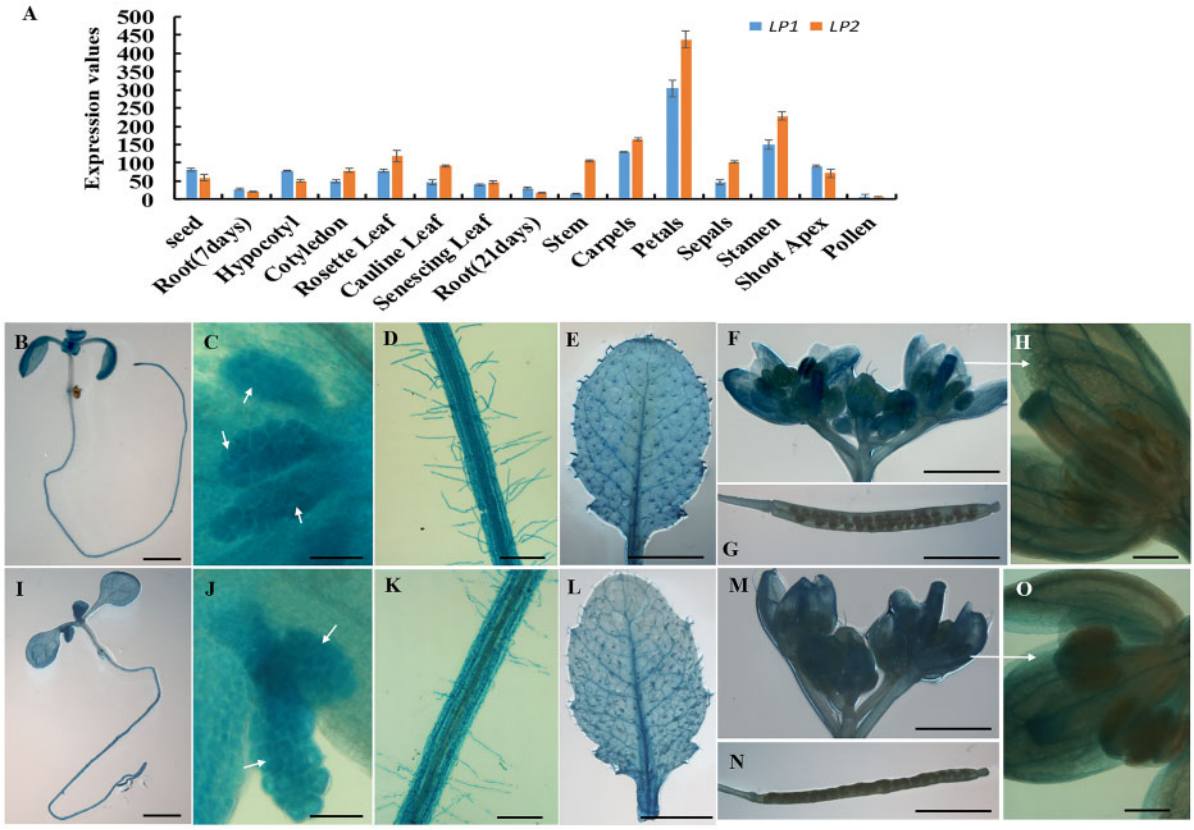

Figure 1 Analysis of expression of *LP1* and *LP2* genes in Arabidopsis tissues. A, Expression profiles of *LP1* and *LP2* genes for 15 different tissue samples through public available microarray data (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Numbers followed by root indicate days after sowing. B–O, Histochemical assay of activity of GUS under the control of *LP1* or *LP2* promoter in the *LP1p:GUS* or *LP2p:GUS* transgenic plants. B, A 6-d-old *LP1p:GUS* transgenic seedling. C, Leaf primordia of 6-d-old *LP1p:GUS* transgenic seedling. D, The root of 6-d-old *LP1p:GUS* transgenic seedling. E, A fifth rosette leaf of 28-d-old *LP1p:GUS* transgenic plant. F, Flower organs of *LP1p:GUS*. G, A fruit of *LP1p:GUS*. H, A flower organ in F. I, A 6-d-old *LP2p:GUS* transgenic seedling. J, Leaf primordia of 6-d-old *LP2p:GUS* transgenic seedling. K, The root of 6-d-old *LP2p:GUS* transgenic seedling. L, A fifth rosette leaf of 28-d-old *LP2p:GUS* transgenic plant. M, Flower organs of *LP2p:GUS*. N, A fruit of *LP2p:GUS*. O, A flower organ in M. Bars = 50 mm in (B, E–G, I, and L–N). Bars = 100 μm in (C, D, H, J, K, and O).

Furthermore, *LP1* single mutant (pYAO:hSpCas9-LP1-sgRNA), *LP2* single mutant (pYAO:hSpCas9-LP2-sgRNA), and *lp1lp2* double mutant (pYAO:hSpCas9-LP1-sgRNA-LP2-sgRNA) were obtained by the CRISPR/Cas9 gene-editing method. As shown in Supplemental Figure S3, A and B, the expressions of *LP1* and *LP2* were significantly upregulated in *LP1* overexpression and *LP2* overexpression plants, respectively. On the other hand, DNA sequence alignment indicated successful gene editing around the target region of *LP1* and *LP2* (Supplemental Figure S3, C–E). Compared with wild-type, *LP1* and *LP2* overexpression plants had longer hypocotyls and increased aspect ratio of the cotyledon, but *lp1* and *lp2* single mutants did not exhibit the obviously altered phenotypes. However, the length of hypocotyls and aspect ratio of the cotyledon in *lp1lp2* double mutant was indeed reduced relative to that in wild-type, implying that LP1 and LP2 have redundant roles in regulating plant development (Figure 2, A–C). To further elucidate the function of LP1 and LP2, we analyzed the phenotypes of the transgenic plants 4 weeks after sowing. *LP1* and *LP2* overexpression plants showed particularly longer leaf blades, whereas the double mutant *lp1lp2* exhibited small-sized orbicular leaves with decreased leaf length (Figure 2, D and E). Additionally, we expressed the *LP1* or *LP2* gene under the control of native promoter in *lp1lp2* mutant and found the phenotype of these transgenic plants was similar to that of wild type, indicating *lp1lp2* mutant could be rescued by expression of *LP1* or *LP2* (Figure 2).

To determine why the shape of cotyledon was changed in *LP1* and *LP2* transgenic plants, we observed the size and shape of epidermal cells and palisade mesophyll cells in the middle portion of the 6-d-old cotyledon. The length of both epidermal cells and palisade mesophyll cells of *LP1* and *LP2* overexpression plants was much longer than that of the wild-type, while *lp1lp2* double mutant exhibited shorter cell length compared with the wild-type. However, the width of epidermal cells and palisade cells of *LP1* and *LP2* transgenic plants was similar to that of wild-type (Figure 3, A–M). As the fifth leaf is considered a good representative of the rosette leaves in Arabidopsis (Tsuge et al., 1996), we also observed cell size and shape of the epidermis and palisade in the middle portion of 28-d-old fifth rosette leaves. *LP1* and *LP2* overexpression plants showed much longer epidermal and palisade mesophyll cells, whereas *lp1lp2* double mutant exhibited shorter epidermal and palisade cells compared with the wild-type (Supplemental Figure S4, A–M). Besides, the aspect ratio of epidermal cells and palisade cells in *LP1* and *LP2* overexpression plants was increased, but in *lp1lp2* double mutant was decreased compared with that of the wild-type (Supplemental Figure S5). These data indicated that LP1 and LP2 promote cell elongation in the leaf-length direction, resulting in longer cotyledon and leaf blades. On the other hand, the altered shape of epidermal cells and palisade cells in the double mutant could be recovered by expressing *LP1* or *LP2* (Figure 3; Supplemental S4). Trichomes on leaves of Arabidopsis are also regarded as a good model for studying polar cell elongation (Payne et al., 2000; Wan et al., 2014). The trichome shape on fifth leaves of the transgenic plants was observed and trichome length was measured, using wild-type as control. Similar to epidermal and palisade cells, the shape of trichomes on leaves of the overexpression transgenic plants was quite different from that of wild-type (Supplemental Figure S6, A–G). The average trichome length on *LP1* and *LP2* overexpression plants was significantly increased, but there was no distinct difference in trichome length between the double mutant and wild-type, indicating that LP1 and LP2 may promote trichome development in Arabidopsis (Supplemental Figure S6, A–G). The larger size of cells is often associated with enhanced endoreduplication. Therefore, the trichome nuclear ploidy level of transgenic plants was studied, using wild-type as control. Fluorescence signals of trichome nuclei on *LP1* and *LP2* overexpression plants were dramatically enhanced compared with wild-type, but there was no significant difference in fluorescence signals of trichome nuclei between the *lp1lp2* double mutant and wild-type, suggesting that the enlarged cell size was accompanied by enhanced endoreduplication (Supplemental Figure S6, H–N). The above results suggested that LP1 and LP2 play positive roles in regulating polar cell elongation redundantly in Arabidopsis.

LP1 and LP2 as transcriptional activators bind to the promoters of *LNG1* and *LNG2* to positively regulate the expression of these genes

To investigate the localization of LP1 and LP2 proteins in cells, *35S:LP1:eGFP* and *35S:LP2:eGFP* vectors were constructed and transferred into Arabidopsis. As shown in Figure 4, A–C and F–H, both the GFP fluorescence and 4′6-diamidino-2-phenylindole (DAPI) staining were detected in cell nuclei of the transgenic plants, indicating that both LP1 and LP2 proteins were localized in the cell nucleus. Furthermore, a yeast two-hybrid system was employed to analyze whether LP1 and LP2 have transcriptional activation activity. The pGBKT7-LP1 and pGBKT7-LP2 fusion vectors were transferred into yeast strains AH109 and Y187, respectively. The experimental results showed that the transformants grew normally on a selection medium (SD/−Trp/−Ade) (Figure 4, D and I), while the transformants turned blue at the presence of IPTG (isopropy-β-D-thiogalactoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Figure 4, E and J). The above results indicated that the reporter gene *LacZ* was activated in yeast cells, implying that LP1 and LP2 may act as transcriptional activators in cells.

To understand the molecular mechanism of LP1 and LP2 regulated polar cell elongation, we analyzed the expression levels of several cell elongation-related genes in leaves of 4-week-old Arabidopsis plants. As shown in Figure 5A, the expression levels of *LNG1* and *LNG2*, the two genes that regulate longitudinal cell elongation in Arabidopsis, were significantly upregulated in the *LP1* and *LP2* overexpression plants,

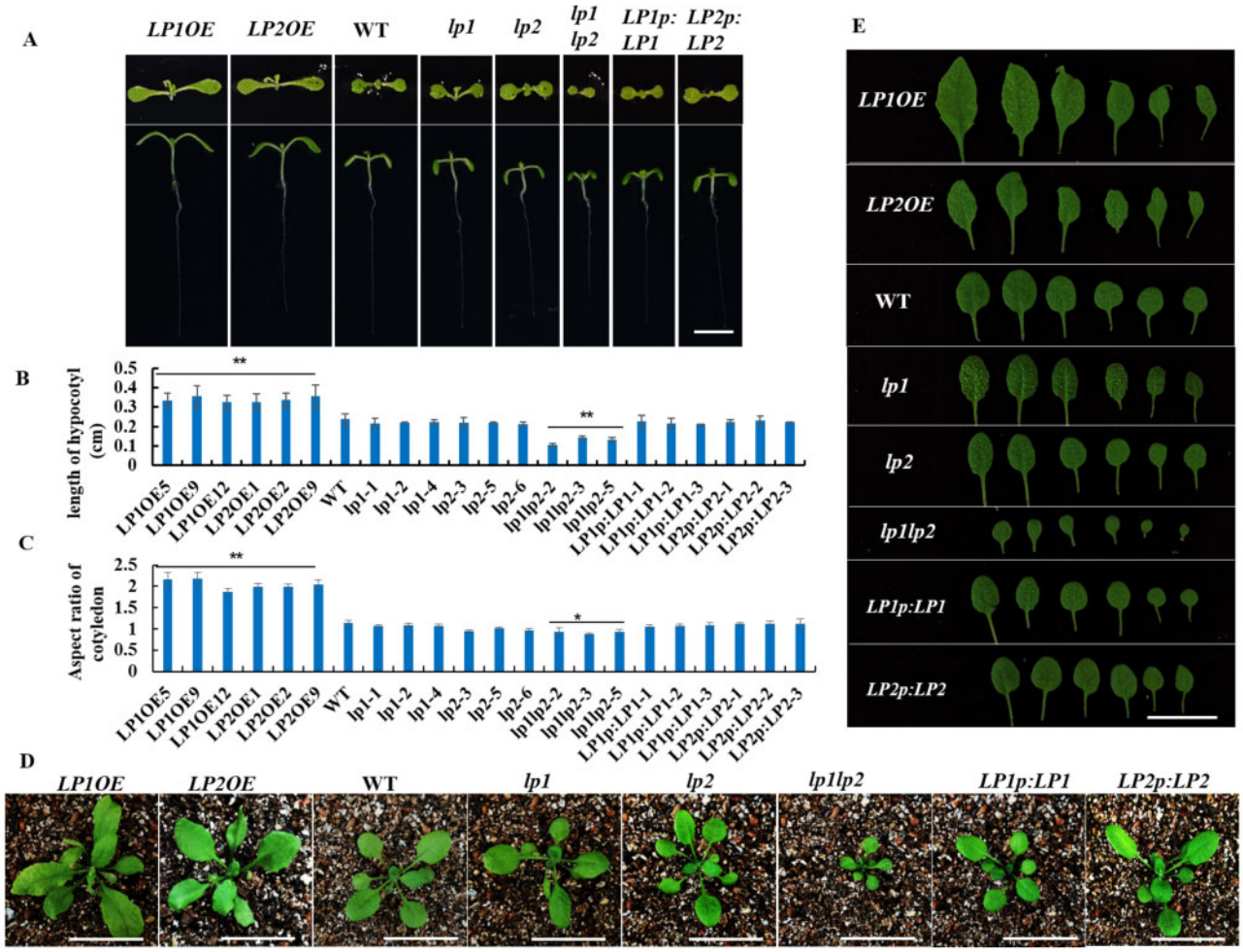

Figure 2 Morphological phenotypic analysis of *LP1 and LP2* transgenic Arabidopsis plants. A, 6-d-old transgenic seedlings, mutants and wild type. Bar = 0.5 cm. B and C, Measurements and statistical analysis of hypocotyl length (B) and aspect ratio of the cotyledon (C) of 6-d-old transgenic lines, mutants, and wild type ($n \geq 30$). Data were dealt with Microsoft Excel, and error bars represent the standard deviations. One or two asterisks represented there was a significant (*$P < 0.05$) or very significant (**$P < 0.01$) difference between wild type and transgenic lines or mutants. D, 28-d-old above ground parts of the transgenic plants, mutants, and wild type. Bar = 2 cm. E, The fourth to eighth rosette leaves of 28-d-old transgenic plants, mutants, and wild type. Bar = 2 cm. WT, wild-type; *LP1OE*, *LP1* overexpression lines (including *LP1OE5*, *LP1OE9*, and *LP1OE12*); *LP2OE*, *LP2* overexpression lines (including *LP2OE1*, *LP2OE2*, and *LP2OE9*); *lp1*, *LP1* single mutant lines (including *lp1-1*, *lp1-2*, and *lp1-4*); *lp2*, *LP2* single mutant lines (including *lp2-3*, *lp2-5*, and *lp2-6*); *lp1lp2*, *LP1*, and *LP2* double mutant lines (including *lp1lp2-2*, *lp1lp2-3*, and *lp1lp2-5*); *LP1p:LP1*, *LP1* overexpression lines under the control of itself promoter in *lp1lp2* background (including *LP1p:LP1-1*, *LP1p:LP1-2*, and *LP1p:LP1-3*); *LP2p:LP2*, *LP2* overexpression lines under the control of itself promoter in *lp1lp2* background (including *LP2p:LP2-1*, *LP2p:LP2-2*, and *LP2p:LP2-3*).

but downregulated in the *lp1lp2* double mutant. In addition, *XTH4/17* (*xyloglucan endotransglucosylase/hydrolases 4/17*) and *EXP8* (*expansin 8*) related to cell elongation were also upregulated in the *LP1* and *LP2* overexpression plants, but downregulated in the *lp1lp2* double mutant (Figure 5A). However, the expression levels of *XTH15/24* and BR biosynthesis genes involved in cell elongation were not significantly changed in the *LP1* or *LP2* transgenic plants, compared with wild type (Supplemental Figure S7). These results indicated that LP1 and LP2 may be involved in modulating the expressions of *LNG1/2*, *XTH4/17*, and *EXP8* and thereby promoting longitudinal cell elongation.

To further analyze whether LP1 and LP2 directly regulate the expression of longitudinal cell elongation related genes, we isolated the promoter fragments of *LNG1* and *LNG2* genes in the Arabidopsis genome. Bioinformatic analysis revealed the potential LP1 and LP2 binding cis-elements that existed in the promoter sequences of these genes. As shown in Figure 5B, *LNG1* promoter sequence contains putative nine E-boxes (CANNTG) located upstream of the transcription start site, while *LNG2* promoter contains 10 E-box elements distributed randomly upstream of the transcription start site. We performed a yeast one-hybrid assay to study whether LP1 and LP2 bind to the promoter sequences of *LNG1* and *LNG2*. The conditions of Y1HGold yeast transformants of pGADT7-LP1/*LNG2p*-pAbAi cultured on SD/−Leu nutritional-deficient medium were the same as the Y1HGold yeast transformants of pGADT7/*GhLNG2p*-pAbAi (negative control). When the transformants were assayed for growth on nutritional selection medium SD/−

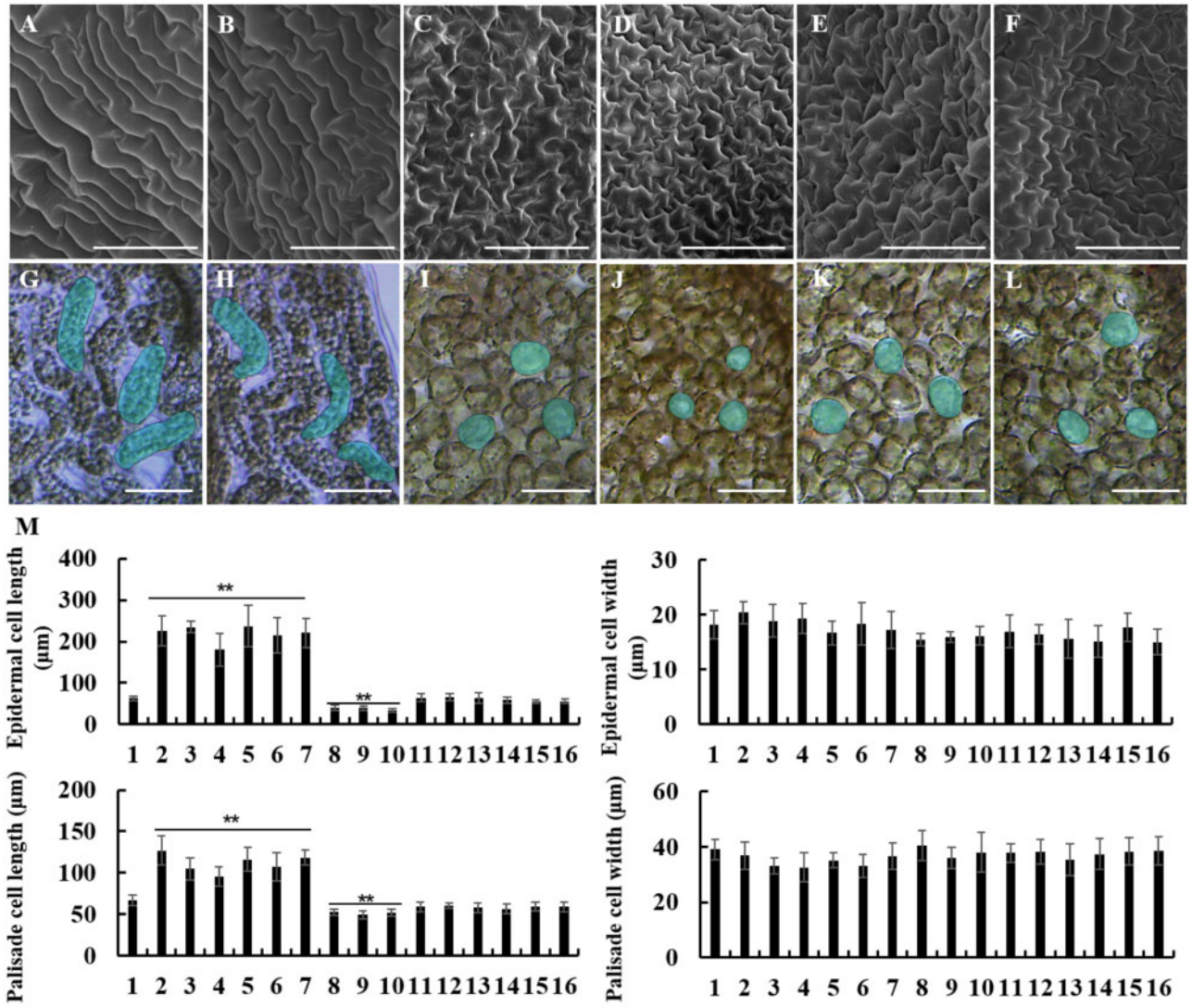

Figure 3 LP1 and LP2 affect longitudinal cell elongation in Arabidopsis. Anatomical analysis was performed with cotyledons of 6-d-old seedlings. Images were taken from the same position in the middle part of an adaxial leaf blade. A–F, Images of epidermal cells in leaves of *LP1* overexpression line (A), *LP2* overexpression line (B), wild-type (C), *lp1lp2* double mutant (D), *LP1p:LP1* line (E), and *LP2p:LP2* line (F) under the scanning electron microscope. G–L, Images of palisade mesophyll cells in leaves of *LP1* overexpression line (G), *LP2* overexpression line (H), wild-type (I), *lp1lp2* double mutant (J), *LP1p:LP1* line (K), and *LP2p:LP2* line (L). M, Measurement and statistical analysis of cell length and width of *LP1* overexpression lines, *LP2* overexpression lines, wild-type, and *lp1lp2* double mutant, *LP1p:LP1* lines and *LP2p:LP2* lines in (A–L) ($n \geq 30$). Data were dealt with Microsoft Excel, and error bars represent the standard deviations. Independent *t* tests demonstrated that there was a significant ($^*P < 0.05$) or very significant ($^{**}P < 0.01$) difference between wild-type and transgenic lines or mutants. 1, wild-type; 2–4, three *LP1* overexpression lines (*LP1OE5*, *LP1OE9*, and *LP1OE12*); 5–7, three *LP2* overexpression lines (*LP2OE1*, *LP2OE2*, and *LP2OE9*); 8–10, three *LP1 and LP2* double mutant lines (*lp1lp2-2*, *lp1lp2-3*, and *lp1lp2-5*); 11–13, three *LP1* overexpression lines (*LP1p:LP1-1*, *LP1p:LP1-2*, and *LP1p:LP1-3*) under the control of *LP1* promoter in *lp1lp2* background; 14–16, three *LP2* overexpression lines (*LP2p:LP2-1*, *LP2p:LP2-2*, and *LP2p:LP2-3*) under the control of *LP2* promoter in *lp1lp2* background. Bars = 100 μm.

Leu with Aureobasidin A (AbA) (150 ng/mL), LP1 could directly bind to *LNG2* promoter region but did not bind to *LNG1* promoter region (Figure 5C). Similarly, the direct binding of LP2 to *LNG1* and *LNG2* promoter region was detected on nutritional selection medium SD/−Leu with AbA (150 ng/ml) (Figure 5D). Furthermore, we also conducted chromatin immunoprecipitation (ChIP) assay to determine whether LP1 and LP2 bind to the promoters of *LNG1* and *LNG2 in vivo*. As shown in Figure 5B, the promoter regions of the target genes were separated into several fragments (chip1–chip8). Compared with CK, the chip7 fragment was substantially enriched in LP1-bound chromatin DNA (Figure 5E), and the chip4 and chip7 fragments were observably enriched in LP2-bound chromatin DNA (Figure 5F). ChIP assays demonstrated that LP1 could bind to the chip7 fragment containing the cis-acting elements of *LNG2* promoter, and LP2 could bind to the chip4 fragment of *LNG1* promoter and the chip7 fragment of *LNG2* promoter *in vivo*.

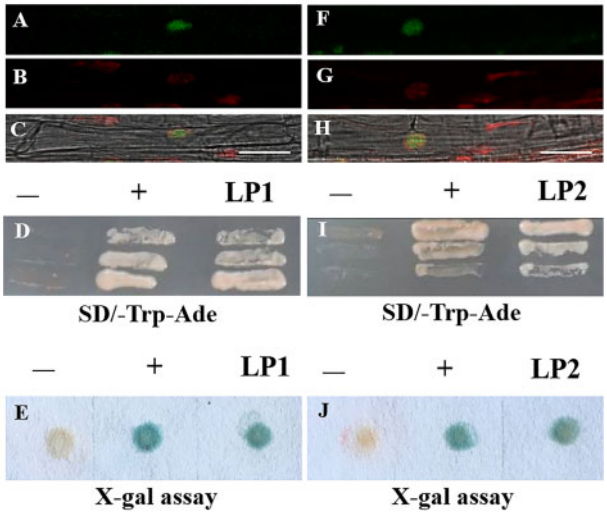

Figure 4 Subcellular localization and transactivation activity assay of LP1 and LP2 proteins. A–C and F–H, Subcellular localization of LP1 and LP2 proteins. Green fluorescence signals were localized to the cell nucleus of the *LP1:eGFP* and *LP2:eGFP* transgenic Arabidopsis hypocotyl cells. A and F, Confocal microscopy of GFP fluorescence in cells expressing LP1:eGFP (A) and LP2:eGFP (F). B and G, Nuclear DAPI staining of the same cells in images (A) and (F). C and H, images (A and B) and images (F and G) superimposed over the bright-field images, respectively. Bars = 25 μm. D, E, I, and J, Transactivation activity assay of LP1 and LP2 proteins in yeast cells. D and I, Yeast AH109 transformants of LP1 (D) and LP2 (I) were streaked on SD/−Trp/−Ade medium. E and J, Flash-freezing filter assay of the β-galactosidase activity in Yeast Y187 transformants of LP1 (E) and LP2 (J). −, negative control; +, positive control.

These results indicated that both LP1 and LP2 directly bind to the promoters of *LNG1* and *LNG2* to positively regulate the expression of these cell elongation-related genes.

Interactions among Arabidopsis bHLH/HLH proteins affect functions of LP1 and LP2

To further study the functions of LP1 and LP2 in longitudinal cell elongation, we next performed yeast two-hybrid (Y2H) screens to identify interaction proteins of LP1 and LP2 using a library of Arabidopsis cDNAs. In Y2H screening for LP1 protein, a total of 20 positive clones were obtained, and interesting clones were further confirmed in the one-to-one interaction analysis. Finally, we found two atypical homologous HLH proteins (IBH1 and IBL1) and LP1 itself among these identified proteins. In Y2H screening for LP2 proteins, we found LP2 also interacted with IBL1, IBH1 and LP2 itself (Figures 6, A and B, and 7, A). We also confirmed these interactions by conducting Luciferase complementation imaging (LCI) assay in transient expression experiments using leaf epidermal cells of *Nicotiana benthamiana* (Figure 6, C and D, and 7, B). Besides, Pull-down assays also displayed that LP1 and LP2 could interact with IBL1 and IBH1 *in vitro* (Figure 6, E and F). Coimmunoprecipitation (CoIP) assays further verified these interactions *in vivo* (Figures 6, G and H, and 7, C). These results demonstrated that LP1 and LP2 not only form homodimers but also form heterodimers with IBL1 and IBH1, thereby possibly influencing LP1 and LP2's functions in cell elongation.

Arabidopsis IBH1 and IBL1 are non-DNA binding proteins, but the ectopic accumulation of IBH1 or IBL1 causes a severe dwarf phenotype by antagonizing BR responses and cell elongation (Zhiponova et al., 2014). Subsequently, we examined the transcriptional activities of IBH1 and IBL1. As shown in Supplemental Figure S8, yeast cells could neither grow on the SD/−Trp/−Ade medium, nor change blue in the X-gal assay, revealing that both IBL1 and IBH1 lack the activity of transcriptional activation. These data indicated that IBH1 and IBL1 may negatively regulate cell elongation by interacting with the LP1 and LP2 and thus interfering with their transcriptional activities. Therefore, a dual-luciferase (LUC) assay system was employed to examine if the protein interaction would affect the transcriptional activation of target genes. The level of the LUC activity controlled by *LNG2* promoter was elevated remarkably when LP1 was expressed, but this activation was impaired observably when the IBL1 or IBH1 was coexpressed with LP1 (Figure 7D). Similarly, the level of the LUC activity regulated by *LNG1* and *LNG2* promoters was enhanced significantly when LP2 was expressed, but this activation was suppressed by the IBL1 and IBH1 (Figure 7D). These data indicated that LP1 and LP2 may form homodimers to activate expressions of *LNG1* and *LNG2*, but IBL1 and IBH1 may repress LP1's and LP2's functions through forming heterodimers (Figure 7E).

Discussion

bHLH/HLH proteins form complicated transcriptional network regulating many biological processes in plants. A number of bHLH/HLH transcription factors regulate cell elongation in Arabidopsis (Oh et al., 2014; Zhiponova et al., 2014). In this study, we identified two bHLH proteins and demonstrated their functionally redundant roles in regulating polar cell elongation by directly regulating two *LNG* genes (*LNG1* and *LNG2*). It has been reported that *LNG1 and LNG2* are two leaf-length regulation genes (Lee et al., 2006b; Lee and Kim, 2018). Our data revealed that overexpression of *LP1* or *LP2* in Arabidopsis individually led to longitudinal polar cell elongation, but *lp1* and *lp2* single mutants exhibited no distinctly altered phenotypes, while double mutant *lp1lp2* showed obviously altered phenotypes, compared with wild-type, implying that LP1 and LP2 have overlapping roles in regulating cell elongation. Furthermore, LP1 and LP2 could directly bind to E-box elements in promoters of *LNG1* and *LNG2*, suggesting LP1 and LP2 promote polar cell elongation possibly by activating expression of the cell elongation-related genes *LNG1* and *LNG2*. The bHLH transcription factor PIF4 also promotes expression of *LNG1* and *LNG2* to induce thermomorphogenic growth in Arabidopsis (Hwang et al., 2017). Our study shows the connections between bHLH proteins and *LNG* genes in the progress of cell elongation, providing evidence of a complicated system of bHLH regulated cell elongation.

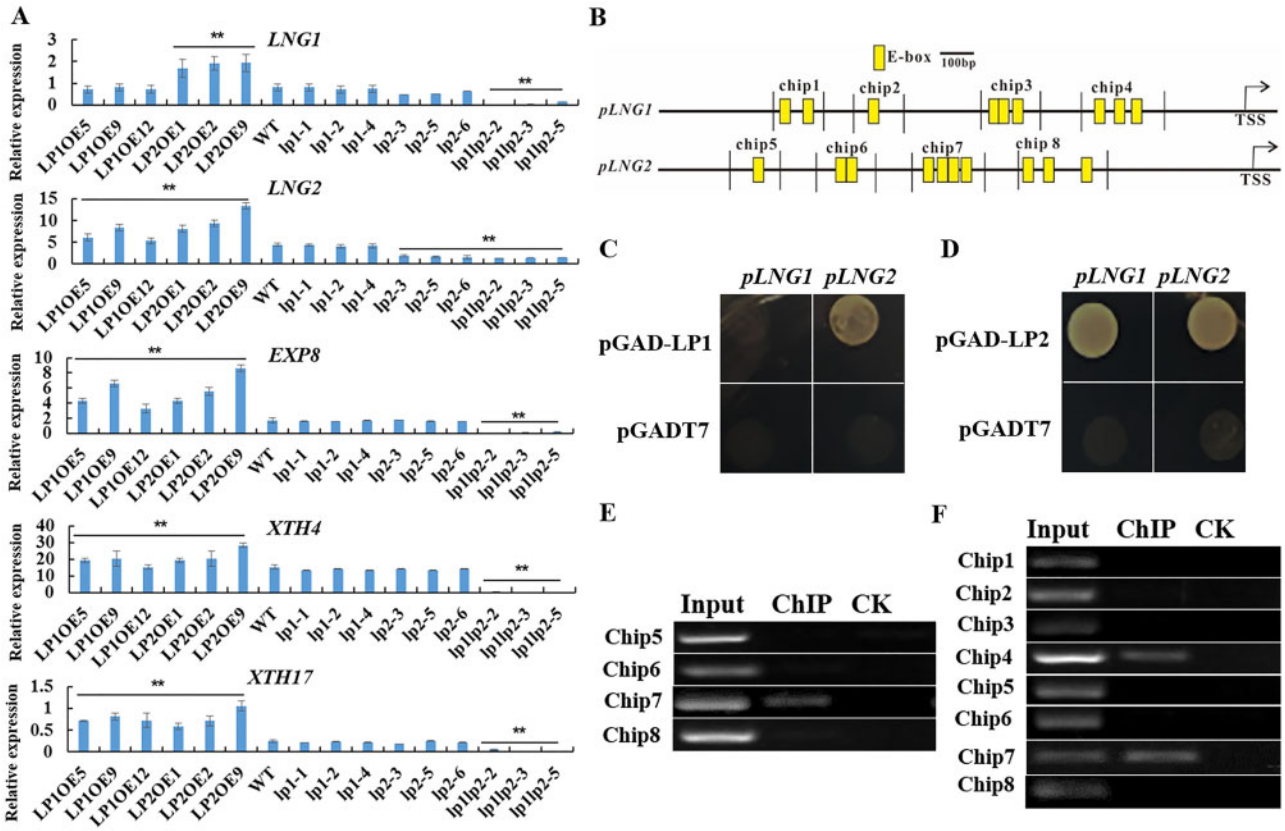

Figure 5 LP1 and LP2 bind to the promoter sequences of *LNG1* and *LNG2*. A, RT-qPCR analysis of the expression of cell elongation-related genes in rosette leaves of wild-type and the transgenic plants. *AtACTIN2* was used as an internal control. Data were processed with Microsoft Excel. Mean values and standard deviation are shown from three biological replicates. Independent *t* tests demonstrated that there was a significant ($^*P < 0.05$) or very significant ($^{**}P < 0.01$) difference in gene expression level between the transgenic lines and wild-type. B, The potential E-box (CANNTG) elements in the promoter sequences of *LNG1* and *LNG2* genes. The cis-elements were predicted by the Plant CARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). TSS, transcription start site. C and D, Yeast one-hybrid assay of LP1 (C) and LP2 (D) interacted with *LNG1* and *LNG2* promoter sequences. Transformants grew on SD/−Leu nutritional selection medium with 150 ng/mL AbA. Y1H Gold yeast transformants of pGADT7/LNGp-pAbAi were used as the negative control. E and F, ChIP assay of LP1 (E) and LP2 (F) proteins binding to the promoters of *LNG1* and *LNG2 in vivo*. ChIP assay was conducted using monoclonal antibodies of LP1 and LP2 proteins. LP1- and LP2-bound chromatin DNA fragments were isolated from rosette leaves of wild type Arabidopsis.

XTH and *EXP* genes promote cell wall expansion by modifying cell wall components or loosening cell walls (Li et al., 2003; Nishitani et al., 2006; Li et al., 2010). Overexpression of *BcXTH1* gene in Arabidopsis resulted in enlarged organs and elongated stem length (Shin et al., 2006). The altered expression of an α-expansin in Arabidopsis modulates leaf growth, indicating the role of expansins in controlling plant organ size (Zenoni et al., 2011). Furthermore, previous studies illustrated that *XTHs* and *EXPs* were regulated by LNGs and bHLH transcription factors in plants. For example, *AtLNG1* overexpressing *N. benthamiana* plants exhibited the increased expression of cell wall modification-related genes *XTH9*, *XTH15* and *XTH33*, resulting in the elongated palisade cells as well as epidermal cells (Lee and Kim, 2018). *LNG* genes have redundant roles in regulating turgor-driven polar cell elongation through activation of *XTH17* and *XTH24* (Lee et al., 2018). Both AtACE1 and AtHBI1 directly promote *AtEXP8*'s expression levels to regulate cell elongation positively (Bai et al., 2012; Ikeda et al., 2012; Fan et al., 2014). Our previous study also suggested that *GhXTH1* and *GhEXPA* participate in regulating rapid fiber cell elongation of cotton (Zhou et al., 2015). In this study, expressions of *XTH4/17* and *EXP8* were significantly altered in the *LP1* and *LP2* overexpression lines and *lp1lp2* double mutant, suggesting these genes may be indirectly regulated by LP1 and LP2, and maybe the target genes of LNGs.

bHLH/HLH proteins include bHLH and HLH proteins, and the difference between the two types of proteins is whether they have N-terminal basic region. The crystal structure of bHLH domain–DNA complex displays the interactions between two HLH regions and each monomer combined half of the DNA recognition sequence (Ma et al., 1994; Shimizu et al., 1997). Both LP1 and LP2 have basic region and HLH regions (Supplemental Figure S1), and have transcriptional activation activity (Figure 4). Moreover, our data revealed that LP1 and LP2 individually form homodimers to recognize and bind to E-box elements in promoters of the target genes, playing positive roles in regulating cell elongation.

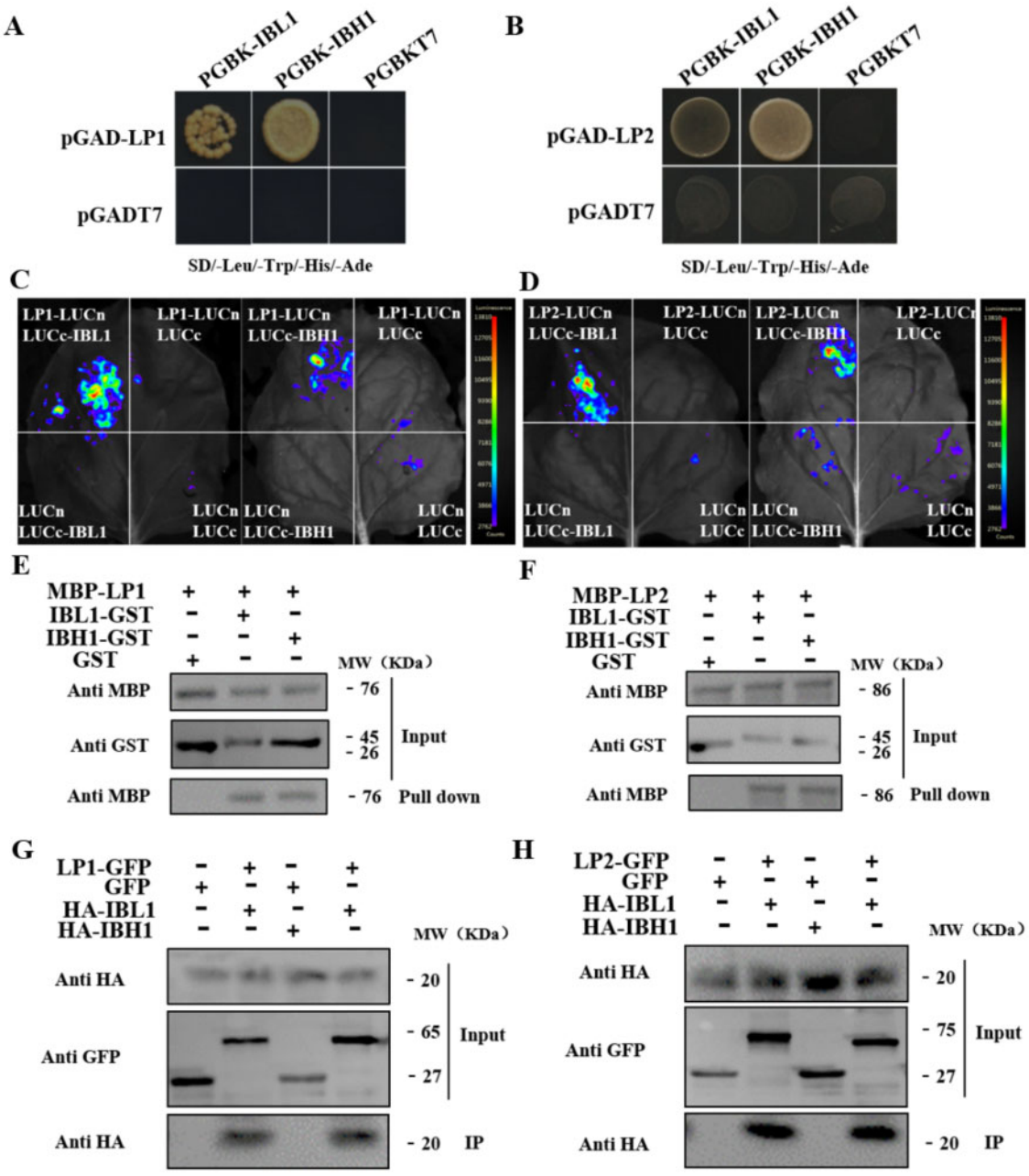

Figure 6 LP1 and LP2 form heterodimers with IBL1 and IBH1. A and B, Yeast two-hybrid assay of the interaction of LP1 (A) or LP2 (B) with IBL1 and IBH1. pGBKT7 and pGADT7 were used as controls. C and D, LCI assay of the interaction of LP1 (C) or LP2 (D) with IBL1 and IBH1. LP1 and LP2 were fused to the amino-terminal of firefly luciferase (LUCn), and IBL1 and IBH1 were fused to carboxyl-terminal of firefly luciferase (LUCc), respectively. The LP1-/LP2-LUCn and LUCc-IBL1/-IBH1 constructs were transiently co-expressed in leaves of *N. benthamiana*, using LUCn and LUCc as the controls. Fluorescence signal intensities represent their binding activities. Right bars indicate the heat map's scales of the signal intensity. E and F, Pull-down assay of the interaction of LP1 (E) or LP2 (F) with IBL1 and IBH1. MBP-LP1 and MBP-LP2 were detected by Western blotting with anti-MBP antibody, and IBH1-GST, IBL1-GST, and GST (control) were detected by Western blotting with anti-GST antibody. G and H, *In vivo* CoIP assay of the interaction of LP1 (G) or LP2 (H) with IBL1 and IBH1. The LP1-/LP2-GFP and IBL1-/IBH1-HA constructs were transiently coexpressed in leaves of *N. benthamiana*, using GFP as the control. LP1-GFP, LP2-GFP, and GFP (control) were detected by Western blotting with anti-GFP antibody, and IBH1-HA and IBL1-HA were detected by Western blotting with anti-HA antibody.

IBL1 and IBH1 are two HLH proteins without containing a basic region, indicating that they cannot recognize DNA sequences. Besides, transactivation activity assay exhibited that IBH1 and IBL1 lack the activity of transcriptional activation, implying that they may function as transcriptional repressors. A previous study also declared that IBH1 could inhibit HBI1's and ACEs' DNA binding and transcriptional activities by interacting with these positive regulators. IBH1 and IBL1 antagonized BR signaling responses and BR controlled cell elongation via repression of IBH1 and IBL1's

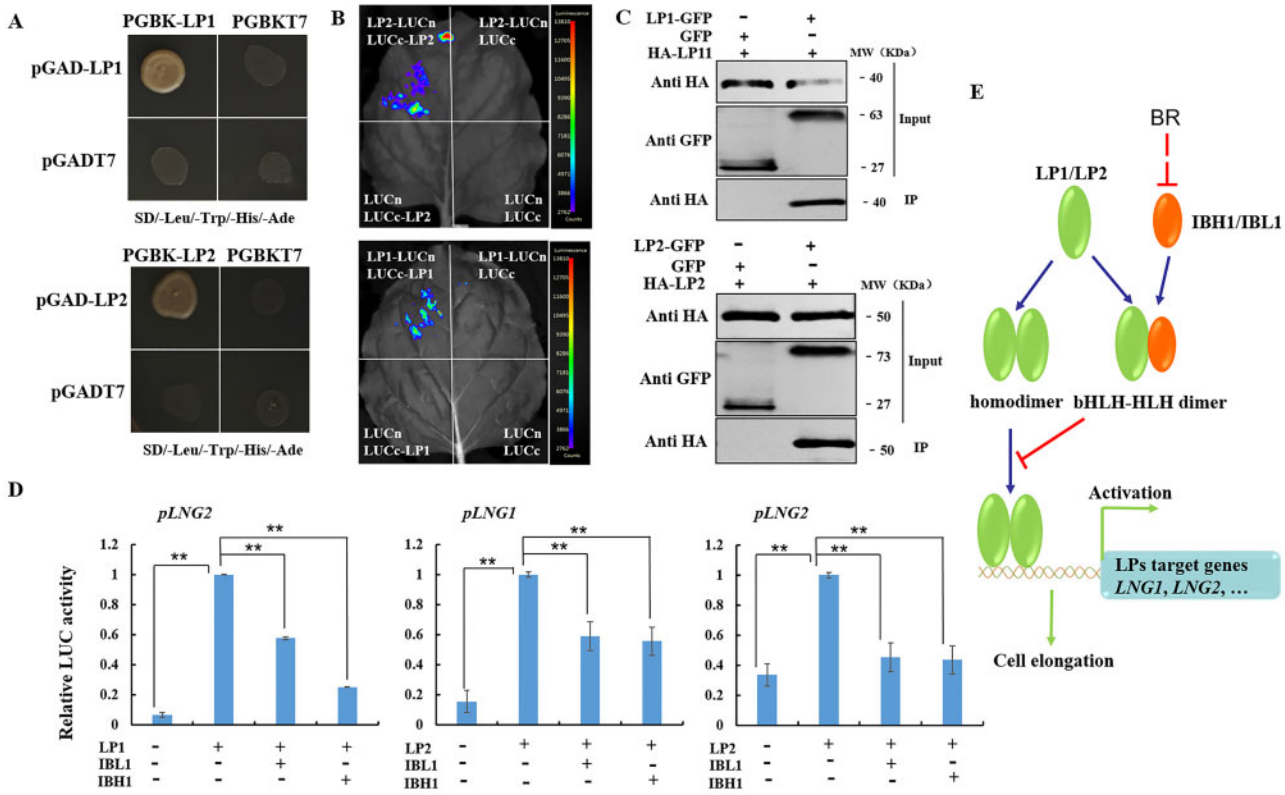

Figure 7 The interactions among bHLH/HLH proteins interfere with transcriptional activation of LP1 and LP2 to the target genes. A, Yeast two-hybrid assay of interactions of LP1 with itself and LP2 with itself. pGBKT7 and pGADT7 were used as controls. B, LCI assay of interactions of LP1 with itself and LP2 with itself. LP1 and LP2 fused to the carboxyl-terminal (LUCc) or amino-terminal (LUCn) of firefly luciferase (LUC), respectively, were transiently co-expressed in leaves of *N. benthamiana*, using LUCn and LUCc as the controls. Fluorescence signal intensities represent their binding activities. The right bar indicated the heat map's scale of the signal intensity. C, *In vivo* CoIP assay of interactions of LP1 with itself and LP2 with itself. The LP1-/LP2-GFP and LP1-/LP2-HA constructs were transiently co-expressed in leaves of *N. benthamiana*, using GFP as a control. LP1-GFP, LP2-GFP, and GFP (control) were detected by Western blotting with anti-GFP antibody, and LP1-HA and LP2-HA were detected by Western blotting with anti-HA antibody. D, Dual-LUC assay of transcriptional activation of LP1 and LP2 to the target genes. *LNG1* and *LNG2* promoters were fused to the *LUC* reporter, respectively, and the promoter activities were determined by a transient dual-LUC transcriptional activation assay in leaves of *N. benthamiana*. The relative LUC activities were normalized to the reference Renilla (REN) luciferase. The corresponding effector (+) and empty vector (−) were co-filtrated. Positive effects of homodimers on activities of *LNG1* and *LNG2* promoters. Inhibitive effects of IBL1 and IBL1 on activation of *pLNG1* and *pLNG2* by LP1 and LP2. Data were dealt with Microsoft Excel, and error bars represent the standard deviations. Independent *t* tests demonstrated that there was a significant ($^*P < 0.05$) or very significant ($^{**}P < 0.01$) difference between the two groups. E, Summary of LP1 and LP2 mediated regulatory mechanism for cell elongation of Arabidopsis. LP1 or LP2 proteins form homodimers that bind to cis-elements in promoters of the target genes to activate the transcription of these genes required for cell elongation. IBH1 or IBL1 may inhibit cell elongation by forming heterodimers with the LP1 and LP2. The expression levels of *IBH1* and *IBL1* are suppressed through BZR1 by responding to BR signaling.

expression (Bai et al., 2012; Fan et al., 2014; Zhiponova et al., 2014). Similarly, our data revealed that heterodimer complexes of LP1/IBH1 and LP1/IBL1 impaired the activation activity of LP1/LP1 homodimer complexes, while heterodimer complexes of LP2/IBH1 and LP2/IBL1 decreased the activation activity of LP2/LP2 homodimer complexes. GhFP1, the homologous proteins of LP1 and LP2 in cotton, also plays positive role in fiber elongation via modulating BR biosynthesis and signaling. GhFP1 could form homodimers to bind to the G-box element, but heterodimer complexes of FP1/IBH2 and FP1/IBH3 couldn't bind to the G-box element (Liu et al., 2020). These data indicate that bHLH proteins have a conserved system to regulate cell elongation via distinct signal channels in different kinds of plants.

To sum up, our results indicated that LP1 and LP2 mediated regulatory mechanisms for activating downstream of *LNG* genes to accelerate longitudinal polar cell elongation. The homodimers of LP1 and LP2 could directly bind to the promoter fragments of *LNG1* and *LNG2* and thus increased the transcript levels of *LNG1* and *LNG2*. Consequently, the accumulation of LNG1 and LNG2 may directly or indirectly promote the expressions of cell wall genes (*XTH4/17* and *EXP8*) to positively regulate longitudinal cell elongation. Furthermore, IBH1 and IBL1 interacted with LP1 and LP2 to disturb transcriptional regulation activities of LP1 and LP2 (Figure 7E). Thus, our study provides insights into understanding the molecular mechanism of LP1/LP2-regulated longitudinal cell elongation in Arabidopsis.

Materials and methods

Construction of vectors

For constructing *LP1p:GUS* and *LP2p:GUS* vectors, a 1,802-bp promoter fragment of *LP1* and a 1,979-bp promoter fragment of *LP2* were inserted into pBI101 vector to fuse with *GUS* gene, respectively. For constructing *LP1p:H2B-GFP* and *LP1p:H2B-GFP* vectors, 1,802-bp promoter fragment of *LP1* and 1,979-bp promoter fragment of *LP2* were inserted into P095 vector to fuse with *H2B-GFP* gene, respectively. For overexpression of *LP1* and *LP2* in Arabidopsis, the coding sequence of *LP1* and *LP2* genes was cloned into pBI121 vector to replace the *GUS* gene respectively under the control of *CaMV 35S* promoter (*pBI-35S:LP1* and *pBI-35S:LP2*). For *pYAO:hSpCas9* constructs, *AtU6-26-sgRNA-SK* was used as an intermediate cloning vector. *LP1* single, *LP2* single and *LP1LP2* double target sites were assembled into the same intermediate construct, respectively. The *AtU6-26-target-sgRNA* vectors were digested by *Spe*I and *Nhe*I and then inserted into the *Spe*I site in *pYAO:hSpCas9* vector to generate the CRISPR/Cas9 system (Yan et al., 2015). For *lp1lp2* double mutant phenotypic rescue constructs, the *GUS* gene in *LP1p:GUS* and *LP2p:GUS* vectors were replaced by the coding sequence of *LP1* and *LP2* genes, respectively. Primers used are listed in Supplemental Table S1.

Arabidopsis transformation

Arabidopsis thaliana Columbia-0 ecotype was used in all experiments. Plants used for transformation were grown in soil at 23°C with a photoperiod of 16-h light/8-h dark. Transformation of Arabidopsis was performed using the previously reported floral dip method (Clough and Bent, 1998). The harvested seeds were germinated on a selective medium containing 50 mg/L kanamycin for screening the transgenic plants. Homozygous lines of T3 generation were used for phenotypic analysis.

Histochemical assay of GUS activity

GUS staining assay was carried out as previously described (Jefferson et al., 1987). Plant tissues were vacuum infiltrated in a substrate solution containing 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D glucuronic acid (X-Gluc), 100 μg/ml chloramphenicol, 2 mM each of potassium ferricyanide and potassium ferrocyanide, and incubated 8 h at 37°C. Then, the samples were washed several times with 80% ethanol.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from collected materials using RNAprep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. A total of 2 μg RNA was used as the template for the synthesis of cDNA first strands using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Gene expression levels were analyzed by real-time PCR using the fluorescent intercalating dye SYBR-Green in a detection system. Arabidopsis *ACTIN2* (AF428330) was used as a standard control. RT-qPCR data are mean values and standard deviations (bar) of three independent experiments with three biological replicates. Primers used are listed in Supplemental Table S2.

Measurement and statistical analysis

Arabidopsis seedlings were cultured in a growth room under a photoperiod of 16-h light/8-h dark at 22°C. The materials were collected from 6-d-old cotyledon and the fifth leaf blades of 28-d-old Arabidopsis plants. Images of leaf palisade cells and trichomes were taken under an optics microscope (Leica, Germany). Length of epidermal cells, palisade cells and trichomes was measured using the Image J software (http://rsbweb.nih.gov/ij/). All experiments were done at a minimum in triplicate, and the data were statistically analyzed by the Student's *t* test. More than 50 cells were used for each biological replicate.

Scanning electron microscopy

For scanning electron microscope images, 6-d-old cotyledon and 28-d-old fifth rosette leaves were attached with colloidal graphite to a copper stub, frozen under vacuum and visualized under a scanning electron microscope (JSM-6360LV, JEOL, Japan).

Nuclear ploidy-level analysis

The fifth rosette leaves on 28-d-old plants were fixed in PEMT (25 mM PIPEs, 2.5 mM EGTA, 0.5 mM $MgSO_4.7H_2O$, 2.5% (v/v) Triton X-100, pH 7.2) buffer with 10% (v/v) formalin and 2% (v/v) glutaraldehyde for 3 h, and then washed at least three times with PEMT buffer for 10 min. The rosette leaves were resuspended with PEMT buffer with 50 mM EGTA and vacuumized for 10 min. After 16 h, the trichomes were removed from the surface of the leaves using a soft brush. The collected trichomes were stained with DAPI (a nucleus-specific dye) for 1 min at room temperature followed by washing three times with PEMT buffer. Fluorescence of the individual nucleus was observed and measured under an SP8 confocal laser scanning microscope (Leica, Germany). DAPI was excited at 405 nm and collected at 430–550 nm bandwidth filter, and the gain was set as 681.

Subcellular localization of proteins

The coding sequences of *LP1* and *LP2* were cloned into the downstream region of enhanced green fluorescent protein (*eGFP*) in an expression vector *pCAMBIA-2300-35S-eGFP* under the control of the *CaMV 35S* promoter. The recombinant vectors were transformed into Arabidopsis. Transgenic plants were used for GFP fluorescence observation. eGFP fusion protein expression was visualized in the hypocotyl cells of the transgenic seedlings stained with DAPI for 1 min at room temperature under an SP5 confocal laser scanning microscope (Leica, Germany). GFP fluorescence was excited at 488 nm and collected at 503–542 nm bandwidth filter, and

the gain was set as 941. DAPI was excited at 405 nm and collected at 461 nm bandwidth filter, and the gain was set as 690. Primers used are listed in Supplemental Table S1.

Yeast one-hybrid assay

Promoter fragments of *LNG1* and *LNG2* were integrated into the linearized pAbAi vector, respectively, to generate recombinant plasmids pAbAi-*LNG1p* and pAbAi-*LNG2p*. The linearized constructs were transferred into Y1HGold competent yeast strain, and then the yeast transformants were tested on SD/-Ura medium with different concentrations of AbA. The coding sequences of *LP1* and *LP2* were cloned into pGADT7 vector and transferred into the Y1HGold yeast strain containing pAbAi-*LNG1p* and pAbAi-*LNG2p*, respectively. Interaction of LP1 and LP2 with the promoter fragments (*LNG1p* and *LNG2p*) were tested, respectively, on SD/−Leu medium with the tested AbA concentration. Primers used are listed in Supplemental Table S1.

Antibody preparation and ChIP assay

The polyclonal antibodies against LP1 and LP2 were generated by inoculation of rabbits with LP1 and LP2 proteins correspondingly. ChIP assay was conducted according to previous descriptions with tiny modification (Fiil et al., 2008). The 28-d-old rosette leaves were cross-linked with 1% formaldehyde. The chromatin DNA fragments (about 400 bp in length) were isolated from cell nuclei of Arabidopsis plants. The LP1- and LP2-binding DNA fragments were immunoprecipitated using rabbit polyclonal anti-LP1 and anti-LP2 antibodies, respectively, and the DNA fragments pulled down without using any antibody were set as control. The bound DNA fragments were then extracted using the phenol–chloroform extracting method and the amount of the bound DNAs was measured by semi-quantitative PCR. The chosen primer pairs can amplify fragments of 150–200 bp within the promoters of *LNG1* and *LNG2*. To ensure the authenticity of ChIP data, the input sample and nonantibody control sample were analyzed with each primer set listed in Supplemental Table S3.

Transcriptional activation activity and yeast two-hybrid assays

The full-length or truncated open reading frames (ORFs) of *bHLH/HLH* genes were cloned into the yeast two-hybrid vectors pGBKT7 and PGADT7, respectively. Each pGBKT7-bHLH/HLH construct was introduced individually into the yeast strain Y187, and each pGADT7-bHLH/HLH construct was transferred into the yeast strain AH109. Both transcriptional activation activity assays and mating reactions were performed according to the BD Matchmaker Library Construction & Screening Kits User Manual (BD Biosciences Clontech, Palo Alto, CA, USA). Primers used are listed in Supplemental Table S1.

LUC complementation imaging assay

LCI assay was conducted as previously reported (Chen et al., 2008). ORFs of *LP1*, *LP2*, *IBL1*, and *IBH1* were cloned into JW771 and JW772 vectors, respectively. Each ORF was fused to the carboxyl-terminal half (cLUC-LP1/LP2/IBH1/IBL1) and the amino-terminal half (LP1/LP2-nLUC) of LUC. cLUC and nLUC empty vectors were used alone as controls. The constructs were transferred into *Agrobacterium tumefaciens* (strain GV3101) carrying helper plasmid, pSoup-P19, which encodes a repressor of co-suppression. The transformant cells were suspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES (2-(N-morpholino) ethanesulfonic acid) pH 5.7, 150 mM acetosyringone) at optical density $(OD)_{600} = 0.8$, and then two transformants were mixed with volume ratio of 1:1. The mixtures were transferred into the fully expanded leaves of *N. benthamiana* plants using a needleless syringe. After infiltration, plants were immediately covered with plastic bags and placed at 23°C for 48 h, and then incubated at 28°C under a photoperiod of 16-h light/8-h dark. The *N. benthamiana* leaves were harvested and sprayed on 0.5 mM D-Luciferin. The materials were kept in dark for 6 min to quench the fluorescence. A low-light cooled CCD imaging apparatus was used to capture the LUC fluorescence image as described (He et al., 2004). Primers used are listed in Supplemental Table S1.

Pull-down assay

The coding sequences of *LP1* and *LP2* were fused with the gene encoding maltose-binding protein (MBP), respectively, and expressed in *Escherichia coli* cells. The fused proteins were purified using amylose resin (NEB, USA). Meanwhile, IBH1 and IBL1 were fused with Glutathione S-Transferase (GST) tag, respectively. The purified MBP-LP1 and MBP-LP2 proteins were incubated with glutathione agarose resin (Merck, Germany) that was combined with IBH1 and IBL1, respectively. The beads were washed five times with column buffer after the mixture was incubated on ice with rotation for 1 h. The proteins were eluted from the beads with 50 µl elution buffer and loaded onto an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) gel. Gel blots were analyzed using an anti-MBP antibody (CWBIO, China). Primers used are listed in Supplemental Table S1.

CoIP assay

The CoIP assay was conducted according to the method described previously (Shan et al., 2014). The LP1-/LP2-GFP were co-expressed with HA-LP1/-LP2, IBL1-HA, and IBH1-HA, respectively, in leaves of *N. benthamiana*. The extraction buffer was used to extract the soluble proteins. Extracts were incubated with anti-GFP antibody (Abcam) overnight at 4°C with gentle rotation. Immune complexes were collected by incubation with prewashed protein A-agarose (Merck, Germany) at 4°C for 4 h, followed by brief centrifugation. Immune complexes were washed three times for 5 min in 1 mL of IP buffer, and resuspended in 2×SDS/PAGE sample buffer. The fusion proteins were detected by immunoblot with anti-HA antibody (Abcam) and anti-GFP antibody (Abcam), respectively.

Dual-LUC assay

The Dual-LUC assay was performed by the method as reported (Liu et al., 2008). The promoters of *LNG1* and *LNG2* were inserted into pGreen-LUC, respectively, to drive the firefly *LUC* reporter gene with the Renilla (REN) *LUC* controlled by the constitutive *35S* promoter on the same plasmid as a reference to normalize infection efficiency. The constructs were transferred into *A. tumefaciens* (strain GV3101) carrying helper plasmid, pSoup-P19. The transformed *Agrobacterium* cells were mixed with the *Agrobacterium* strains containing the effectors or the empty vector control, in a volume ratio of 1:2. After infiltration into leaves, the *N. benthamiana* plants were immediately covered with plastic bags and placed at 23°C for 48 h, and then incubated at 28°C under a photoperiod of 16-h light/8-h dark. The infected area of leaves was collected for total protein extraction. The extracted proteins were assayed with the Dual-Luciferase Reporter Assay System (Promega, USA) following the manufacture's manual, and the fluorescent values of LUC and REN were detected with a luminometer, successively. The value of LUC was normalized to that of REN. Three biological repeats were measured for each combination. Primers used are listed in Supplemental Table S1.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: LP1 (At2g42300), LP2 (At3g57800), LNG1 (AT5G15580), LNG2 (AT3G02170), IBL1 (AT4G30410), IBH1 (At2g43060), AIF2 (At3g06590), EXP8 (At2g40610), EXP1 (AT1G69530), XTH4 (At2g06850), XTH15 (AT4G14130), XTH17 (AT1G65310), XTH24 (AT4G30270), ACTIN2 (AT3g18780), BZR1 (At1g75080), PIF4 (AT2G43010), CPD (AT5G05690), DET2(AT2G38050), BRI1 (AT4G39400), BEE2(AT4G36540), CYP90D1 (AT3G13730), AN (AT1G01510), PRE1 (AT5G39860), GhFP1 (Gh_A11G2808).

Supplemental data

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

Supplemental Figure S1. Sequence alignment of LP1 and LP2 with a cotton homolog GhFP1.

Supplemental Figure S2. Expression pattern of *LP1* and *LP2* in leaf primordia of Arabidopsis.

Supplemental Figure S3. Identification of *LP1* and *LP2* transgenic Arabidopsis plants.

Supplemental Figure S4. LP1 and LP2 participate in regulating longitudinal cell elongation in Arabidopsis.

Supplemental Figure S5. Measurement and statistical analysis of aspect ratio of cells in *LP1* and *LP2* transgenic Arabidopsis.

Supplemental Figure S6. LP1 and LP2 affect the development of trichomes in Arabidopsis.

Supplemental Figure S7. RT-qPCR analysis of the expressions of *XTH15/24* and BR biosynthesis genes in rosette leaves of transgenic Arabidopsis plants.

Supplemental Figure S8. Assay of transcriptional activity of IBL1 and IBH1 proteins.

Supplemental Table S1. Primers used in construction of vectors.

Supplemental Table S2. Primers used in RT-qPCR analysis.

Supplemental Table S3. Primers used in ChIP assay.

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Conflict of interest statement. The authors declare no competing interests.

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