

# *Arabidopsis thaliana* AHL family modulates hypocotyl growth redundantly by interacting with each other via the PPC/DUF296 domain

Jianfei Zhao<sup>a,b</sup>, David S. Favero<sup>a,b</sup>, Hao Peng<sup>b</sup>, and Michael M. Neff<sup>a,b,1</sup>

<sup>a</sup>Molecular Plant Sciences Graduate Program and <sup>b</sup>Department of Crop and Soil Sciences, Washington State University, Pullman, WA 99164

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The *Arabidopsis thaliana* genome encodes 29 *AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED (AHL)* genes, which evolved into two phylogenetic clades. The AHL proteins contain one or two AT-hook motif(s) and one plant and prokaryote conserved (PPC)/domain of unknown function #296 (DUF296) domain. Seedlings lacking both *SOB3/AHL29* and *ESC/AHL27* confer a subtle long-hypocotyl phenotype compared with the WT or either single-null mutant. In contrast, the missense allele *sob3-6* confers a dramatic long-hypocotyl phenotype in the light. In this study, we examined the dominant-negative feature of *sob3-6* and found that it encodes a protein with a disrupted AT-hook motif that abolishes binding to AT-rich DNA. A loss-of-function approach demonstrated different, yet redundant, contributions of additional AHL genes in suppressing hypocotyl elongation in the light. We showed that AHL proteins interact with each other and themselves via the PPC/DUF296 domain. AHLs also share interactions with other nuclear proteins, such as transcription factors, suggesting that these interactions also contribute to the functional redundancy within this gene family. The coordinated action of AHLs requires an AT-hook motif capable of binding AT-rich DNA, as well as a PPC/DUF296 domain containing a conserved Gly-Arg-Phe-Glu-Ile-Leu region. Alteration of this region abolished *SOB3/AHL29*'s physical interaction with transcription factors and resulted in a dominant-negative allele *in planta* that was phenotypically similar to *sob3-6*. We propose a molecular model where AHLs interact with each other and themselves, as well as other nuclear proteins, to form complexes which modulate plant growth and development.

enhanceosome | seedling establishment

Since the colonization of land plants, plant genomes have rapidly expanded and diversified from their common ancestor through the process of evolution. During the long period of natural selection that shaped the morphology and adaptivity of plants to their environment, some genes that regulate important growth and developmental processes of ancient plant species have been amplified and gradually expanded into multimember gene families (1–4). One such family, the *AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED (AHL)* genes, exists in all plant species that have been sequenced thus far, from the moss *Physcomitrella patens* (5) to both modern dicot and monocot plants, such as *Arabidopsis thaliana* (6), *Oryza sativa* (7, 8), *Sorghum bicolor* (9), and *Populus trichocarpa* (10). This high conservation through evolution suggests that the AHL gene family is important for plant growth and development.

The AHL proteins contain two conserved structural units, the AT-hook motif and the plant and prokaryote conserved (PPC) domain, the latter being also annotated as the domain of unknown function #296 (DUF296). The AT-hook motif enables binding to AT-rich DNA and has been identified in various gene families both in prokaryotes and eukaryotes including the high mobility group A (HMGA) proteins in mammals (11). The AT-hook motif contains a conserved palindromic core sequence, Arg-Gly-Arg, which binds the minor groove of AT-rich B-form DNA (12). On binding with DNA, the Arg-Gly-Arg core sequence

adopts a concave conformation with close proximity to the backbone of the DNA, and the side chains of both arginine residues firmly insert into the minor groove (12).

The PPC/DUF296 domain is ~120 amino acids in length and exists as a single protein in Bacteria and Archaea (13). Crystal structures of several bacterial and archaeal PPC/DUF296 proteins have been determined (14, 15). All of the above prokaryotic PPC/DUF296 proteins share the same tertiary structure with five  $\beta$ -strands forming an antiparallel  $\beta$ -sheet, which partially surrounds a single  $\alpha$ -helix. The solved crystal structures suggest that the prokaryotic PPC/DUF296 proteins form a trimer (14). In land plants, the PPC/DUF296 domain is found in AHL proteins, where it is located at the carboxyl end relative to the AT-hook motif(s) (13). In *Arabidopsis* AHL1, a hydrophobic region at the C-terminal end of its PPC/DUF296 domain is essential for its nuclear localization (13). However, there are currently no known biological functions for this domain in regulating plant growth and development.

Based on gene overexpression and biochemical studies, members of the AHL family have been proposed to regulate diverse aspects of growth and development in plants. Overexpression of either of two *Arabidopsis* AHL genes, *SUPPRESSOR OF PHYTOCHROME B-4 #3 (SOB3/AHL29)* or *ESCAROLA (ESC/AHL27)* represses hypocotyl elongation for seedlings grown in the light, but not in darkness (16). As adults, these overexpression plants develop larger organs, including expanded leaves and enlarged flowers and fruits, together with delayed flowering and senescence (16). The AHL genes modulate several aspects of plant growth and

## Significance

Members of the *AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED (AHL)* family are involved in various plant biological processes. Our findings reveal a molecular model whereby the AHLs interact with each other via the plant and prokaryote conserved (PPC)/domain of unknown function #296 (DUF296) domain to form homo-/hetero-complexes, possibly trimers. The AHL complex also interacts with other nuclear proteins to form a macromolecular complex that modulates plant growth and development. The coordinated action of AHLs requires an AT-hook motif capable of binding AT-rich DNA, as well as a PPC/DUF296 domain containing a conserved six-amino-acid region. Our proposed model provides a better understanding of the roles of AHL genes in regulating plant growth and development, which may in turn lead to better seedling establishment and increased yield.

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<sup>1</sup>To whom correspondence should be addressed. E-mail: mmneff@wsu.edu.

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development, including hypocotyl elongation in the light (*AHL22*, *ESC/AHL27*, and *SOB3/AHL29*) (16, 17), flower development (16–20), and root growth (21). Overexpression of other *AHL* gene members, such as *HERCULES* (*HRC/AHL25*), also enhances adult leaf and stem growth (22). Several *AHL* genes are involved in the homeostasis of phytohormones or the mediation of their responses. For example, overexpression of either *AT-HOOK PROTEIN OF GA FEEDBACK REGULATION 1* (*AGF1/AHL25*) or *AGF2/AHL15* suggests that these genes play a role in maintaining the negative feedback of *GA 3-oxidase* in gibberellin signaling (23). Based on yeast-one-hybrid and transactivation analysis, *Catharanthus roseus AHLs* have been shown to regulate the jasmonic acid response of AP2 transcription factors (24). Transcript accumulation of *AHL21* was dramatically suppressed in cytokinin-treated *Arabidopsis* roots (25). Overexpression studies also implicate *AHL* genes (*ESC/AHL27* and *AHL20*) in the regulation of plant innate immune responses (19, 26). Gene overexpression and related biochemical analyses, however, can be misleading due to neomorphic or hypermorphic phenotypes; in other words, those reactions caused by gene misexpression or biologically irrelevant protein levels. Therefore, it is important to complement gene overexpression and biochemical studies with loss-of-function genetic analysis to unveil the biological functions of the *AHL* genes in plants.

Our previous loss-of-function study showed that two *Arabidopsis AHLs*, *SOB3/AHL29* and *ESC/AHL27*, repress hypocotyl growth redundantly in light-grown seedlings (16). Single loss-of-function mutants for either *SOB3/AHL29* (*sob3-4*) or *ESC/AHL27* (*esc-8*) have WT phenotypes. In contrast, the *sob3-4 esc-8* double mutant exhibits slightly increased hypocotyl growth under continuous white, red, far-red, and blue light, providing the first *AHL* loss-of-function analysis and demonstrating that at least *SOB3/AHL29* and *ESC/AHL27* act as negative regulators of hypocotyl elongation. In support of these observations, an *AHL18-AHL22-ESC/AHL27-SOB3/AHL29* RNAi line also exhibits a long-hypocotyl phenotype in white, blue, red, and far-red light (17). These studies suggest functional redundancy among multiple *AHL* genes in *Arabidopsis*. In contrast, genetic analyses of single *AHL* loss-of-function mutants in monocots demonstrated that they regulate inflorescence and floral organ growth, as well as palea formation (27, 28). Although we are beginning to understand the roles of *AHL* genes in plant growth and development, especially with regard to hypocotyl elongation, the molecular mechanism underlying their functional redundancy in *Arabidopsis* and other plants is not known.

In contrast to the subtle phenotypes shown by *sob3-4 esc-8* (16) and the quadruple RNAi line (17), the missense activation-tagged allele *sob3-6*, which was identified in our previous study, confers a much more severe long-hypocotyl phenotype in the light (16). Analysis of this dominant-negative *sob3-6* allele, which is caused by a single amino acid change in the conserved core of the AT-hook, has led to the hypothesis that this DNA-binding domain, as well as protein-protein interactions, is necessary for the biological function of this gene family.

In this study, we show that the *sob3-6* allele encodes a full-length protein that no longer binds AT-rich DNA. Loss-of-function analysis with triple- and quadruple-null mutants demonstrates that multiple *AHL* genes, but not all, suppress hypocotyl growth in the light. We also show that multiple WT *AHLs*, as well as the *SOB3-6* mutant protein, can associate with each other in the nucleus and that the PPC/DUF296 domain mediates this interaction. Overexpression of this domain also leads to a dominant-negative long hypocotyl phenotype, which is similar to that of *sob3-6*. In addition, a yeast two-hybrid (Y2H) library screen identified non-*AHL*, nuclear-localized, DNA-binding proteins that physically interact with *AHLs*. We demonstrate that a conserved six-amino-acid region in the PPC/DUF296 domain of *SOB3/AHL29* is necessary for transcriptional activation in yeast and

interaction with at least one transcription factor. Removal of this region also confers a dominant-negative long hypocotyl phenotype when expressed in plants. Based on these results, we propose a molecular model whereby *AHLs* interact with themselves and each other, as well as other nuclear proteins, to form complexes similar to the human enhanceosome, which modulate plant growth and development.

## Results

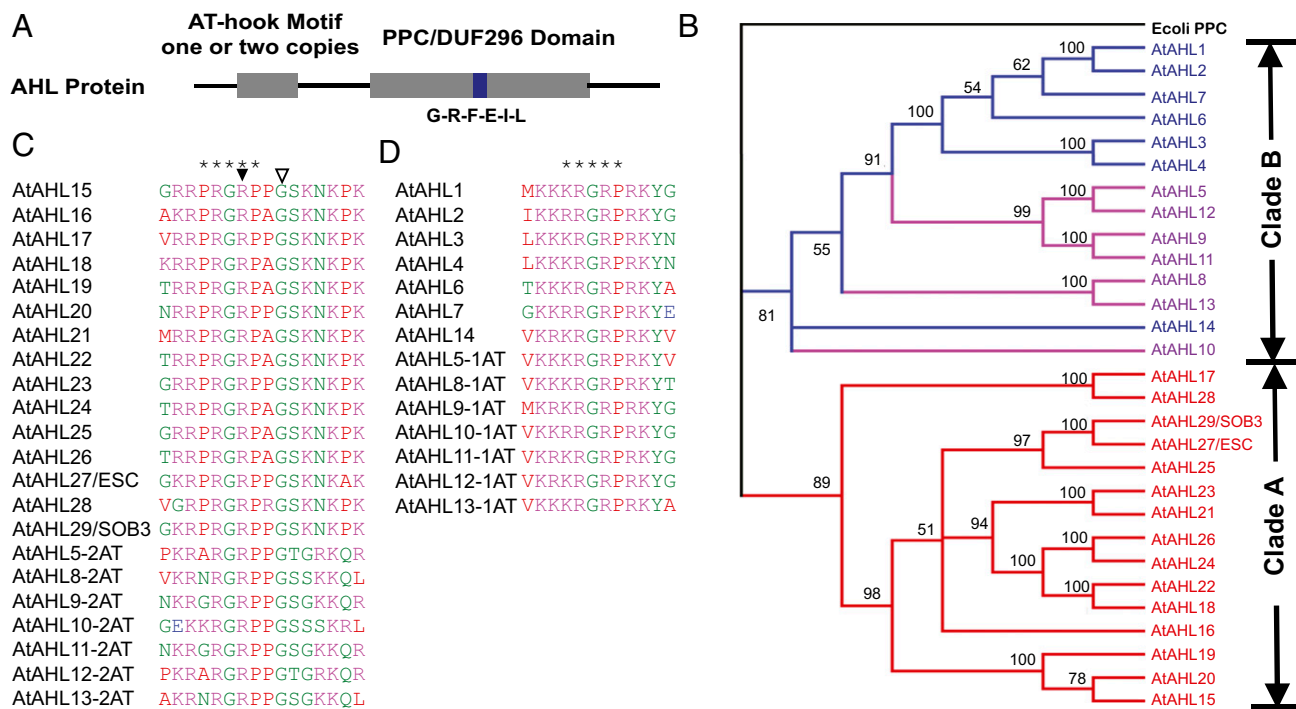
**AHL Gene Family in *A. thaliana*.** The *A. thaliana* genome encodes 29 *AHL* genes (Fig. 1A). A Bayesian phylogenetic analysis showed that these 29 *Arabidopsis AHL* genes evolved into two phylogenetic clades (clades A and B; Fig. 1B). Clade A *AHLs* are intronless with only one AT-hook motif and a single PPC/DUF296 domain. Clade B consists of intron-containing *AHLs* with either one or two AT-hook motif(s) and a single PPC/DUF296 domain (Fig. 1B). The type 1 and 2 AT-hook motifs are distinguished by the sequences that flank the conserved Arg-Gly-Arg core, especially those at the carboxyl end. The consensus sequence at the carboxyl end of the conserved core in type 1 AT-hook motifs in *AHL* proteins is Gly-Ser-Lys-Asn-Lys, whereas Arg-Lys-Tyr-X is found at the carboxyl end of the core in type 2 AT-hook motifs (Fig. 1C and D). Within each clade, the *Arabidopsis AHLs* tend to coexpress with other members (Fig. S1) (29, 30).

The PPC/DUF296 domain of *SOB3/AHL29* is predicted to share the same order of secondary structural elements,  $\beta_1$ - $\alpha$ - $\beta_2$ - $\beta_3$ - $\beta_4$ - $\beta_5$ - $\beta_6$ - $\beta_7$ - $\beta_8$ - $\beta_9$ , as observed in their prokaryotic counterparts (Fig. S2). We predicted the tertiary structure of the PPC/DUF296 domain from *SOB3/AHL29* via homology modeling (Fig. S3). It is also predicted to adopt a similar tertiary structure as observed in the crystal structures of prokaryotic PPC/DUF296 proteins. The PPC/DUF296 proteins form a trimer, where the  $\beta$ -sheets mediate interactions among the three PPC monomers (14). This structure suggests that the PPC/DUF296 domains in *Arabidopsis AHL* proteins could associate with each other as their counterparts do in prokaryotes.

***sob3-6* Is a Dominant-Negative Allele Disrupting *AHL* Protein Interactions with DNA.** To obtain loss-of-function alleles, we treated activation-tagged *SOB3-D* seeds with ethyl methanesulfonate (EMS) and screened for intragenic suppressors of the short-hypocotyl phenotype conferred by overexpression of *SOB3/AHL29* (16). In addition to the *sob3-4* null allele, we identified two additional missense mutant alleles, *sob3-5* and *sob3-6*, which produced longer hypocotyls in white light than the WT (Fig. 2A) (16). The *sob3-5* allele has a Gly<sup>80</sup> to Gln mutation, which is adjacent to the carboxyl end of the core sequence of the type 1 AT-hook motif (Fig. 1C). The *sob3-6* mutation is at the second arginine (Arg<sup>77</sup> to His) in the conserved AT-hook core motif (Fig. 1C). Mutations in this Arg-Gly-Arg core motif have been shown to abolish DNA binding by the AT-hook motif in non-*AHL* proteins (31, 32).

Due to the nature of the mutation, the *SOB3-6* protein is likely to have minimal or no AT-rich DNA binding capability. To test this hypothesis, we performed an EMSA with *SOB3* and *SOB3-6* proteins using a probe sequence taken from the AT-rich *PRA2* promoter from pea (*Pisum sativum*) (17, 19). *SOB3* readily binds with the biotin-labeled oligonucleotide *PRA2* probe (Fig. 2B). Incubation with an anti-*SOB3* antibody resulted in a supershift of the *PRA2* probe, indicating the presence of *SOB3* in the protein-DNA complex. In addition, binding of the *PRA2* probe by *SOB3* could be competed away by adding a cold (unlabeled) competitive probe. In contrast, no DNA-binding could be detected when using *SOB3-6* protein instead of *SOB3* (Fig. 2B and Fig. S4A).

*sob3-6* was identified as an intragenic suppressor of the short-hypocotyl phenotype conferred by the gain-of-function *SOB3-D* mutant, leading to a light-grown hypocotyl that is even longer than the WT (16). The observation that the *sob3-6* allele confers



**Fig. 1.** The *AHL* gene family in *A. thaliana*. (A) Topologies of the *AHL* proteins. A conserved six-amino-acid region in the PPC/DUF296 domain is highlighted by the blue box. (B) Phylogeny of the *AHL* gene family using Bayesian analysis. Numbers near branches indicate Bayesian posterior probabilities for given clades. *AHL* genes containing only one type 1 AT-hook motif are shown in red. *AHL* genes containing one type 2 AT-hook motif are shown in blue. *AHL* genes containing two AT-hook motifs (one each of type 1 and 2) are shown in purple. Two forms of the AT-hook motif, types 1 (C) and 2 (D), were identified in the *Arabidopsis* *AHL* proteins. The stars indicate the sequences of the AT-hook motifs. The arrowhead and open arrowhead represent the *sob3-6* mutation (R-to-H) and *sob3-5* mutation (G-to-Q) in *SOB3/AHL29*, respectively.

the opposite phenotype of *SOB3-D* suggests that the Arg<sup>77</sup> to His missense mutation disrupts AT-hook motif function, rendering *sob3-6* a negative allele. The *sob3-6* negative allele was initially identified as exhibiting a long-hypocotyl phenotype in white light-grown heterozygous *sob3-6/SOB3-D* plants (16). Because one copy of *sob3-6* was sufficient to suppress the *SOB3-D* gain-of-function phenotype, we hypothesized that this missense mutation functions as a dominant-negative allele. We tested this hypothesis by overexpressing the *sob3-6* cDNA under the constitutive *Cauliflower Mosaic Virus (CaMV) 35S* promoter in WT *Arabidopsis*. Multiple independent T1 (hemizygous) and T2 transgenic lines recapitulated the long-hypocotyl phenotype of the original *sob3-6* allele when grown in white light, with some lines conferring an even longer hypocotyl phenotype than the original *sob3-6* mutant line (Fig. 2A).

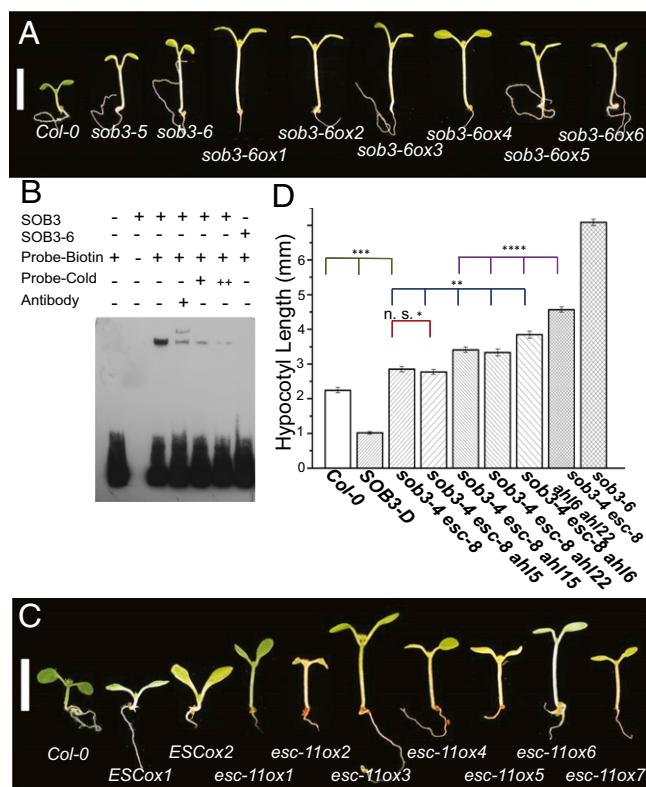
We also overexpressed another *AHL*, *ESC/AHL27*, driven by the *CaMV 35S* promoter in *Arabidopsis*. The *ESC/AHL27* overexpression lines exhibited similar suppressed hypocotyl growth as seen in *SOB3-D* seedlings (16) (Fig. 2C). We further created the same *sob3-6*-like mutation (Arg<sup>91</sup> to His) in the AT-hook motif of the *ESC/AHL27* gene (designated as *esc-11*). Multiple independent T1 and T2 *esc-11* overexpression lines (also driven by the *CaMV35S* promoter) exhibited long hypocotyls in white light, recapitulating the *sob3-6* phenotype (Fig. 2C) and demonstrating that multiple *AHLs* with a *sob3-6*-like mutation function as dominant-negative alleles.

**AHL Genes Contribute Differently to the Suppression of Hypocotyl Elongation in White Light.** The *sob3-6* and *esc-11* overexpression lines both exhibited longer hypocotyls than the *sob3-4 esc-8* double-null mutant. This observation suggests that other *AHLs* with similar functions are also affected by these dominant-negative alleles. To test this hypothesis and further examine the function of additional *AHLs* with regard to suppression of

hypocotyl elongation in the light, we generated four *AHL* triple-null mutant combinations (Fig. 2D and Fig. S4 B–E). Three triple-null mutants (*ahl6 sob3-4 esc-8*, *ahl15 sob3-4 esc-8*, and *ahl22 sob3-4 esc-8*) exhibited longer hypocotyls than the *sob3-4 esc-8* double-null, although they varied with regard to the severity of the phenotype ( $P < 4.5E-4$ ). A fourth triple-null mutant, *sob3-4 esc-8 ahl5*, had a similar light-grown hypocotyl length as the *sob3-4 esc-8* double-null (Fig. 2D). We also made the *sob3-4 esc-8 ahl6 ahl22* quadruple-null mutant. The *sob3-4 esc-8 ahl6 ahl22* quadruple null conferred an even longer hypocotyl phenotype than both triple-null lines when grown in white light, although this phenotype was still shorter than that of *sob3-6* ( $P < 3.2E-7$ ; Fig. 2D). Together these data demonstrate that these *AHL* genes contribute differently to white light-mediated suppression of hypocotyl elongation.

**AHL Proteins Form Homo- and Hetero-Complexes.** The dominant-negative long-hypocotyl phenotype of *sob3-6* and *esc-11* overexpressing seedlings suggests a model whereby these mutant proteins physically interact with other *AHL* family members and/or other shared interacting partners, rendering the complex functionally inactive due to abolished DNA binding by the mutated AT-hook motif. To test this hypothesis, we first performed a targeted GAL4-based Y2H assay (Fig. 3A). Using the lowest concentration of 3-amino-1, 2, 4-triazol (3-AT) that prevented transcriptional autoactivation by the *AHL* baits, we demonstrated that *SOB3/AHL29* and *ESC/AHL27* interact with themselves as well as with each other (Fig. 3A and Fig. S5A).

We further examined the *AHL*-*AHL* interactions *in planta* using a bimolecular fluorescence complementation (BiFC) assay via transient expression in onion epidermal cells (33, 34). In this experiment, two halves of the enhanced YFP (EYFP) (nEYFP, N terminus half of EYFP; cEYFP, C terminus half of EYFP)



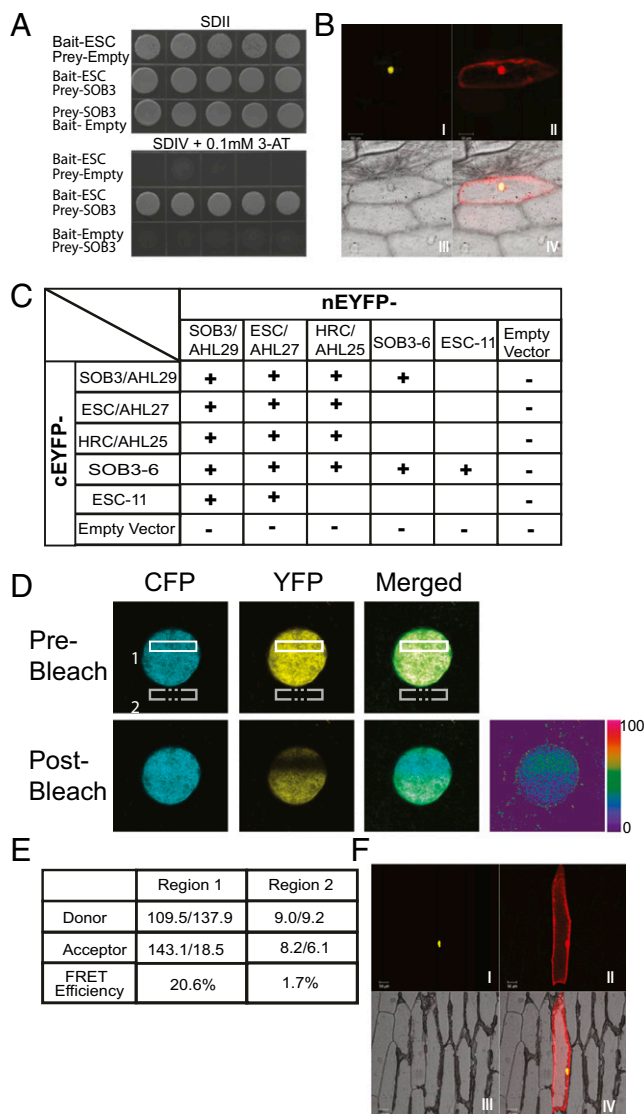
**Fig. 2.** *sob3-6* is a dominant-negative allele involved in regulating hypocotyl elongation in light growth. (A) Hypocotyl growth of WT *Col-0*, *sob3-5*, *sob3-6*, and multiple independent transgenic lines overexpressing the *sob3-6* allele, growing in  $30 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  white light. (B) SOB3 bound to the biotin-labeled pea *PRA2* probe in EMSA. A super shift was detected with the addition of the anti-SOB3 antibody. With increasing concentrations of cold probe, the binding of *PRA2* probe by SOB3 could be competed away. However, SOB3-6 did not bind to the *PRA2* probe in EMSA. (C) Hypocotyl growth of *Col-0*, *ESC/AHL27* overexpression lines (*ESCox1* and *ESCox2*) and multiple *esc-11* overexpression lines, growing in  $30 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  white light. (Scale bar, 5 mm.) (D) Six-day-old *Arabidopsis* seedlings were grown in  $25 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  white light at  $25^\circ\text{C}$ . The error bar denotes the SEM. In a Student *t* test (unpaired two-tailed *t* test with unequal variance). \*not significant with  $P = 0.39$ . \*\* $P < 4.5\text{E}-4$ . \*\*\* $P < 7.5\text{E}-7$ . \*\*\*\* $P < 3.2\text{E}-7$ . *Col-0*,  $n = 24$ ; *SOB3-D*,  $n = 29$ ; *sob3-4 esc-8*,  $n = 34$ ; *sob3-4 esc-8 ahl5*,  $n = 50$ ; *sob3-4 esc-8 ahl15*,  $n = 47$ ; *sob3-4 esc-8 ahl22*,  $n = 32$ ; *sob3-4 esc-8 ahl6*,  $n = 31$ ; quadruple null,  $n = 38$ ; *sob3-6*,  $n = 32$ .

were translationally fused to the 5' end of *SOB3/AHL29* and *ESC/AHL27* (Fig. 3B and Fig. S5 B–F). A separate plasmid expressing a monomeric red fluorescent protein (mRFP) was also cotransformed with the nEYFP and cEYFP fusions as an indicator of successful transformation. The mRFP protein was expressed in the cytoplasm and in the nucleus. The reconstructed yellow fluorescence signal could be detected in the transformed onion epidermal cells and colocalized with the red fluorescent signal only in the nucleus (Fig. 3B). No yellow fluorescence was observed with the negative controls using empty BiFC vectors (Fig. S5 G–L).

We also used the BiFC assay to demonstrate interactions among SOB3/AHL29, ESC/AHL27, and another clade A AHL, HRC/AHL25 (Fig. 3C). In addition, we used the Y2H and BiFC assays to test and demonstrate that two clade B AHLS, AHL5 and AHL12, interact with SOB3/AHL29 and ESC/AHL27, as well as with themselves (Fig. S5 M–P). AHL5 and AHL12 each contain two AT-hook motifs (one each of type 1 and 2) and one PPC/DUF296 domain. Thus, the tested AHL proteins in both

clades can associate with each other and with themselves, regardless of the number and type of the AT-hook motif(s).

To further characterize the AHL protein complex *in planta*, we performed a BiFC-FRET assay in onion epidermal cells (Fig. 3D). Three copies of SOB3/AHL29 were fused separately to the two halves of the EYFP, as well as to intact cyan fluorescent protein (CFP). The resulting three plasmids were simultaneously transformed into onion epidermal cells (Fig. 3D). Yellow fluorescence

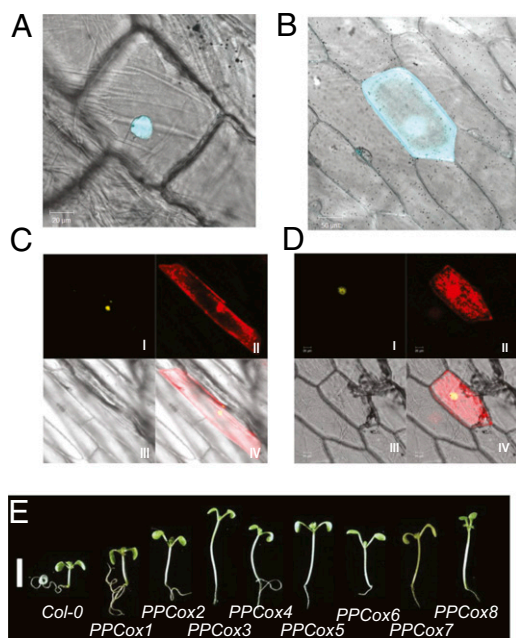


**Fig. 3.** The AHL proteins interact with each other and themselves. SOB3/AHL29 interacts with ESC/AHL27 in Y2H (A) and BiFC (B) assays. Yeast transformed with the indicated prey and bait plasmids were plated on SDII, as well as on SDIV media (supplemented with 3-AT of indicated concentration), and checked for growth after 5 d in  $30^\circ\text{C}$ . Five independent biological replicates are shown. Onion epidermal cells, transformed with plasmids expressing nEYFP-SOB3 and cEYFP-ESC, were checked for reconstructed yellow fluorescence signal (I) and red fluorescence signal (II), observed in bright field (III) and merged (IV). The protein-protein interactions tested by BiFC and/or Y2H were summarized as in C. Interaction among three copies of SOB3/AHL29 proteins was examined by BiFC-FRET assay (D). Onion epidermal cells were transformed with plasmids expressing nEYFP-SOB3, cEYFP-SOB3, and CFP-SOB3. (E) Fluorescent intensities (before bleach/after bleach) were examined individually in region 1 and control region 2 with laser bleaching at the acceptor's (YFP) excitation wavelength. (F) SOB3-6 and ESC-11 interact with each other in the BiFC assay.

could be observed in the nucleus due to reconstitution of EYFP by the physical interaction between the two SOB3/AHL29 proteins that were each fused either to nEYFP or cEYFP. Cyan fluorescent signal could also be detected in the nucleus due to the nuclear localization of SOB3/AHL29. A laser, set at the acceptor's excitation wavelength, was then used to bleach a selected region in the nucleus. Following this treatment, the intensity of yellow fluorescence diminished, whereas the cyan fluorescence increased, suggesting that, before bleaching, FRET was occurring between the donor (CFP) and the acceptor (reconstructed EYFP) (Fig. 3E). The high efficiency of FRET indicated that the three SOB3/AHL29 proteins form at least a trimer complex *in planta*.

**sob3-6 and esc-11 Mutations Do Not Abolish AHL Physical Interactions or Nuclear Localization.** We further used the Y2H and BiFC assays to test whether SOB3-6 and ESC-11 proteins interact with their WT forms in yeast and *in planta*. In both assays, the mutant proteins interacted with WT SOB3 and ESC proteins and with themselves (Fig. 3F and Fig. S5Q). In addition, these interactions still occurred in the nucleus (Fig. 3F), demonstrating that the dominant-negative effect of these alleles is not acting through the disruption of nuclear localization and further that disruption of the AT-hook motif did not abolish the ability of AHLs to form homo- or hetero-complexes.

**AHLs Interact via the PPC/DUF296 Domain.** We fused the PPC/DUF296 domain from SOB3/AHL29 to the carboxyl end of CFP to examine its subcellular localization via transient expression in onion epidermal cells. The PPC/DUF296 domain of SOB3/AHL29 localized to the nucleus of onion epidermal cells (Fig. 4A and B). This finding is consistent with the report that the C-terminal hydrophobic region of the PPC/DUF296 domain is



**Fig. 4.** The PPC/DUF296 domain mediates interactions between AHL proteins. The SOB3 PPC/DUF296 domain tagged with CFP localizes to the nucleus in transformed onion epidermal cells (A) in contrast to free CFP (B). The SOB3 PPC/DUF296 domain interacts with SOB3 in the BiFC assay (C). The PPC/DUF296 domain of SOB3/AHL29 also interacts with SOB3-6, which contains a disrupted AT-hook motif, in the BiFC assay (D). (E) Compared with WT *Col-0* (Left), overexpression of the PPC/DUF296 domain in SOB3 leads to longer hypocotyl growth in the light in multiple independent transformation events.

essential for the nuclear localization of AHL1 (13). The SOB3/AHL29 PPC/DUF296 domain also interacted with full-length SOB3/AHL29 (Fig. S5 R and S) and ESC/AHL27 (Fig. S5T) proteins in Y2H assays. A role for the PPC/DUF296 domain in mediating protein-protein interactions *in planta* was further supported by BiFC analysis (Fig. 4C). The SOB3/AHL29 PPC/DUF296 domain also interacted with SOB3-6 and ESC-11 mutant proteins in both assays (Fig. 4D and Fig. S5 U and V).

To test the hypothesis that the dominant-negative nature of *sob3-6* is caused by the association of its PPC/DUF296 domain with other AHL proteins, we overexpressed this domain under the control of the constitutive *CaMV 35S* promoter in WT *Arabidopsis* and examined the hypocotyl phenotype in white light (Fig. 4E). Multiple independent T1 seedlings exhibited a long-hypocotyl phenotype in white light. Many lines recapitulated the phenotypes of *sob3-6/esc-11* overexpression, as well as the original *sob3-6* line (Fig. 2A and C), demonstrating that overexpression of the SOB3 PPC/DUF296 domain alone functions in a dominant-negative manner (Fig. 4E). Together these results support the hypothesis that the AHLs suppress hypocotyl elongation in *Arabidopsis* seedlings by associating with each other via the PPC/DUF296 domain and that this suppression requires functional AT-hook motifs that recognize AT-rich chromosomal regions.

**AHLs Interact with Other Nuclear-Localized Non-AHL Proteins.** To further test the hypothesis that AHL proteins interact with each other and possibly have shared interacting partners, we performed a blind Y2H library screen using multiple AHL proteins as the bait. We identified multiple AHLs as interacting partners (Table 1 and Table S1) supporting the AHL-AHL physical interactions identified in our study. Interestingly, using just the SOB3/AHL29 PPC/DUF296 domain alone as bait in the library screen, we identified SOB3/AHL29, as well as AHL3, as interaction partners. In addition, we also identified several transcription factors that interact with the AHLs (Table 1, Table S1, and Fig. S6 A–H), suggesting that the redundancy among AHLs is not only attributable to their physical interactions with each other but also due to other common interacting partners. We also found that core histones (histones H2B, H3, and H4) in

**Table 1. Combined interactors identified in Y2H library screens and/or targeted Y2H assays**

Interaction	AGI number
<b>AHLs interacted with AHLs</b>	
AHL1 (as bait)—AHL8	
AHL5 (as bait)—AHL3	
AHL27 (as bait)—AHL23	
AHL27 (as bait)—AHL20	
PPC/DUF296 of SOB3 (as bait)—AHL29/SOB3	
PPC/DUF296 of SOB3 (as bait)—AHL3	
<b>Transcription factors interacted with AHLs</b>	
AP2/EREBP	At5G61890
NAC transcription factor (ATAF2)	At5G08790
Zinc-finger protein	At1G09520, At2G36930
TCP transcription factor 4	At3G15030
TCP transcription factor 13	At3G02150
TCP transcription factor 14	At3G47620
Transcriptional coactivator (KIWI)	At5G09250
<b>Methyltransferase interacted with AHLs</b>	
Methyltransferase	AT5G10830
<b>Histones interacted with AHLs</b>	
Histone H2B	At1G07790
Histone H3.3	At5G10980, At4G40040
Histone H4	At3G53730

nucleosomes interact with multiple AHLs in the Y2H analysis (Fig. S6 I–K and Table S1).

**Conserved Region in the PPC/DUF296 Domain of SOB3/AHL29 Is Essential for Its Activation of Transcription and Physical Interaction with Non-AHL Interactors.** Alignment of amino acid sequences of the PPC/DUF296 domain from the AHLs of several sequenced plant genomes reveals a conserved six-amino-acid region, Gly-Arg-Phe-Glu-Ile-Leu, which is predicted to exist as one  $\beta$ -strand in its secondary structure (Fig. S2). We deleted this six-amino-acid region from the coding sequence of *SOB3/AHL29* and designated it as *sob3- $\Delta$ G*. The deletion did not affect the nuclear localization of the mutant SOB3- $\Delta$ G protein (Fig. 5A). In addition, it did not disrupt interactions with WT SOB3/AHL29 or ESC/AHL27 (Fig. 5B and Fig. S6 L and M). The PPC/DUF296 domain of SOB3/AHL29 also interacted with SOB3- $\Delta$ G in both assays (Fig. 5C and Fig. S6 N and O). These results, together with the observed interactions of SOB3- $\Delta$ G with both SOB3-6 and the ESC protein with the same deletion (ESC- $\Delta$ G) (Fig. S6 P–R), suggest that protein stability, nuclear localization, and association among AHL proteins do not depend on this region.

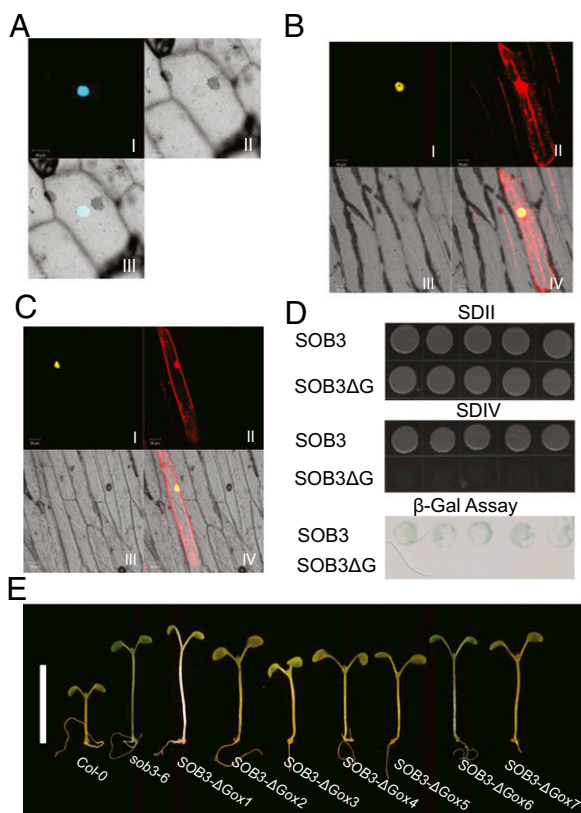
AHL25 from *Arabidopsis* has been shown to activate transcription in a yeast one-hybrid assay (23). We tested SOB3/AHL29 for transcriptional activation in yeast (Fig. 5D and Fig. S7A). With

no 3-AT supplemented in synthetic dropout IV (SDIV) minimal media, yeast expressing SOB3/AHL29 as a Y2H bait exhibited strong autoactivation activity (Fig. 5D). Interestingly, the SOB3/AHL29 PPC/DUF296 domain alone tagged with the DNA-binding domain used in the Y2H assay also conferred autoactivation (Fig. S5S). This observation suggests that the PPC/DUF296 domain is responsible for transcriptional activation by the AHL proteins in yeast.

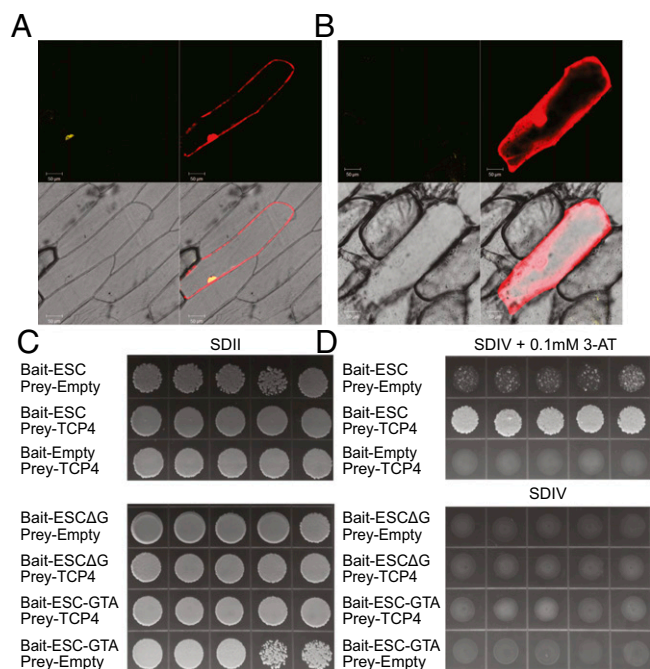
In contrast, SOB3- $\Delta$ G demonstrated a complete loss of transcriptional activation compared with the WT SOB3 (Fig. 5D and Fig. S7A). Yeast transformed with WT SOB3/AHL29 or SOB3- $\Delta$ G survived on synthetic dropout II (SDII) media. When transferred to SDIV media without 3-AT, only the yeast transformed with WT SOB3/AHL29 survived, due to activation of transcription by the AHL protein. Neither growth on SDIV media nor  $\beta$ -gal activity was observed in the five biological yeast replicates that were transformed with SOB3- $\Delta$ G (Fig. 5D). We also created another mutant allele (designated as SOB3-GTA) in which the six amino acids were substituted with alanine residues. SOB3-GTA also lost transcriptional activity in yeast (Fig. S7A). Similar alleles created for ESC/AHL27 (ESC- $\Delta$ G and ESC-GTA) also demonstrated loss of transcriptional activity in yeast in contrast with the WT (Fig. S7B). Therefore, this six-amino-acid region in the PPC/DUF296 domain is essential for the activation of transcription by AHL proteins.

If transcriptional activation is necessary for biological function, then removal of this six-amino-acid region would alleviate, or even abolish, SOB3/AHL29 suppression of hypocotyl elongation in the light. To test this hypothesis, we overexpressed SOB3- $\Delta$ G driven by the *CaMV 35S* promoter in WT *Arabidopsis*. Surprisingly, we observed a long-hypocotyl phenotype in multiple independent hemizygous lines (Fig. 5E), which is similar to seedlings overexpressing *sob3-6*, *esc-11*, or the SOB3 PPC/DUF296 domain alone (Figs. 2 A and C and 4E). Alleles of *sob3-6* and SOB3- $\Delta$ G, as well as the SOB3 PPC/DUF296 domain alone, are likely to have similar efficacy with regard to promoting hypocotyl growth in the light (Fig. S7C). Overexpression of SOB3-GTA by the *CaMV 35S* promoter also recapitulated a similar long-hypocotyl phenotype (Fig. S7D). Thus, SOB3- $\Delta$ G and SOB3-GTA function as dominant-negative alleles, even though the mutant proteins still bear functional AT-hook motifs and could bind with the AT-rich DNA *PRA2* probe (Fig. S7E). This finding suggests that interactions with transcription factors mediated by this Gly-Arg-Phe-Glu-Ile-Leu region in the PPC/DUF296 domain are necessary for the biological function of AHL proteins.

The dependence on this six-amino-acid region in the PPC/DUF296 domain of SOB3/AHL29 for transcriptional activation in yeast, together with the observed interactions with transcription factors identified in this study, implies that this region may play a key role in mediating the physical interaction between SOB3/AHL29 and transcription factor(s) *in planta*. To test this hypothesis, we first used Y2H and BiFC assays to examine the physical interaction with the transcription factor TCP4. TCP4 interacted with WT SOB3/AHL29 in both the BiFC (Fig. 6A) and Y2H assays (Fig. S6C). However, TCP4 no longer interacted with SOB3- $\Delta$ G (Fig. 6B). TCP4 also interacted with WT ESC/AHL27 but not with ESC- $\Delta$ G or ESC-GTA (Fig. 6C and D and Fig. S6D), even though all three forms of ESC were expressed at similar levels (Fig. S8A). Similarly, we also tested interactions with another transcription factor, TCP13 (previously also known as TCP10). TCP13 has been shown to localize in the nucleus (35), as well as in the chloroplast (36). From the results of our BiFC assay, as well as targeted Y2H analysis, TCP13 interacted with the WT SOB3/AHL29 protein and ESC/AHL27 (Figs. S6A and B and S8B and C). However, TCP13 no longer interacted with SOB3- $\Delta$ G (Fig. S8D and E).



**Fig. 5.** Transcription activation by SOB3/AHL29 depends on a conserved region in the PPC/DUF296 domain. (A) Subcellular localization of SOB3- $\Delta$ G protein in onion epidermal cells. Protein-protein interaction analysis of SOB3- $\Delta$ G with full-length SOB3/AHL29 in BiFC (B). Protein-protein interaction analysis of SOB3- $\Delta$ G with the SOB3/AHL29 PPC/DUF296 domain in BiFC (C). (D) Comparison of transcription activation by SOB3/AHL29- and SOB3- $\Delta$ G-containing yeast growing on SDIV plates and in a  $\beta$ -gal assay. Five independent biological replicates are shown. (E) Multiple T2 generation, 6-d-old seedlings overexpressing SOB3- $\Delta$ G under the *CaMV 35S* promoter exhibited a longer hypocotyl phenotype in  $23 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  white light. (Scale bar, 1 cm.)



**Fig. 6.** Interactions of AHLs with transcription factors depends on a six-amino-acid conserved region in the PPC/DUF296 domain. (A) SOB3/AHL29 interacted with the transcription factor TCP4, and (B) this interaction was abolished when the conserved six amino acids in the PPC/DUF296 domain was removed in the BiFC assay. (C and D) ESC/AHL27 interacted with TCP4, and the interaction was abolished when the same six-amino-acid region was removed or substituted with alanine residues in a Y2H assay.

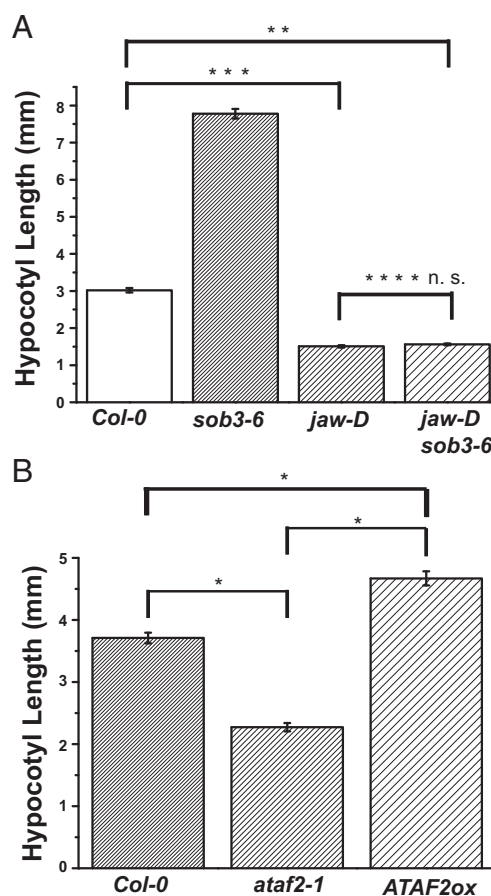
**Non-AHL Interactors Modulate Hypocotyl Growth in the Light.** To understand the roles of the AHL-interacting transcription factors in hypocotyl elongation in the light, we first examined members of the *TCP* gene family. *Jaw-D*, in which several *TCP* genes were specifically knocked down (especially *TCP4*) (37), exhibited a shorter hypocotyl phenotype (Fig. 7A), which is consistent with a previous report (38), suggesting *TCPs* play a role in regulating hypocotyl elongation. We also examined roles of other AHL interactors in hypocotyl elongation in the light. We over-expressed the transcription factor ATAF2, one member of the plant-specific NAC gene family, driven by the *CaMV 35S* promoter in WT *Arabidopsis*. The homozygous single-locus insertion overexpression line (*ATAF2ox*) confers a longer hypocotyl than the WT in the light, whereas the loss-of-function line, *ataf2-1*, showed suppressed hypocotyl growth in the light (Fig. 7B). Another plant-specific AHL interacting partner that was identified in this study, *Gibberellic Acid-Stimulated Arabidopsis 4* (*GASA4*), also has been implicated in regulating hypocotyl growth in the light (39).

To further understand the biological meaning of the physical interactions between the *TCPs* and AHLs, we crossed *jaw-D* with the activation-tagged *sob3-6* allele. The *jaw-D sob3-6* double mutant line exhibited a similar hypocotyl length as *jaw-D* ( $P = 0.13$ ; Fig. 7A). This observation suggests that the long-hypocotyl phenotype conferred by the activation-tagged *sob3-6* allele in the light requires the presence of functional *TCP* proteins. We examined transcript accumulation of *SOB3* (or *sob3-6*) in these lines and observed similar levels of gene expression in each case. These data indicate that the observed genetic epistasis is not due to *TCPs* affecting transcription accumulation of these alleles of *sob3* (Fig. S9 A and B). Collectively, these observations demonstrate a direct role for the participation of physical interacting partners in AHL-mediated hypocotyl elongation in the light.

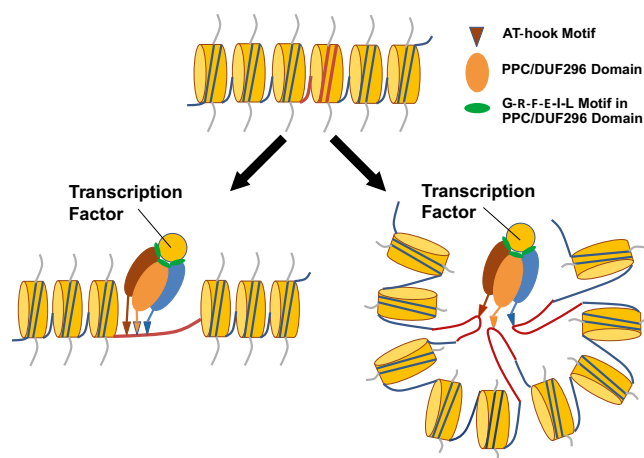
**Molecular Model for AHL Proteins.** Based on our results, as well as those in the literature, we propose a working model that describes the physical interactions among AHLs, other nuclear proteins, and AT-rich chromosomal DNA (Fig. 8). The AHL proteins form a homo-/hetero-complex via the PPC/DUF296 domain, possibly resulting in a trimer (Fig. 8). The multiple AT-hook motifs of the AHL homo-/hetero-complex then allow it to bind AT-rich DNA regions of the same or different chromosomes. Finally, the AT-rich DNA-anchored AHL complex, aided by the Gly-Arg-Phe-Glu-Ile-Leu region, recruits other nuclear non-AHL proteins, such as transcription factors, to form a DNA-AHL-transcription factor (DNA-AHL-TF) complex, which modulates hypocotyl growth in the light.

## Discussion

A unique missense allele, *sob3-6*, caused by an amino acid change in the core of the AT-hook motif, confers a dramatic long-hypocotyl phenotype in the light (16). In this study, we determined that *sob3-6* functions as a dominant-negative allele due to abolished AT-rich DNA-binding ability (Fig. 2). Over-expression of the SOB3/AHL29 PPC/DUF296 domain also led to a similar long-hypocotyl phenotype in light-grown seedlings through a dominant-negative process (Fig. 4). These dominant-negative alleles demonstrated the importance of the AT-hook



**Fig. 7.** Hypocotyl analysis of seedlings grown in continuous white light. (A) Six-day-old seedlings were grown in  $20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  of white light at  $25^\circ\text{C}$ . *Col-0*,  $n = 147$ . *sob3-6*,  $n = 63$ . *jaw-D*,  $n = 70$ . *jaw-D sob3-6*,  $n = 232$ . (B) Seedlings (3.5 d old) were grown in  $10 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  of white light at  $25^\circ\text{C}$ . *Col-0*,  $n = 48$ . *ataf2-1*,  $n = 48$ . *ATAF2ox*,  $n = 48$ . The error bar denotes SEM. In a Student *t* test (unpaired two-tailed *t* test with unequal variance): \* $P < 1.6E-9$ . \*\* $P < 1.7E-54$ . \*\*\* $P < 1.3E-55$ . \*\*\*\*Not significant,  $P = 0.13$ .



**Fig. 8.** The *AHL* gene family regulates plant growth and development by forming an AHL complex. The AHL proteins associate with themselves and each other to form a homo-/hetero-trimeric complex via the PPC/DUF296 domains. The complex uses its AT-hook motifs to anchor itself to AT-rich DNA regions (red-color region) and recruit either transcription factors or other non-AHL proteins in order to regulate plant growth and development. In the proposed molecular model, the hypocotyl-growth-promoting functions of associated transcription factors are rendered nonfunctional by their physical interaction with the DNA-AHL-TF complex.

motif(s) in AHL proteins and implied a biological function for the PPC/DUF296 domain. The severity of the phenotype conferred by these dominant-negative mutations also suggests that other *AHL* genes are involved in regulating hypocotyl growth in *Arabidopsis* seedlings.

Our previous study showed that *SOB3/AHL29* and *ESC/AHL27* redundantly suppress hypocotyl elongation in light-grown *Arabidopsis* seedlings (16). As a part of this study, we characterized hypocotyl growth in four triple-null lines, each in which a different *AHL* gene (*AHL5*, *AHL6*, *AHL15*, or *AHL22*) was removed from the *sob3-4 esc-8* double mutant background (Fig. 2D). We determined that the removal of *AHL5* (unlike the other three *AHL* genes) did not have an impact on hypocotyl elongation. Of the other three *AHLs*, removal of *AHL6* had the greatest impact, demonstrating that *AHL* genes have different quantitative contributions to seedling elongation in the light. The *sob3-4 esc-8 ahl6 ahl22* quadruple-null line exhibited an even longer hypocotyl than the relevant triple-null controls, although it was still shorter than the dominant-negative *sob3-6* mutant (Fig. 2D). This difference suggests that there are additional proteins, possibly *AHLs*, involved in this developmental process.

Given that not all *Arabidopsis AHLs* contribute to light-grown hypocotyl elongation (e.g., *AHL5*), and the probability that some may act redundantly, a dominant-negative approach may be useful for revealing the biological role of other members in this gene family. It is important to note, however, that it would be best if the dominant-negative proteins were expressed according to their endogenous pattern, using the native promoter (possibly with enhancer elements), or via TILLING (40), TALEN (41), or CRISPR/Cas (42) approaches. Such genetic approaches could be very powerful in the case of genomes with even larger *AHL* gene families, such as *Glycine max* (43) and *Brassica rapa* (44), which each have >50 *AHLs*.

In our proposed molecular model, we suggest that the genetic redundancy among AHL proteins is not only due to interactions among themselves, mediated by the PPC/DUF296 domain, but also due to shared, nuclear-localized, non-AHL interactors (Fig. 8). The predicted tertiary structure of the PPC/DUF296 domain of *SOB3/AHL29* suggests trimeric oligomerization, which is similar to that observed in seven determined crystal structures of

this domain from Bacteria and Archaea [Protein Data Bank (PDB) ID: 2H6L, 2DT4, 2HX0, 2NMU, 2P6Y, 3HTN, and 3HWU] (14, 15). Trimer formation in a proposed AHL complex is also supported by the physical interactions observed among AHLs in this study (Figs. 3 and 4 and Fig. S5). The 29 *AHL* genes encoded by the *A. thaliana* genome allow flexibility in complex formation and subsequent regulation of the related biological processes in plant growth and development.

According to our proposed model, a trimeric AHL complex would have three or more (up to six) AT-hook motifs, depending on the composition of its subunits. The number of AT-hook motifs, as well as their types (type 1 or 2), would determine the AHL complex's affinity for AT-rich DNA. It is important to note that the AT-hook motifs in AHL proteins are identical to the ones in human HMGA proteins. The human genome encodes three HMGA proteins, each of which contains three AT-hook motifs (45). Among the three AT-hook motifs in an HMGA protein, the middle one is identical to the AT-hook motif in *SOB3/AHL29* (Fig. S10). These AT-hook motifs also confer the highest affinity to AT-rich DNA (12, 46). The flanking AT-hook motifs of HMGAs are similar to the type 2 AT-hook motif of AHLs. These flanking HMGA AT-hook motifs both have decreased DNA-binding affinity compared with type 1 (12). With these different affinities in mind, trimeric AHL complexes could possess a variety of combinations of AT-hook motifs that in turn could impart to the complex a wide range of binding affinities for AT-rich chromosomal regions. Such AHL complexes could regulate a variety of biological processes.

The human HMGA proteins have been proposed to promote the assembly of an enhanceosome by binding to the AT-rich promoter region of genes and further recruiting other nuclear proteins, such as chromatin remodeling machinery and transcription factors, to positively or negatively regulate gene expression (47–49). In the case of virus-induced transcription of the human *IFN- $\beta$*  gene, HMGA has been proposed to first bind AT-rich DNA in a 1:1 (protein:DNA) ratio and subsequently alter chromatin status, as well as serve as a platform for the recruitment of eight transcription factors. This process leads to the assembly of the nuclear macromolecular complex (50, 51). The 1:1 HMGA:DNA binding ratio indicates that three AT-hook motifs of one HMGA are necessary to target HMGA to the corresponding chromosome region and induce assembly of the enhanceosome.

Similar promoter binding capabilities have been reported for several AHL proteins. *AHL15* and *AHL25* bind to the promoter of *GA 3-oxidase 1* in *Arabidopsis* (23). *AHL21* binds to the promoter of the *ETTIN/auxin response factor 3* gene (20). *AHL22* binds to the promoter of the *Flowering Locus T* gene (18). In *Catharanthus roseus*, five AHLs bind to the promoter of the *APETALA2* (*AP2*) transcription factor *ORCA3* gene (24). These observations suggest that trimeric AHL complexes could also be targeted to promoter regions through their AT-hook motifs.

The three HMGA AT-hook motifs necessary for AT-rich DNA binding correspond to the three motifs (at minimum) in a proposed trimeric AHL complex. This molecular model explains the dominant-negative long-hypocotyl phenotypes conferred by the *sob3-6* allele (which does not bind AT-rich DNA) or expression of the PPC domain alone (which lacks an AT-hook motif). Both dominant-negative alleles encode proteins containing an intact PPC/DUF296 domain, which could still physically interact with WT AHL proteins and form an AHL complex with attenuated DNA-binding activity (Figs. 2 and 4). A similar dominant-negative phenomenon has also been observed with a human HMGA protein bearing a nonfunctional AT-hook motif (32), which resembles the *sob3-6* allele in this study.

We demonstrated that the conserved Gly-Arg-Phe-Glu-Ile-Leu region of the PPC/DUF296 domain is necessary for physical interaction with the transcription factors TCP4 and TCP13 (Fig. 6 and Figs. S6 A–D and S8). We also showed that expression of



*SOB3* alleles that are mutated in this region confers a dominant-negative long-hypocotyl phenotype (Fig. 5E and Fig. S7D). Homology modeling of the SOB3/AHL29 PPC/DUF296 domain suggests that this conserved region is located at the exterior of each monomer and could combine to form an exposed quaternary domain after trimerization (Fig. S3D). This prediction suggests that the quaternary domain composed of these three six-amino-acid regions could serve as the site of physical interaction between the AHL complex and transcription factors (Fig. 8). However, the exact biological importance of this domain with regard to physical interactions with transcription factors is still unknown.

Some members of the *AHL* gene family are negative regulators of hypocotyl elongation in the light (16, 17) (Fig. 2D). In contrast, we identified AHL-interacting transcription factors (ATAF2 and TCPs) that are positive regulators of this event (37, 38) (Fig. 7). In the proposed molecular model, the hypocotyl growth-promoting function of associated transcription factors is rendered nonfunctional by their physical interaction in the DNA-AHL-TF complex. For example, the overexpression of WT *AHLs* (e.g., *SOB3-D*, *SOB3ox*, and *ESCOx*) leads to an increased accumulation of DNA-AHL-TF complexes resulting in a short hypocotyl phenotype (16) (Fig. 2). When the relevant *AHL* genes are knocked out in higher-order combinations (e.g., *sob3-4 esc-8 ahl6 ahl22*), or lose their ability to bind DNA (e.g., *sob3-6* and *esc-11*), this leads to a decreased accumulation of DNA-AHL-TF complexes resulting in a long hypocotyl phenotype (16) (Fig. 2A and C), possibly by releasing the transcription factors from the complex and allowing them to promote hypocotyl elongation.

Similarly, mutating the conserved Gly-Arg-Phe-Glu-Ile-Leu region of the PPC/DUF296 domain (e.g., *SOB3-ΔG* and *SOB3-GTA*) disrupts interactions with certain transcription factors and also leads to a decreased accumulation of DNA-AHL-TF complexes resulting in a long hypocotyl phenotype via growth promotion from the free transcription factors (Fig. 5E and Fig. S7D). The *jaw-D sob3-6* double mutant demonstrates the importance of some TCP transcription factors in contributing to the long-hypocotyl phenotype conferred by the *sob3-6* disruption of the DNA-AHL-TF complexes (Fig. 7A), presumably due to the lack of free TCPs for promoting hypocotyl elongation. Hence, the *sob3-6* phenotype is gone and the double mutant exhibits a similar hypocotyl length as observed in the *jaw-D* single mutant.

How exactly the physical interaction with the DNA-AHL complex impairs the functions of their associated transcription factors remains to be explored. It is possible that the association with DNA-AHL complex interferes with the normal binding of transcription factors to the promoter regions of the genes that they regulate, subsequently impairing their biological functions. It is also possible that the association with the DNA-AHL complex alters posttranslational modifications associated with

the transcription factors, in turn rendering the associated transcription factors nonfunctional. On the other hand, in the *sob3-6* and *esc-11* dominant-negative lines, the abolishment of DNA-binding ability for the AHL complex possibly destabilizes the association between AHLs and transcription factors; therefore, transcription factors that would normally be part of the DNA-AHL-TF complexes are released and function to promote hypocotyl growth in the light.

Through a combination of overexpression and loss-of-function analysis, we determined that multiple members of the *AHL* gene family suppress hypocotyl elongation redundantly in light-grown seedlings. The physical interactions among the AHL proteins via the PPC/DUF296 domain, as well as their interactions with other common nuclear proteins, such as transcription factors, may be important for their biological functions. However, biological functions of each *AHL* gene in the *Arabidopsis* genome still need to be determined. Multiple lines of evidence suggest that functional similarities exist between the HMGA and AHL proteins in that they both form nuclear macromolecular complexes on AT-rich chromatin regions. Further experiments should be conducted to reveal binding sites of AHL proteins in the *Arabidopsis* genome. The biological importance of the protein-protein interactions between the AHL complex and other nuclear proteins also needs to be further determined.

## Materials and Methods

Detailed descriptions of plant materials, seedling growth conditions and hypocotyl measurement, plasmid construction, phylogenetic analysis, protein expression and purification, EMSA, bimolecular fluorescence complementation assay, and yeast two-hybrid assays are provided in *SI Materials and Methods*. Briefly, all *Arabidopsis* lines are in *Columbia* background. Sequences of the *AHL* genes and the transfer DNA insertion lines were obtained from the *Arabidopsis* Biological Research Center. Surface-sterilized *Arabidopsis* seeds were sown on media containing 1.0% (wt/vol) phytigel, 1.5% (wt/vol) sucrose, and 0.5× Linsmaier and Skoog-modified basal medium and incubated for 5 d at 25 °C in continuous white light under the indicated intensity. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific).

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