

ATBS1 INTERACTING FACTORs negatively regulate *Arabidopsis* cell elongation in the triantagonistic bHLH system

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We recently demonstrated that cell elongation in plants is regulated by a triantagonistic bHLH system, in which three bHLH proteins, Activator of Cell Elongation 1 (ACE1), *Arabidopsis* IL1 binding BHLH 1 (AtIBH1) and Paclobutrazol Resistance 1 (PRE1), competitively regulate the expression of genes for cell elongation. Here we show that ATBS1 Interacting Factor 2 (AIF2), AIF3 and AIF4 interact with PRE1 and ACE1, similar to AtIBH1, and also negatively regulate cell elongation in the triantagonistic bHLH system. The expression of each *AIF* is constitutive or induced by light, but *AtIBH1* expression is dependent on BR signaling and developmental phase. These results indicate that AIFs and AtIBH1 may play different roles in cell elongation in different signaling pathways.

In plants, multiple phytohormones, namely brassinosteroids (BR), gibberellins (GA) and auxin, positively regulate cell elongation throughout the plant life cycle.¹ Three atypical bHLH proteins of the PRE sub-family, Paclobutrazol Resistance 1 (PRE1), PRE3/Activation-Tagged BRI1 Suppressor 1 (PRE3/ATBS1) and PRE6/KIDARI, positively regulate organ elongation in response to GA, BR and light signaling, respectively.²⁻⁴ These PRE sub-family proteins interact with other bHLH proteins, including *Arabidopsis* IL1 Binding bHLH 1 (AtIBH1), ATBS1 Interacting Factors (AIFs) and Long Hypocotyl in Far-Red (HFR1), which negatively regulate organ elongation.^{2,4,5} We recently showed that AtIBH1, PRE1 and newly identified bHLH transcriptional factors, named Activators for Cell Elongation 1 (ACE1), ACE2, ACE3 and CIB5, constitute a triantagonistic bHLH system. In this system, AtIBH1, PRE1 and ACEs competitively regulate cell elongation under BR, GA and developmental phase dependent signals.⁶ ACE1 directly activates the expression of enzyme genes for cell elongation by interacting with their promoter regions, while AtIBH1 interacts with ACEs, interfering with the binding of ACEs to the promoters of their target genes, and thereby inhibiting cell elongation. PRE1 interacts with AtIBH1 and counteracts the ability of AtIBH1 to affect ACEs. Therefore, PRE1 restores the transcriptional activity of ACE1, resulting in induction of cell elongation.⁶

To identify other negative regulators that interact with PRE1 and function like AtIBH1 in the triantagonistic system, we performed a yeast two-hybrid (Y2H) screen using PRE1 as a bait

and a library composed of only cDNAs of *Arabidopsis* transcription factor genes.⁷ From this screen, we found that AIF2, AIF3 and AIF4, but not AIF1, interact with PRE1 (Table 1). We confirmed the interaction of PRE1 with AIF2, AIF3 and AIF4 by individual Y2H assays (Fig. 1A) and found that those AIFs also interacted with ACE1 (Fig. 1B). Interestingly, AIF1, which is closely related to AIF2/3/4, does not interact with either PRE1 or ACE1 in our Y2H assays (Fig. 1A and B). The AIFs are closely related to AtIBH1 and were shown to interact with PRE3/ATBS1 (Fig. 1C).^{3,4} Wang et al. showed that AIF1 regulates cell elongation negatively but PRE3/ATBS1, which interacts with AIF1, regulates it positively in response to BR signaling.⁴ Overexpression of *AIF1* was shown to induce a dwarf phenotype, similar to the phenotype of *AtIBH1*-overexpressing plants.⁴⁻⁶

We prepared transgenic plants that ectopically expressed *AIF2*, *AIF3* and *AIF4* (*P35S:AIF2*, *P35S:AIF3*, *P35S:AIF4*), respectively (Fig. 2A and B). All the *AIF* overexpressing plants exhibited drastic dwarfism, and had round-shaped, dark green leaves and short petioles and siliques (Fig. 2B). These phenotypes were similar to the phenotype of *P35S:AtIBH1* plants,^{5,6} suggesting that AIF2, AIF3 and AIF4 have similar biological functions to AtIBH1, which negatively regulates cell elongation. In addition, we analyzed the phenotype of transgenic plants that express the chimeric repressors for AIFs, which have the plant-specific SRDX repression domain fused to AIF2 and AIF3 (*P35S:AIF2-SRDX* and *P35S:AIF3-SRDX*; Fig. 2A).⁸ We found that both

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Table 1. TFs that interact with PRE1, as identified by yeast two-hybrid screening

Times isolated	Isolated TF	TF family	Description
6	At1g09250 (AIF4)	bHLH	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
2	At3g06590 (AIF2)	bHLH	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
2	At2g43060 (AtIBH1)	bHLH	<i>Arabidopsis</i> IL11 binding bHLH 1
1	At3g17100 (AIF3)	bHLH	basic helix-loop-helix (bHLH) DNA-binding family protein
14	Three other bHLHs	bHLH	basic helix-loop-helix (bHLH) DNA-binding family protein

P35S:AIF2-SRDX and *P35S:AIF3-SRDX* had round-shaped and dark green leaves and exhibited similar phenotypes to *P35S:AIF2* and *P35S:AIF3* plants (Fig. 2B and C). These results indicate that these AIFs act as transcriptional repressors, similar to AtIBH1, because fusion of the SRDX to a native repressor results in a similar phenotype to its ectopic expression.⁹

The basic motif of AIF proteins lacks the amino acids necessary for binding to the E- and G-box; therefore, it has been suggested that AIFs might not be DNA-binding bHLH proteins.^{4,10} Our results suggest that the functional mechanisms of AIF2, AIF3 and AIF4 are likely to be the same as that of AtIBH1, binding to another bHLH protein to interfere with its function. AIFs likely interfere with the DNA binding of ACE1 and inhibit cell elongation by suppressing the activation activity of ACE1. Moreover, we showed that AIFs interact with PRE; therefore, similar to its inhibition of AtIBH1, PRE is also likely to inhibit AIFs.

In this study, we show that *Arabidopsis* AIF2, AIF3 and AIF4 function as negative regulators of cell elongation and are likely to act redundantly with AtIBH1. AIFs may constitute a triantagonistic bHLH system with ACE1 and PRE1. AIF2/3/4, however, may regulate cell elongation differentially from AtIBH1, because the AIFs and *AtIBH1* have different expression patterns (Fig. 2D).^{5,6} For example, *AIF4* expression was induced by light treatment but *AIF2* and *AIF3* expression was not, suggesting that *AIF4* may regulate cell elongation in response to light signaling (Fig. 2D). Also, BR and developmental phase did not affect *AIF* expression. These *AIF* expression patterns indicated that *AtIBH1* and *AIFs* might have different mechanisms regulating their expression. Therefore AtIBH1 and each AIF may negatively regulate cell elongation under different signaling pathways, but may act through similar functional mechanisms, suppressing cell elongation via the inhibition of ACE1 DNA binding. Further studies of the systems that control the expression and post-transcriptional regulation of AIFs will provide more information on plant cell elongation.

Materials and Methods

Construction of plasmids. For construction of *P35S:AIF2*, cDNAs of *AIF2*, with the stop codon, were amplified by a pair of primers with an *attB1/B2* site (*attB1_At3g06590*; 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAT GGC GTC TCT GAT CTC AGA TAT TGA ACC-3', *attB2_At3g06590*; 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCA AAT CGG TGG AGG AGC TGA GCC GTA GGA-3') and cloned into the pDONR207 vector (Life Technologies Inc.) by BP cloning. Cloned cDNAs were transferred into pDEST_35S_HSP_GWB5 by LR clonase. For construction of *P35S:AIF3* and *P35S:AIF4*, cDNAs of *AIF3* and *AIF4* with the stop codon were amplified by a pair of primers (*At3g17100N*; 5'-gAT GGA GTC TAT ATC TCC GGT ATC GAA TCA -3', *At3g17100.2SC*; 5'-GGG GTC GAC TAA CCA AGC ATG TGT GTT TGT CCC CCT TC-3', *At1g09250N*; 5'-GAT GGT GGA GTC TCT GTT CCC GAG CAT CGA-3', *AIF4oxsal*; 5'-GGG TCG ACT TAA GTT CCG GTC AAC GTC GTC CGT GGT GC-3') and cloned into the P35S vector. Constructions for CRES-T, cDNAs of *AIF2* and *AIF3* without the stop codon were amplified by a pair of primers (*At3g06590N*; 5'-gAT GGC GTC TCT GAT CTC AGA TAT TGA ACC-3', *At3g06590C*; 5'-AAT CGG TGG AGG AGC TGA GCC GTA GGA GGA-3', *At3g17100N*; 5'-gAT GGA GTC TAT ATC TCC GGT ATC GAA TCA-3', *At3g17100C*; 5'-ACC AAG CAT GTG TGT TTG TCC CCC TTC GTG-3') and cloned into the SmaI site of the p35SSRDXG vector.¹¹ Cloned cDNAs were transferred into pBCKH by LR clonase.

Yeast two-hybrid screening. The yeast two-hybrid assays were performed as described previously.⁷ For bait construction, *PRE1* cDNA with the stop codon was amplified by a pair of primers with an *attB1/B2* site (*attB1_AT5G39860.1*; 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAT GTC GAA CAG AAG ATC AAG GCA ATC TTC-3', *attB2_AT5G39860.1*; 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA CAT GAG TAG GCT TCT AAT AAC GGC GGC-3') and *ACE1* cDNA with the stop codon was amplified by a pair of primers with an *attB1/B2* site (*attB1_ACE1*; 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAT GGA TTT AAG TGC GAA AGA TGA GTT TTC A-3', *attB2_ACE1*; 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TGG CTC AAC CTT CAT ATT TGC AGC TGG AAG-3') and cloned into the pDONR207 vector (Life Technologies Inc., USA) by BP cloning. Cloned cDNAs were transferred into pDEST_BTM116 by LR clonase.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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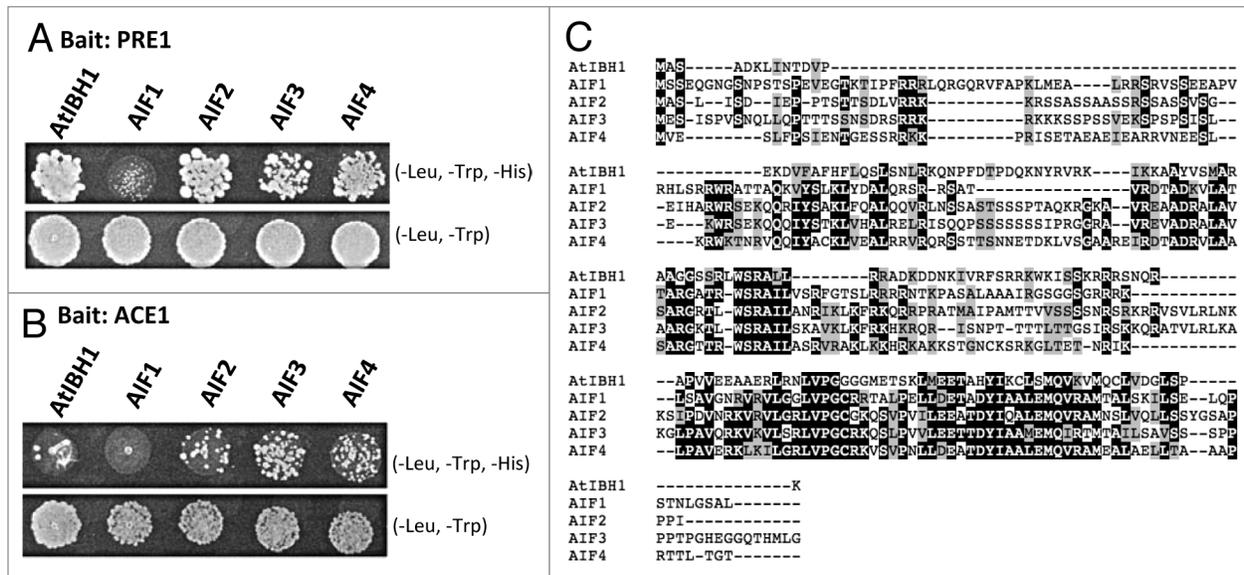


Figure 1. Interaction between PRE1 and AIFs and between ACE1 and AIFs in yeast two-hybrid assays on -L-W-H and -L-W media using PRE1 (A) or ACE1 (B) as bait. (C) Alignment of amino acid sequences of AtIBH1 and each AIF.

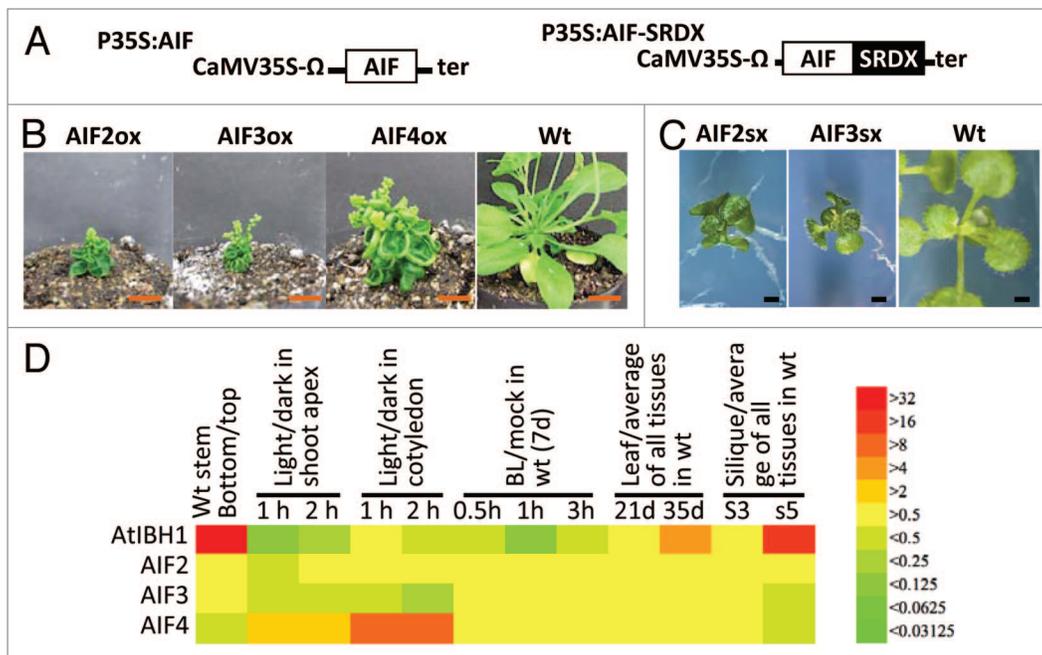


Figure 2. Functional analysis of AIF2, AIF3 and AIF4. (A) Schematic representation of the constructs. (B) Rosette plants of *P35S:AIF2* (AIF2ox), *P35S:AIF3* (AIF3ox), *P35S:AIF4* (AIF4ox) and wild-type (wt) plants. Bar = 1 cm. (C) Fourteen-day-old seedlings of *P35S:AIF2-SRDx* (AIF2sx) and *P35S:AIF3-SRDx* (AIF3sx). Bar = 1 mm. (D) The fold change of expression of *AtIBH1* and *AIFs* in different tissues (the bottom / top part of stem, young and old leaves and young and old siliques shoot), different light conditions in two tissues (shoot apex and cotyledon) and treatment with brassinolide (BL). Color represents the fold-change of expression to control as shown in right.

References

- Depuydt S, Hardtke CS. Hormone signalling crosstalk in plant growth regulation. *Curr Biol* 2011; 21:R365-73; PMID:21549959; <http://dx.doi.org/10.1016/j.cub.2011.03.013>.
- Hyun Y, Lee I. KIDARI, encoding a non-DNA Binding bHLH protein, represses light signal transduction in *Arabidopsis thaliana*. *Plant Mol Biol* 2006; 61:283-96; PMID:16786307; <http://dx.doi.org/10.1007/s11103-006-0010-2>.
- Lee S, Lee S, Yang KY, Kim YM, Park SY, Kim SY, et al. Overexpression of PRE1 and its homologous genes activates Gibberellin-dependent responses in *Arabidopsis thaliana*. *Plant Cell Physiol* 2006; 47:591-600; PMID:16527868; <http://dx.doi.org/10.1093/pcp/pcj026>.

4. Wang H, Zhu Y, Fujioka S, Asami T, Li J, Li J. Regulation of Arabidopsis brassinosteroid signaling by atypical basic helix-loop-helix proteins. *Plant Cell* 2009; 21:3781-91; PMID:20023194; <http://dx.doi.org/10.1105/tpc.109.072504>.
5. Zhang LY, Bai MY, Wu J, Zhu JY, Wang H, Zhang Z, et al. Antagonistic HLH/bHLH transcription factors mediate brassinosteroid regulation of cell elongation and plant development in rice and Arabidopsis. *Plant Cell* 2009; 21:3767-80; PMID:20009022; <http://dx.doi.org/10.1105/tpc.109.070441>.
6. Ikeda M, Fujiwara S, Mitsuda N, Ohme-Takagi M. A triantagonistic basic helix-loop-helix system regulates cell elongation in Arabidopsis. *Plant Cell* 2012; 24:4483-97; PMID:23161888; <http://dx.doi.org/10.1105/tpc.112.105023>.
7. Mitsuda N, Ikeda M, Takada S, Takiguchi Y, Kondou Y, Yoshizumi T, et al. Efficient yeast one-/two-hybrid screening using a library composed only of transcription factors in *Arabidopsis thaliana*. *Plant Cell Physiol* 2010; 51:2145-51; PMID:20980269; <http://dx.doi.org/10.1093/pcp/pcq161>.
8. Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M. Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. *Plant J* 2003; 34:733-9; PMID:12787253; <http://dx.doi.org/10.1046/j.1365-313X.2003.01759.x>.
9. Ikeda M, Ohme-Takagi M. A novel group of transcriptional repressors in Arabidopsis. *Plant Cell Physiol* 2009; 50:970-5; PMID:19324928; <http://dx.doi.org/10.1093/pcp/pcp048>.
10. Toledo-Ortiz G, Huq E, Quail PH. The Arabidopsis basic/helix-loop-helix transcription factor family. *Plant Cell* 2003; 15:1749-70; PMID:12897250; <http://dx.doi.org/10.1105/tpc.013839>.
11. Mitsuda N, Hiratsu K, Todaka D, Nakashima K, Yamaguchi-Shinozaki K, Ohme-Takagi M. Efficient production of male and female sterile plants by expression of a chimeric repressor in Arabidopsis and rice. *Plant Biotechnol J* 2006; 4:325-32; PMID:17147638; <http://dx.doi.org/10.1111/j.1467-7652.2006.00184.x>.