eLIFE
elifesciences.org

RESEARCH ARTICLE

Cell elongation is regulated through a central circuit of interacting transcription factors in the Arabidopsis hypocotyl

Eunkyoo Oh, Jia-Ying Zhu, Ming-Yi Bai, Rafael Augusto Arenhart, Yu Sun, Zhi-Yong Wang*

Department of Plant Biology, Carnegie Institution for Science, Stanford, United States

Abstract As the major mechanism of plant growth and morphogenesis, cell elongation is controlled by many hormonal and environmental signals. How these signals are coordinated at the molecular level to ensure coherent cellular responses remains unclear. In this study, we illustrate a molecular circuit that integrates all major growth-regulating signals, including auxin, brassinosteroid, gibberellin, light, and temperature. Analyses of genome-wide targets, genetic and biochemical interactions demonstrate that the auxin-response factor ARF6, the light/temperature-regulated transcription factor PIF4, and the brassinosteroid-signaling transcription factor BZR1, interact with each other and cooperatively regulate large numbers of common target genes, but their DNA-binding activities are blocked by the gibberellin-inactivated repressor RGA. In addition, a tripartite HLH/bHLH module feedback regulates PIFs and additional bHLH factors that interact with ARF6, and thereby modulates auxin sensitivity according to developmental and environmental cues. Our results demonstrate a central growth-regulation circuit that integrates hormonal, environmental, and developmental controls of cell elongation in Arabidopsis hypocotyl.

DOI: 10.7554/eLife.03031.001

Auxin/IAA

Auxin is essential for cell elongation responses to shade, warm temperature, and the circadian clock as well as tropic growth responses to light and gravity

The ability of auxin to regulate cell elongation also depends on developmental context and the status of other hormonal and environmental signals.

Gibberellins/GAs

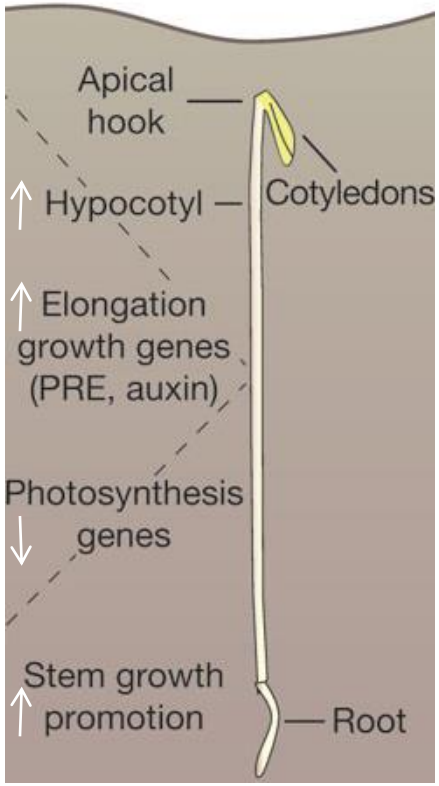
Gibberellins are also involved in the promotion of cell expansion. GAs promote cell expansion cooperating with PIF factors, negative regulators of light-mediated processes.

Brassinosteroids/BRs

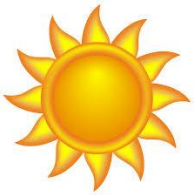
Brassinosteroids are a group of plant steroid hormones, originally characterized for their function in cell elongation, although it is becoming clear that they play major roles in plant growth and developmental processes.

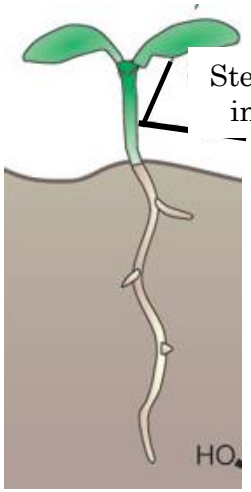
Cell expansion in hypocotyl growth

Skotomorphogenesis



Photomorphogenesis





DELLA

GAs

PIF

Aux, Br

The main Players

ARF6

Encodes a member of the auxin response factor family. Mediates auxin response via expression of auxin regulated genes. Acts redundantly with ARF8 to control stamen elongation and flower maturation. Involved in cell expansion

BZR1

Encodes a positive regulator of the brassinosteroid (BR) signalling pathway that mediates both downstream BR responses and negative feedback regulation of BR biosynthesis.

PIF4

Encodes a bHLH protein that interacts with active phyB protein. Negatively regulates phyB mediated red light responses. Involved in shade avoidance response. Protein abundance is negatively regulated by phyB.

ARF6 and its closed homolog ARF8

were previously shown to redundantly regulate

hypocotyl elongation in *Arabidopsis*

In this study

Genome-wide analyses of target genes of ARF6 (ChIP-Seq),

that regulates hypocotyl elongation,

demonstrate that the majority of ARF6 target genes

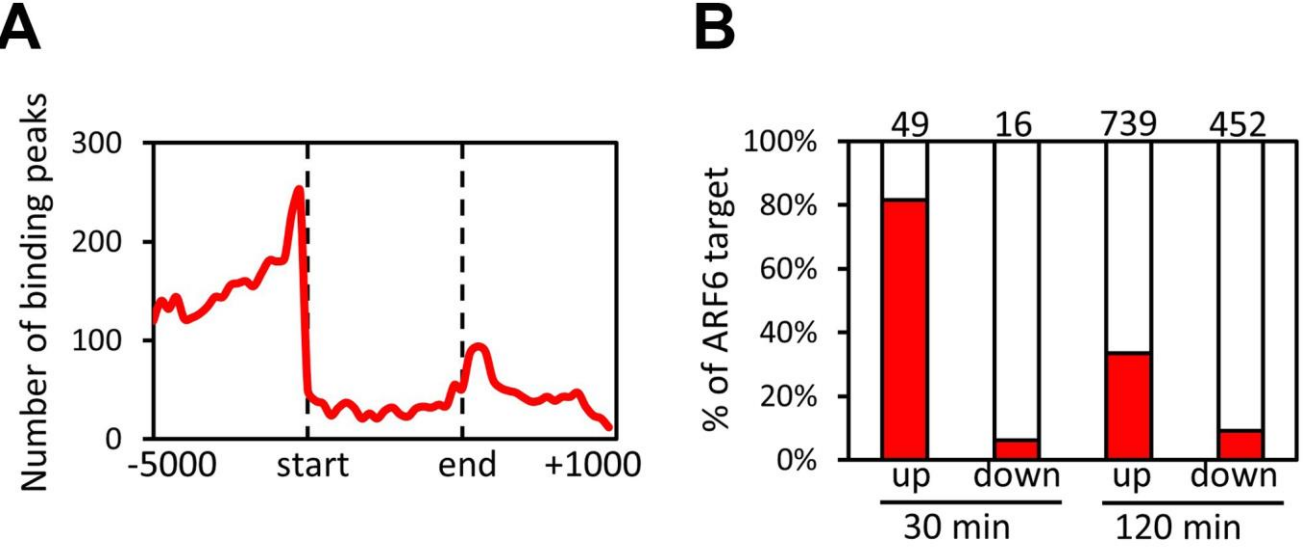
are also targets of BZR1 and/or PIF4

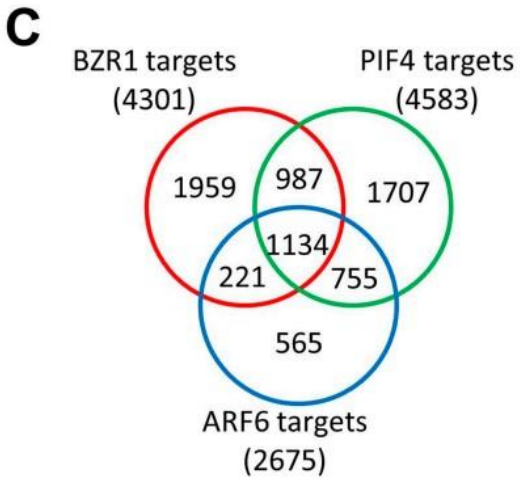
ChIP-Seq analysis of target genes of ARF6

An ARF6-Myc fusion protein was expressed from the *ARF6* promoter in transgenic *arf6-2;arf8-3* plants, and rescued the short-hypocotyl phenotype of the *arf6;arf8* double mutant

(A) ARF6-Myc regulated by ARF6 native promoter restored short hypocotyl of the *arf6;arf8* double mutant. Seedlings were grown in the dark for 6 days. Representative seedlings are shown

Complementation assay with the ARF6-MYC chimeric protein





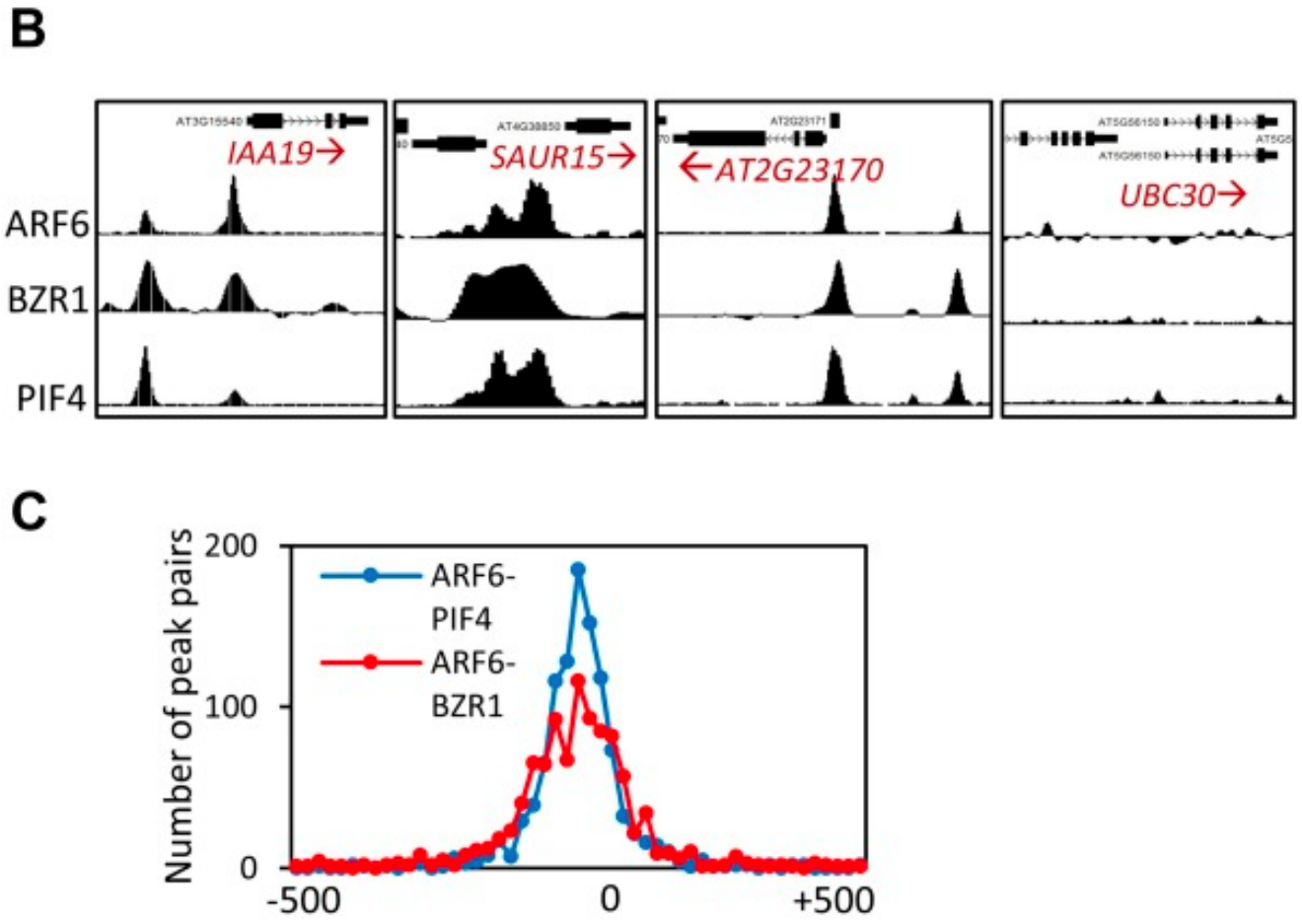
ARF6 ChIP-Seq analyses.

A) Distribution of ARF6 binding peaks relative gene structure.

B) Most of the early auxin-activated genes are ARF6 targets.

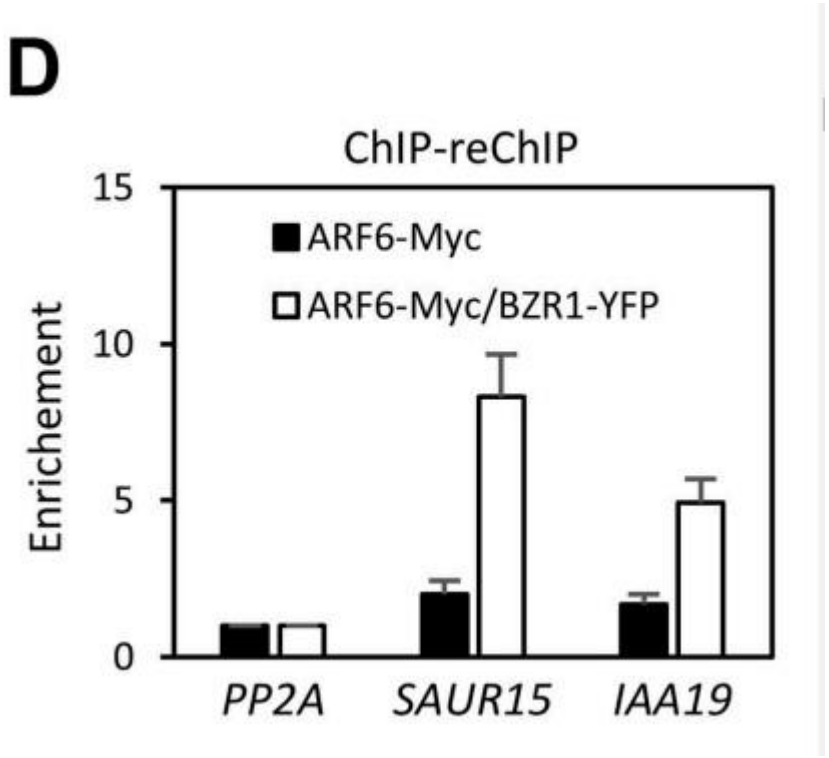
Performed BZR1 ChIP-Seq (BZR1p::BZR1-CFP and 35S::YFP control) in the same conditions (dark); PIF4 ChIP-Seq from previous data

C) Venn diagram shows **significant overlap among binding target genes of BZR1, PIF4 and ARF6**.



(**B**) **Representative ARF6, BZR1, and PIF4 binding peaks** in the promoters of common target genes (*IAA19*, *SAUR15* and *AT2G23170*). *UBC30* promoter as a negative control. (**C**) **Distance distribution of ARF6 and PIF4 binding peaks or ARF6 and BZR1 binding peaks** in the common target genes.

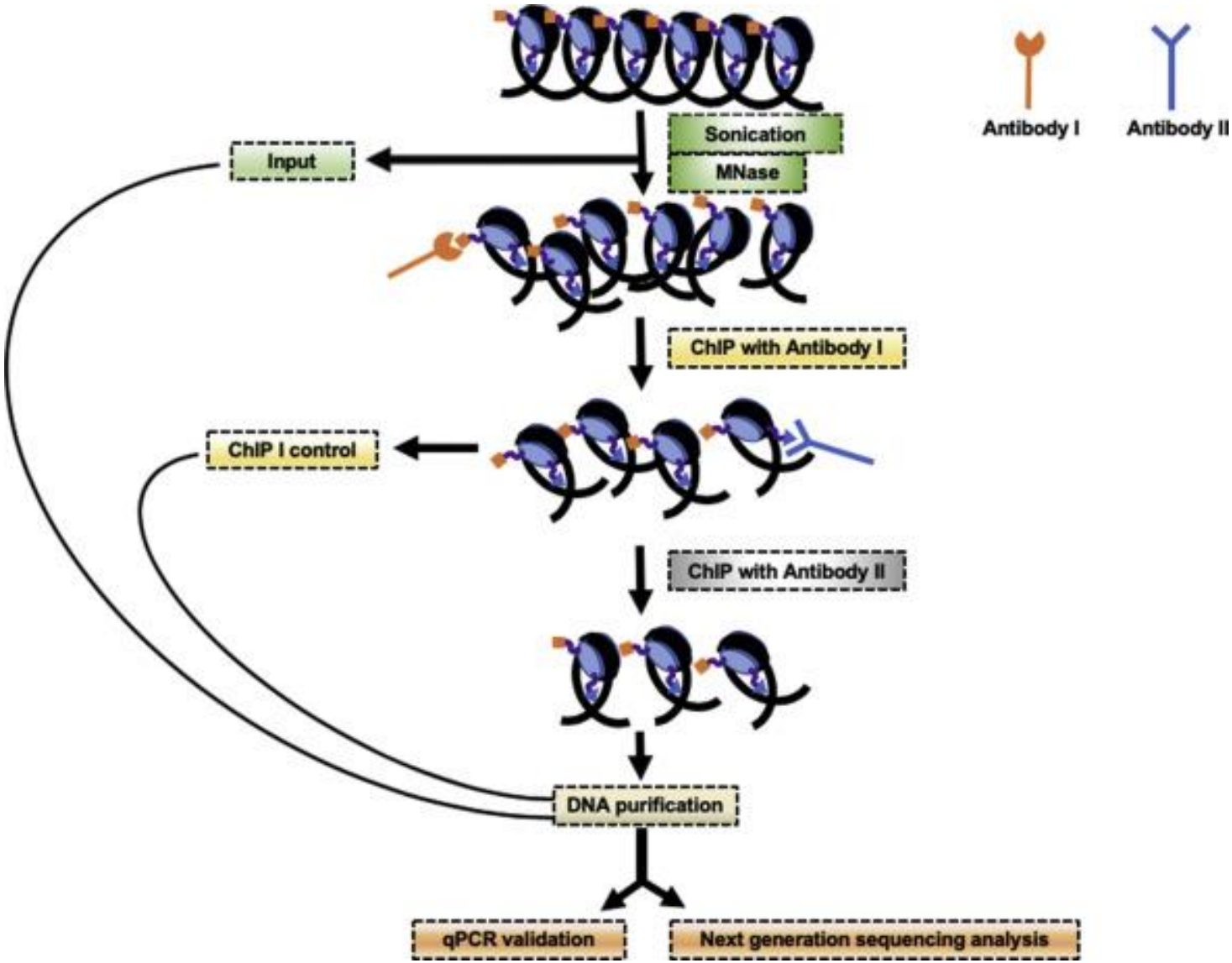
These TFs bind to same or nearby genomic locations.



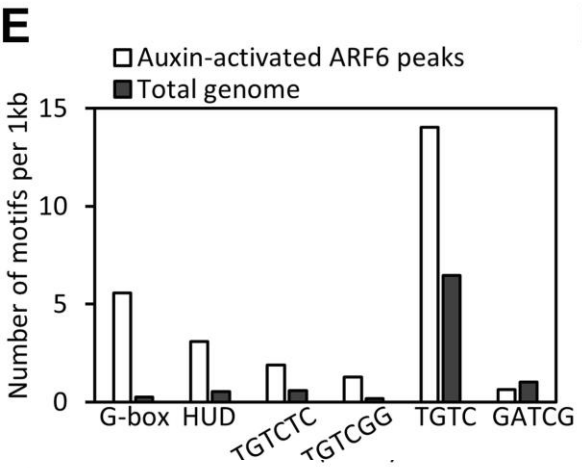
Two common targets, SAUR15 and IAA19, were recovered by sequential immunoprecipitation in plants expressing both BZR1-YFP and ARF6-Myc, but not in plants expressing ARF6-Myc only

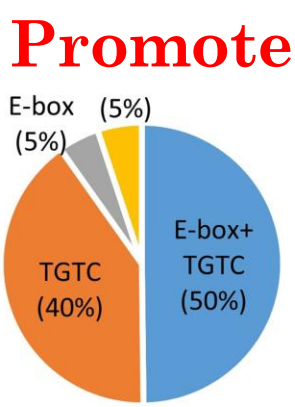
(D) ChIP-reChIP assay shows that BZR1 and ARF6 co-occupy shared target promoters. The enrichment of precipitated DNA was calculated as the ratio between transgenic plants and WT control, normalized to that of the PP2A coding region as an internal control. Error bars indicate the SD of 3 biological repeats.

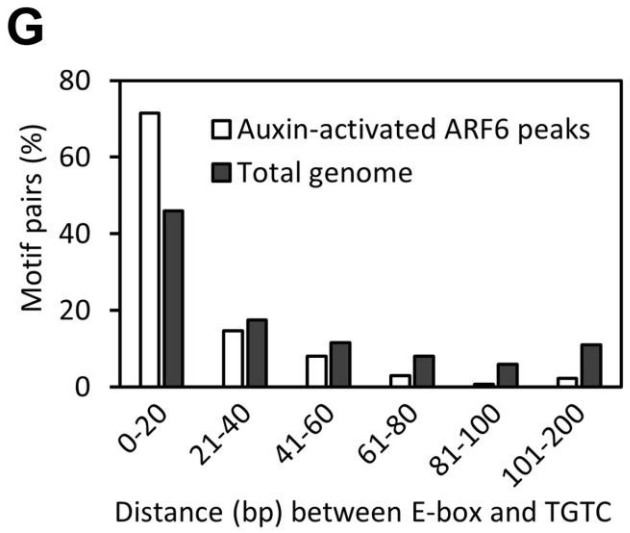
ChIP-reChIP assay

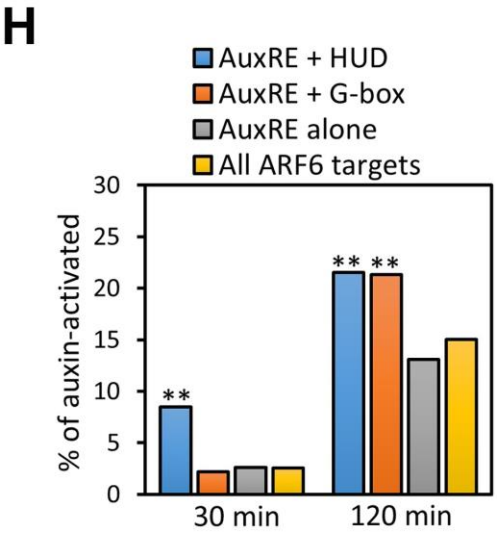


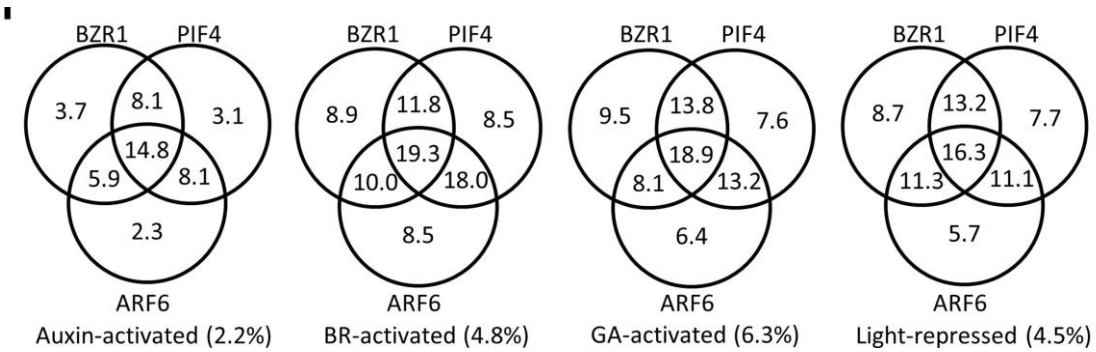
Promoter Analysis











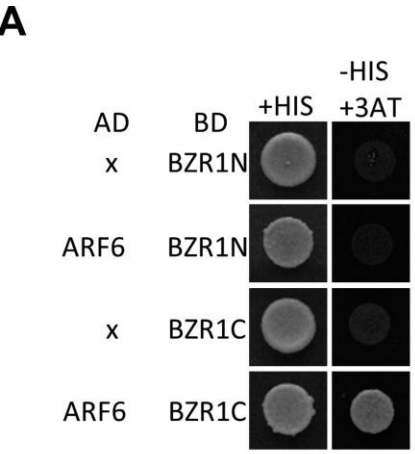
box, HUD (CACATG), AuxRE and TGTCGG are enriched in the ARF6 binding peaks associated with IAA-activated genes. GATCG is shown as a negative control. (**F**) % of IAA-activated ARF6 binding peaks that have both E-box and AuxRE, only TGTC, or only E-box motifs. (**G**) Distance between E-box and AuxRE found in the ARF6 peaks associated with IAA-activated genes or total genome. (**H**) **ARF6 binding peaks having both E-box motifs and AuxRE have higher probability (%) of being associated with IAA-activated genes than the ARF6 binding peaks having only AuxRE**. **p<0.01. (**I**) Venn diagram shows that genes activated by IAA, BR, or GA and genes repressed by light are enriched in the common binding targets of BZR1, PIF4 and ARF6. Numbers in Venn diagram: % of corresponding genes in each section. Numbers in (): % of genes in total genome.

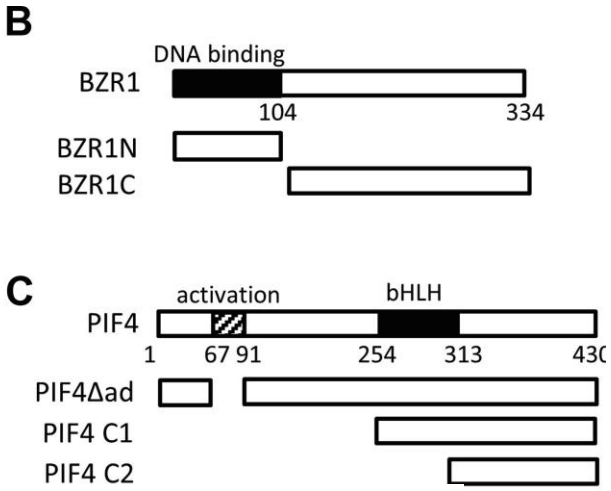
These results suggest that the major growth signals
—auxin, BR, GA, and light—
converge at shared genomic target promoters
containing
combinatorial cis-elements for these factors

This raises the hypothesis
of direct interactions among these factors

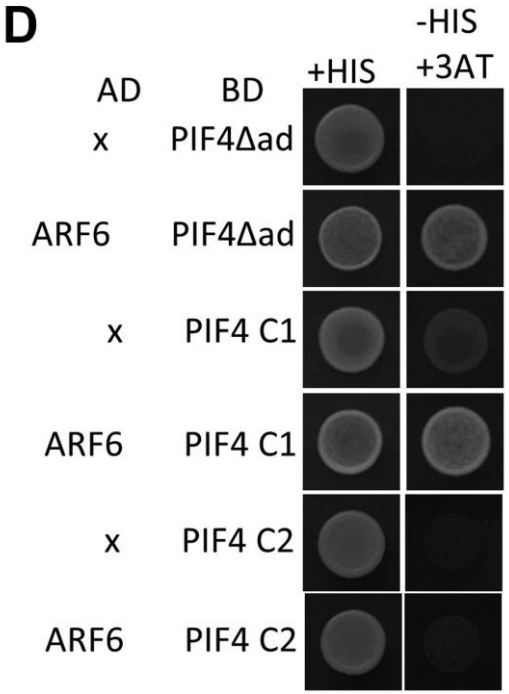
BZR1 and PIF4 interact with ARF6

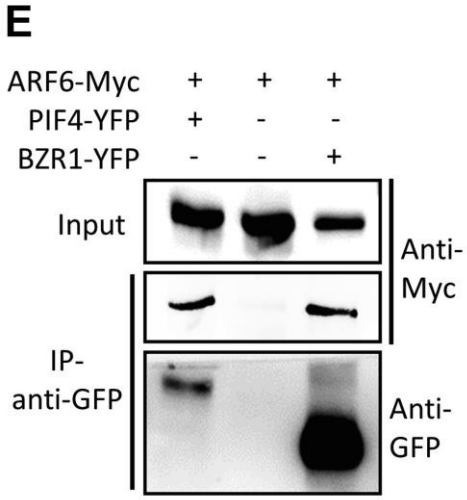
BZR1 ARF6

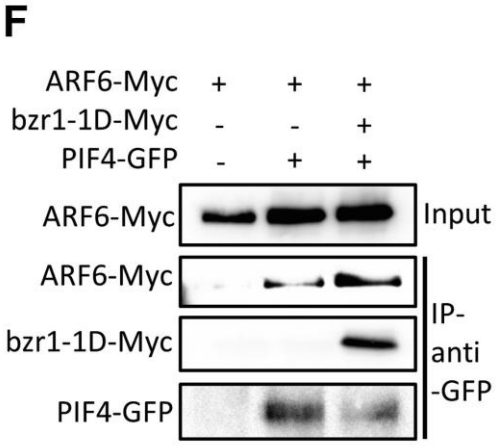




PIF4 ARF6



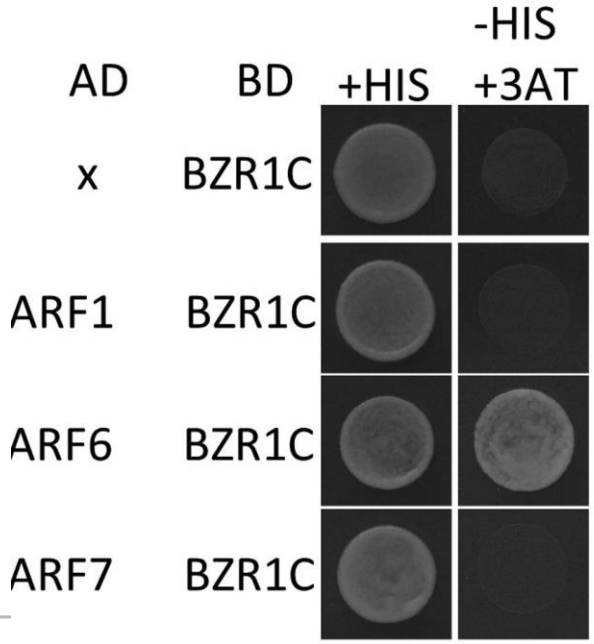


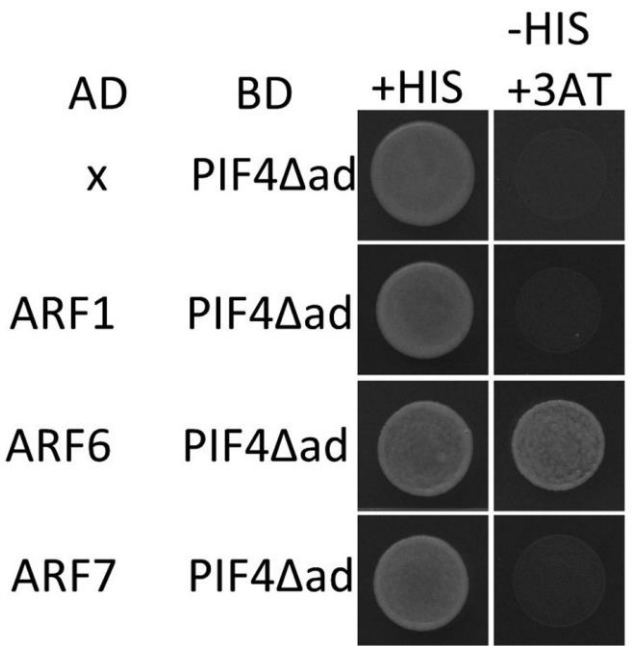


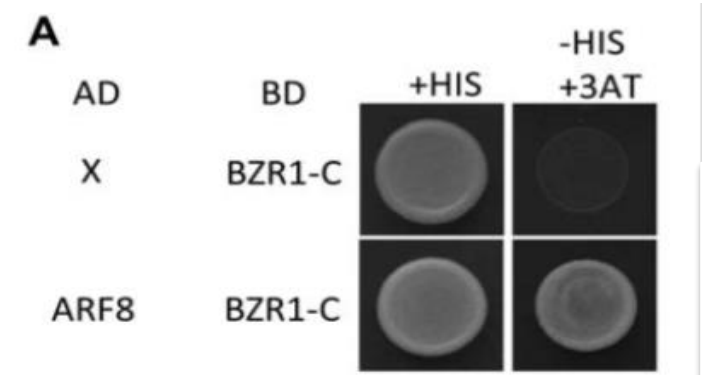
ARF6 interacts with BZR1 in 2-H assay. (**B, C**) Fragments of BZR and PIF4 used. (**D**) ARF6 interacts with PIF4 in 2-H assay.

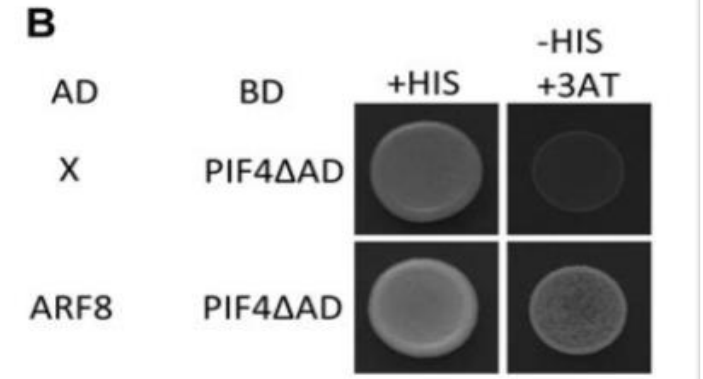
bzr1-1D protein is constitutively active irrespective of BR signaling

(**E**) **ARF6 interacts with BZR1 and PIF4 in vivo**. Transgenic plants expressing the indicated fusion proteins were used for IP using anti-GFP ab, the blots were probed with anti-Myc ab. (**F**) **BZR1 enhances the ARF6–PIF4 interaction**. Protoplasts transfected to express ARF6-Myc alone or together with PIF4-GFP and *bzr1-1*D-Myc, and the extracted proteins were immunoprecipitated by anti-GFP ab. Gel blots probed with anti-Myc or anti-GFP ab.









Both BZR1 and PIF4 specifically interacted with the ARF6 homolog ARF8, but not with ARF1 and ARF7

Function of BZR1-ARF6-PIF4 interaction in regulating gene expression

RNA-Seq analyses using WT & *iaa3/shy2-2* mutant seedlings treated with mock or BL for 4 hr

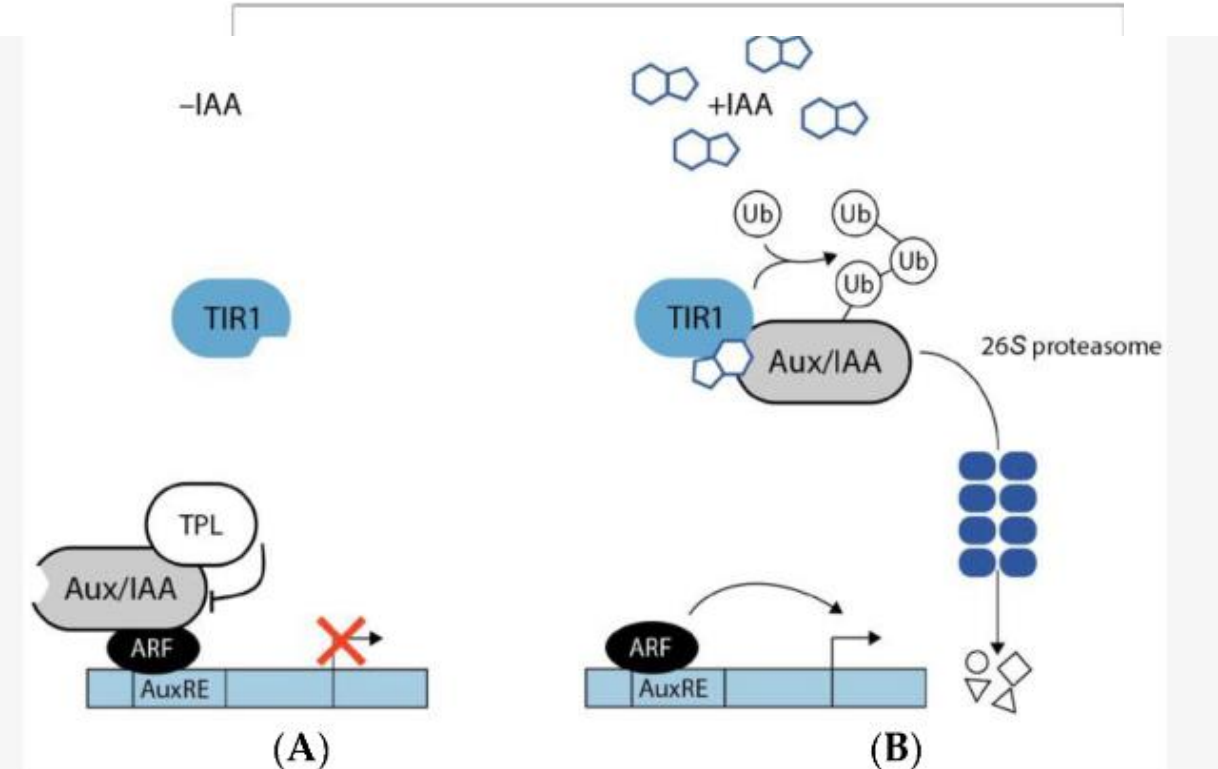
***iaa3/shy2-2* mutant:**

gain of function mutation

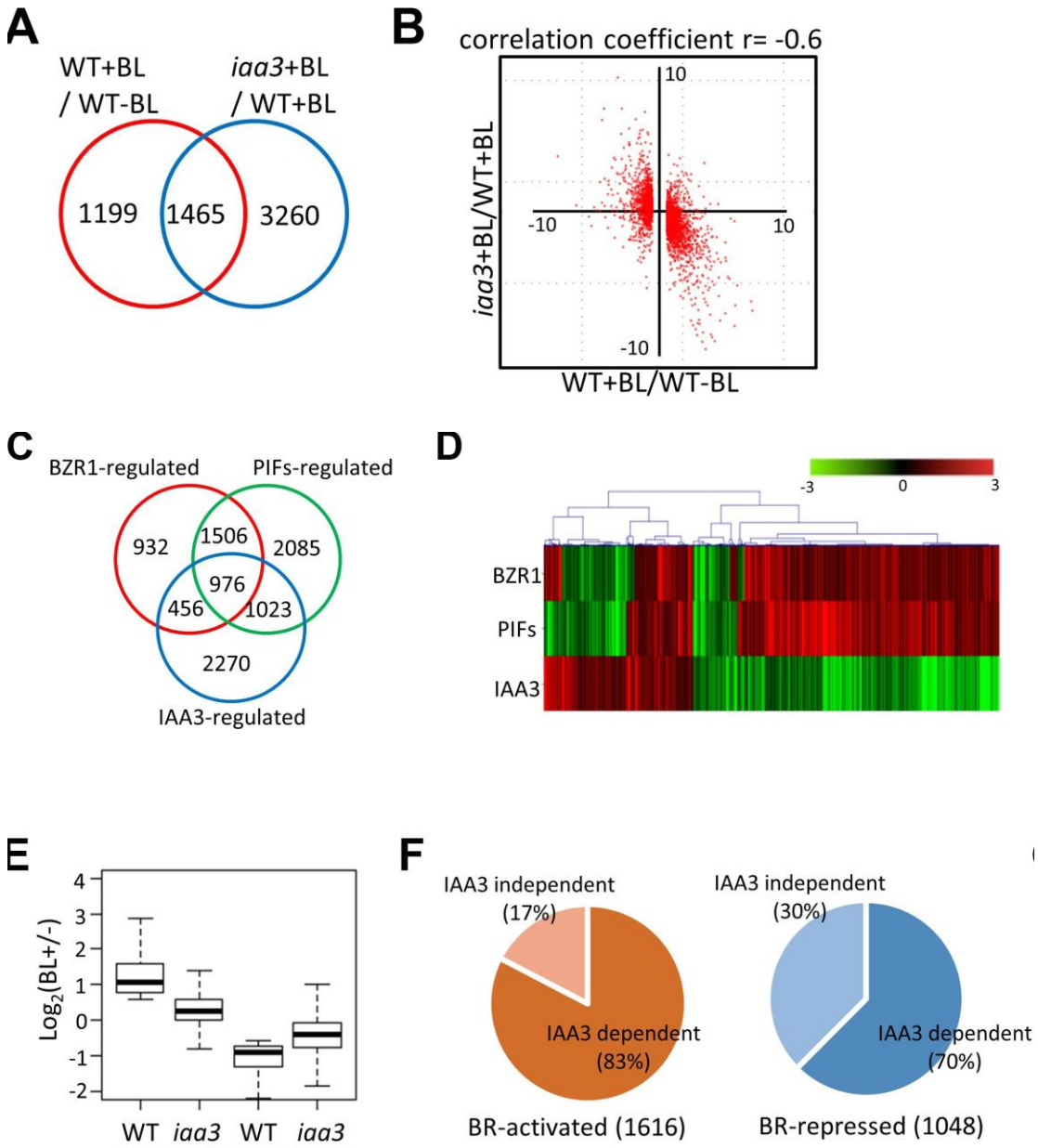
Auxin insensitivity

IAA3 stabilised

IAA3 interacts with and inactivates ARF6 & ARF8

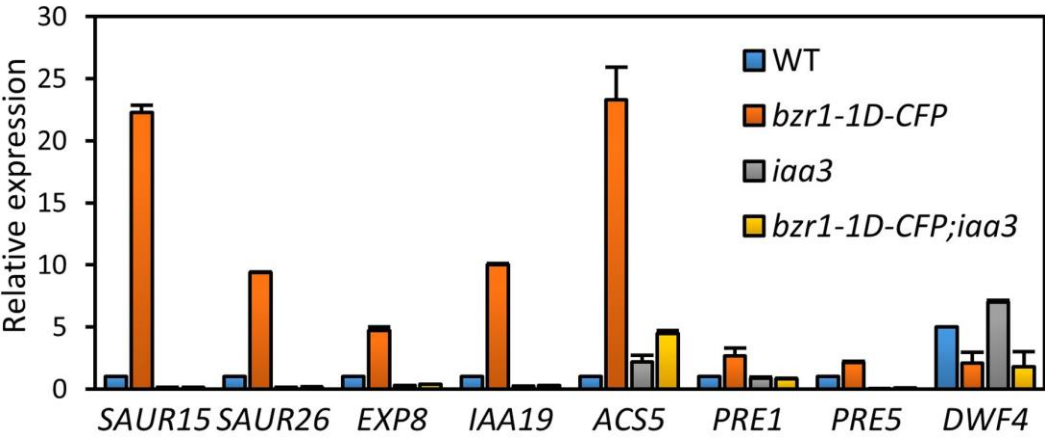


ARF6, BZR1, and PIF4 synergistically induce gene expression

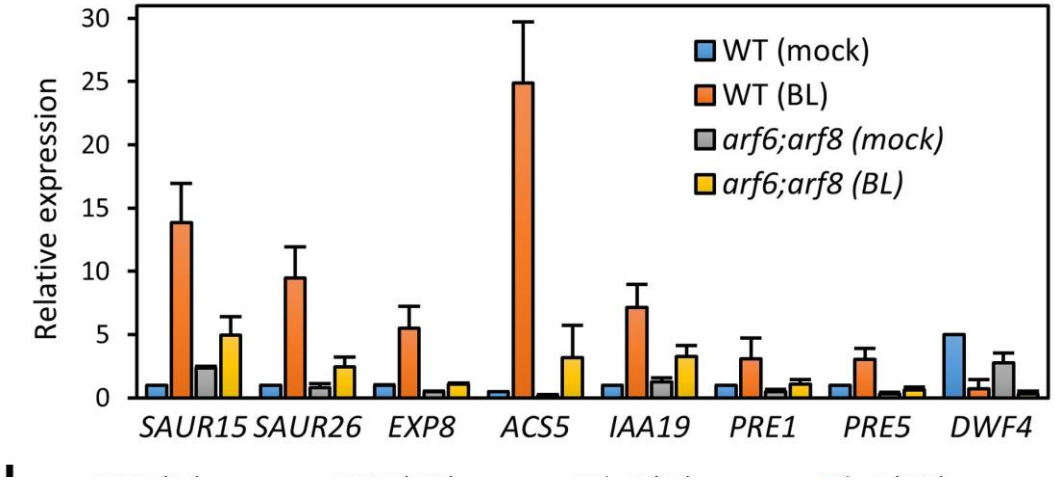


(A) Significant overlap between BR-regulated genes and IAA3-regulated genes. (B) Scatter plot of FC values in the 1465 overlapping set of IAA3- and BR-regulated genes. **Many BR-regulated genes (1465, 55%) were also affected by *iaa3*, and mostly in opposite ways (correlation coef = −0.6)**

(C) Significant overlap among BZR1-, PIFs-, and IAA3-regulated genes. (D) Heat map of the **976 genes co-regulated by BZR1, PIFs, and IAA3.** Scale bar = FC. (E) Box plot of the 1616 BR-activated or the 1048 BR-repressed genes in the WT and *iaa3*. (F) % of IAA3-dependent and IAA3-independent BR-regulated genes. **Genes that were not significantly affected by BR treatment in *iaa3* are defined as IAA3-dependent BR-regulated genes.**



H



qRT-PCR analysis of BZR1-regulated genes.

The effect of *arf6arf8* is similar to but weaker than *iaa3*, consistent with additional ARF factors playing over-lapping role with ARF6 and ARF8 and being suppressed by iaa3

IAA responsive genes in the seedlings (I) grown on medium +/- IAA.

Activation of the BR- and IAA-induced genes by *bzr1-1D* abolished or diminished by *iaa3*, but the repression of BR-repressed DWF4 unaffected by *iaa3*

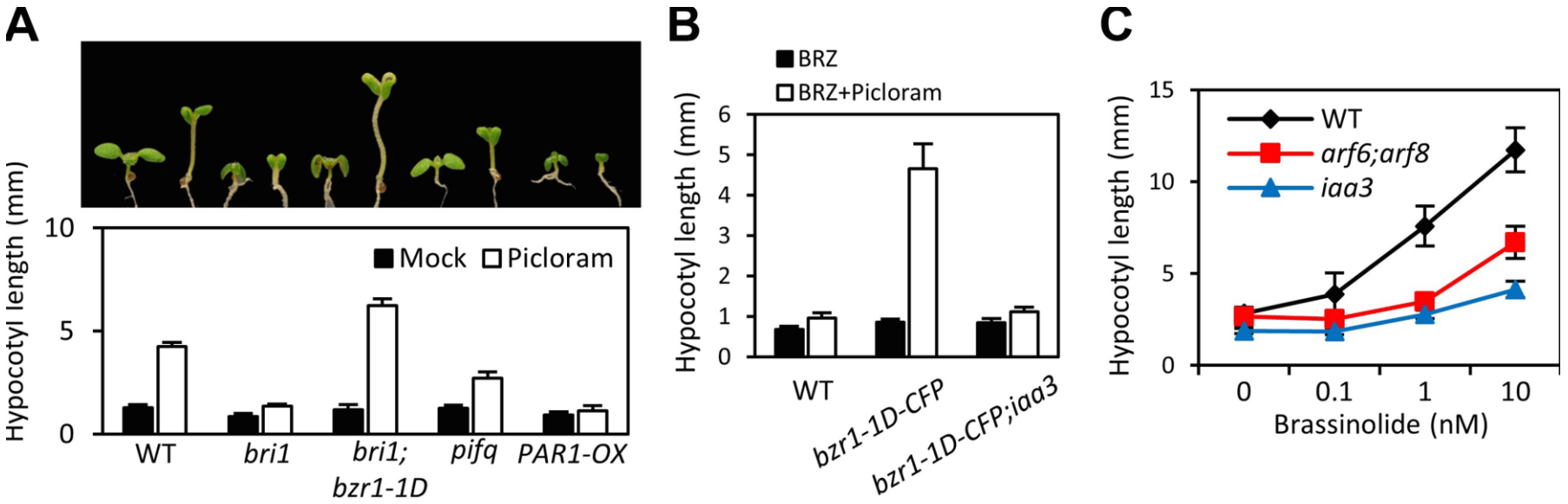
Similar results are obtained from two independent biological repeats. Error bars indicate the SD of three technical repeats

These results indicate that BR activation of genes for hypocotyl elongation is dependent on auxin activation of ARFs, whereas BR feedback repression of BR biosynthesis genes is independent of IAA signaling (*DWF4*).

Taken together, genome- and gene-expression analyses show that BZR1, PIFs, and ARFs interdependently regulate the expression of large numbers of genes, integrating BR, light, and auxin signals into a common set of transcriptome

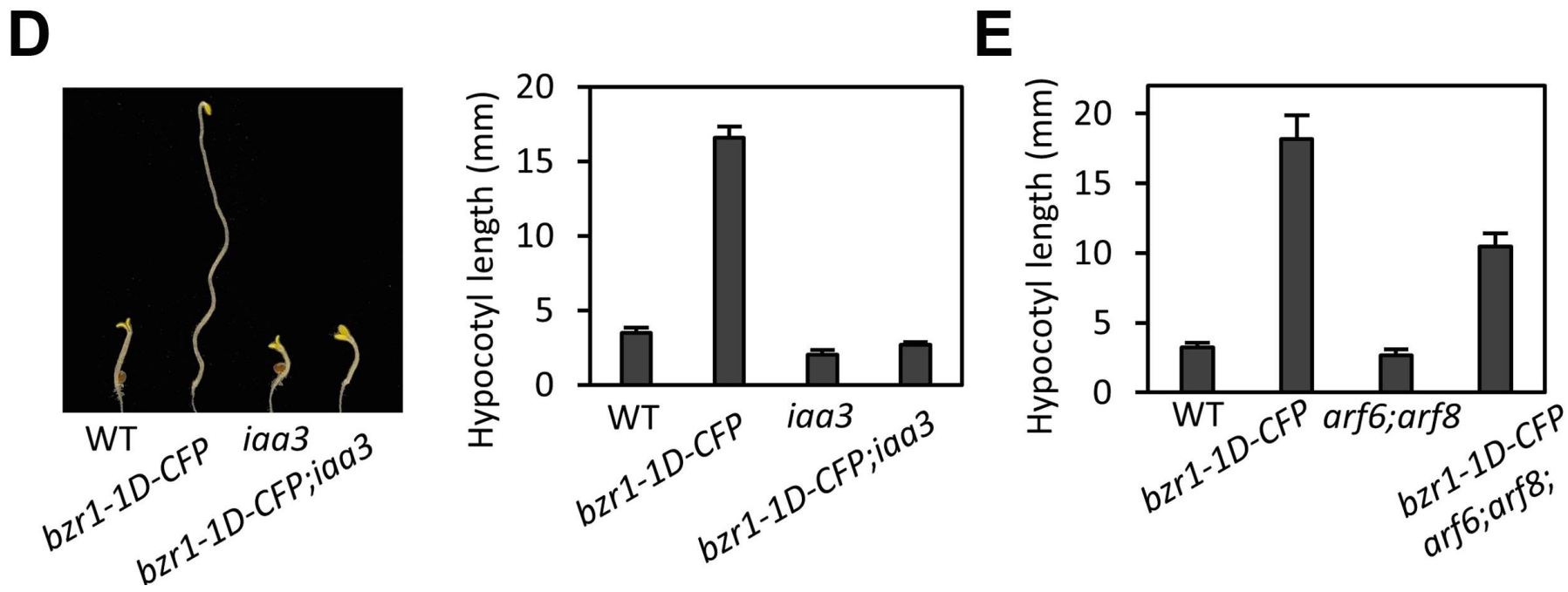
ARF6, BZR1, and PIF4
interdipendently and synergistically promote hypocotyl elongation

To understand the functional importance of the interactions between ARF6, BZR1, and PIF4, analysis of the effects of genetic alteration of each component on the growth responses to changes in the other activities



Hypocotyl elongation of the BR receptor mutant *bri1* shows diminished response to auxin (***4A***), but the auxin-insensitive phenotype of *bri1* was fully rescued by *bzr1-1D* (***4A***), indicating that BZR1 mediates BR enhancement of IAA response. The hypersensitivity of *bzr1-1D* to auxin was abolished by the *iaa3* mutation (***4B***), suggesting that ARF activity is required for BZR1 function. Consistently, both *iaa3* and *arf6;arf8* were less sensitive to BR than WT. To determine whether PIFs are required for IAA response, checked the hypocotyl response to auxin in pifq. Compared with WT, pifq was less sensitive to auxin (4A). **These results indicate that ARF, BZR1 and PIFs are interdependent in promoting hypocotyl elongation, consistent with their cooperative regulation of a core set of genes involved in hypocotyl cell elongation.**

(A) BZR1 and PIFs are required for auxin promotion of hypocotyl elongation. Seedlings were grown on 5 μM artificial auxin picloram or mock. (B) Hypersensitivity of bzr1-1D to auxin is abolished in iaa3/shy2-2. Seedlings were grown on the medium containing BT inhibitor (BRZ) with or without 5 μM artificial auxin picloram. (C) ARF6 and ARF8 are required for BR promotion of hypocotyl elongation. Seedlings were grown on 2 μM BRZ plus various concentration of BL in the dark.



the *bzr1-1D;arf6;arf8* triple mutant and *bzr1-1D;iaa3* double mutant showed shorter hypocotyls on the medium containing BR inhibitor BRZ than the *bzr1-1D* single mutant (Figure 4D,E), indicating that ARF6/8 are required for BZR1 promotion of hypocotyl elongation.

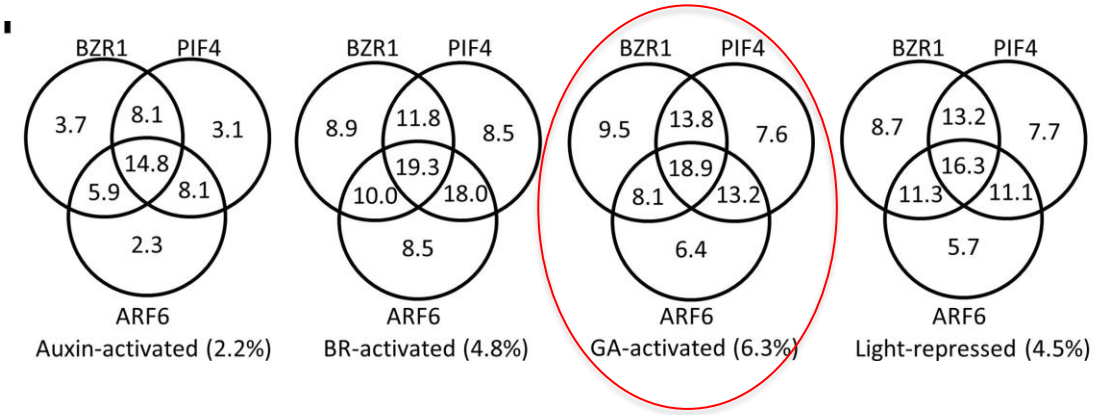
(**D**) The *iaa3/shy2-2* mutation inhibits BZR1 promotion of hypocotyl elongation. Representative seedlings are shown in left panel and quantification of hypocotyl lengths are shown in right graph. Seedlings were grown on the 2 μM BRZ in the dark. (**E**) ARF6 and ARF8 are required for BZR1 promotion of hypocotyl elongation. Seedlings were grown on the 2 μM BRZ medium in the dark. All error bars in (**A**–**E**) indicate SD (n = 10 plants).

Auxin and GA crosstalk through RGA interaction with ARF6

RGA interacts with ARF6 and blocks ARF6 binding to DNA

GA regulates cell elongation through the degradation of DELLA proteins, which inactivate BZR1 and PIFs.

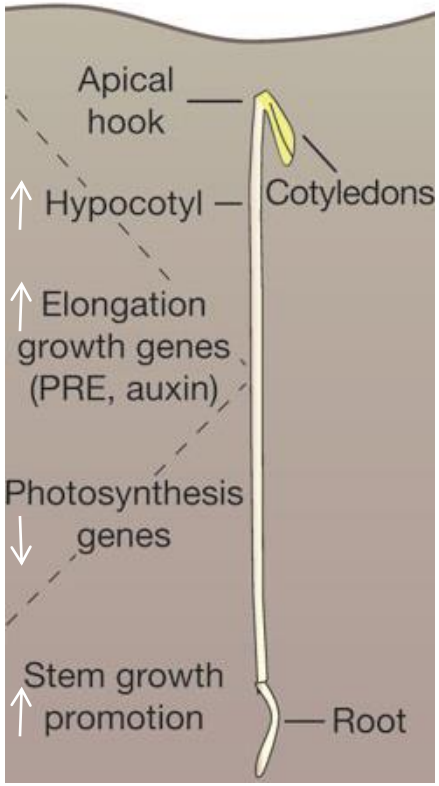
A comparison of ARF6 targets with BZR1 and PIF4 targets revealed that GA-activated genes are enriched in the common targets of ARF6, BZR1, and PIF4, suggesting that ARF6 is also involved in GA response.



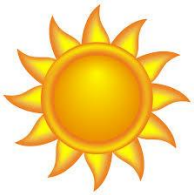
Testing whether ARF6 directly interacts with the DELLA protein RGA

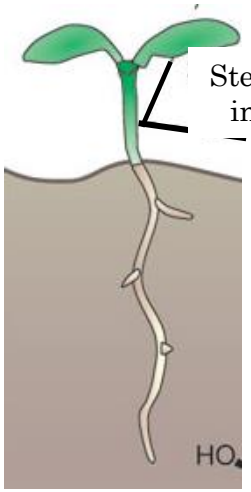
Cell expansion in hypocotyl growth

Skotomorphogenesis



Photomorphogenesis



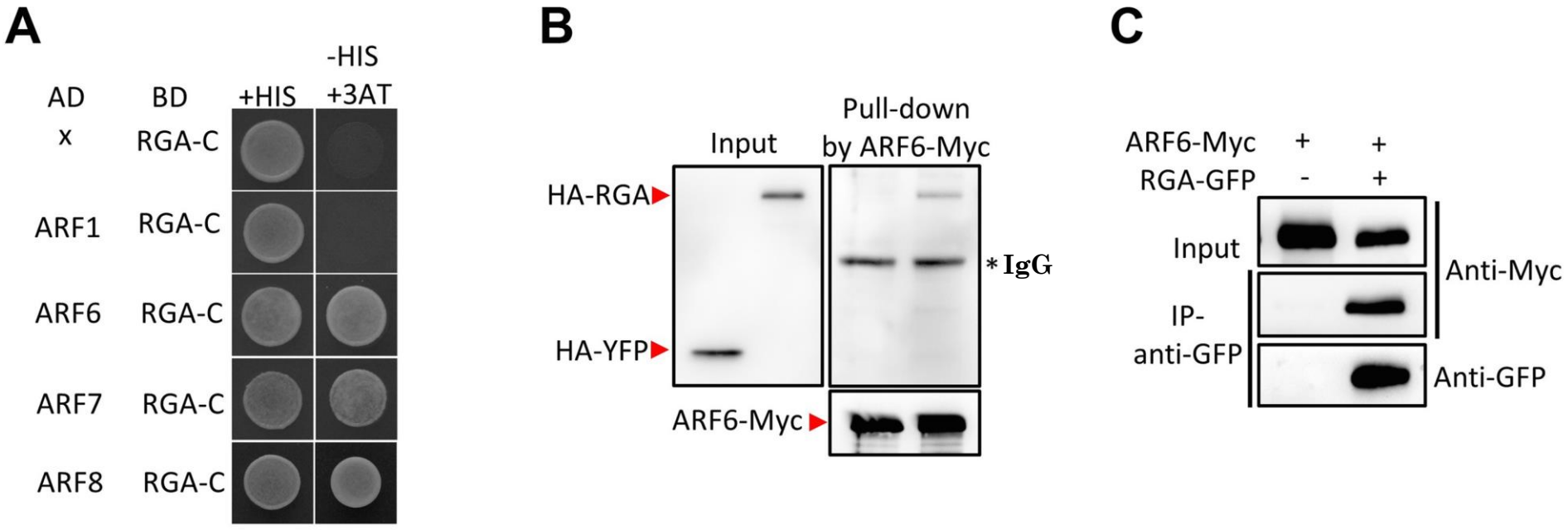


DELLA

GAs

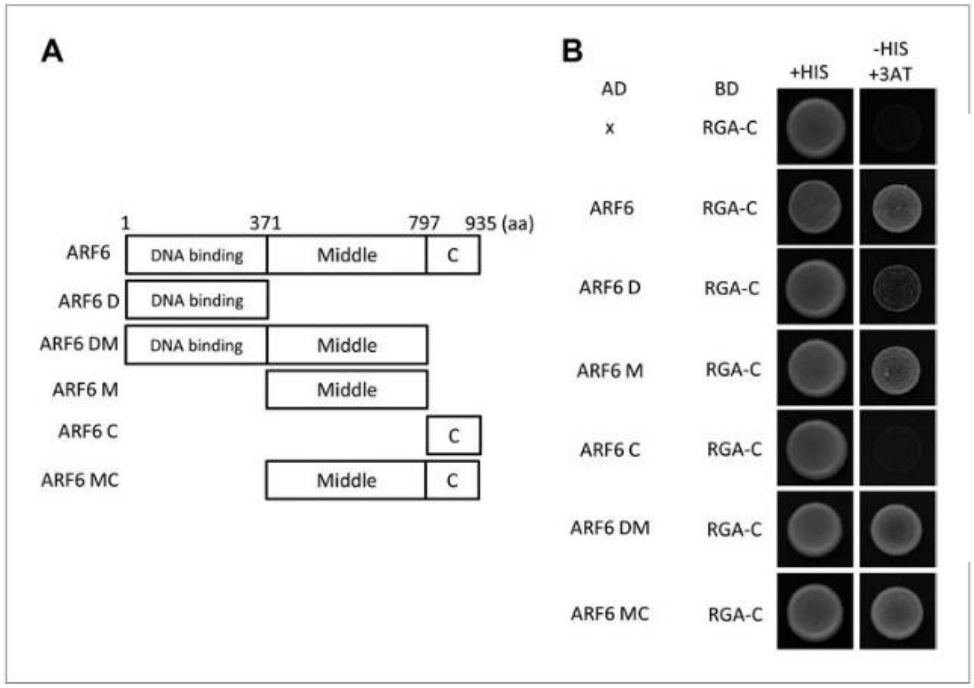
PIF

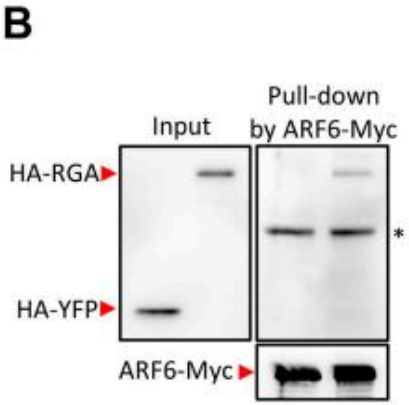
Aux, Br



ARF6 directly interacts with the DELLA protein RGA

(A) Y2-H. RGA interacts with ARF6, ARF7, and ARF8.
(B) RGA interacts with ARF6 *in vitro*. In vitro-translated HA-YFP and HA-RGA proteins were incubated with in vitro-translated ARF6-Myc protein bound to magnetic beads, and the pulled-down proteins analyzed with anti-HA antibody.
(C) RGA interacts with ARF6 *in vivo*. Protein from protoplasts transfected with ARF6-Myc or ARF6-Myc and RGA-GFP immunoprecipitated with anti-GFP ab, analyzed with anti-GFP or anti-Myc ab.





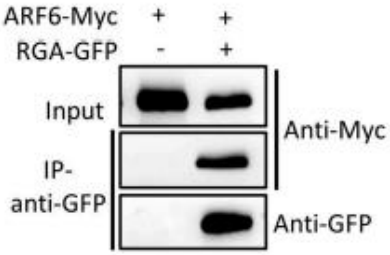


Figure 6—figure supplement 1.

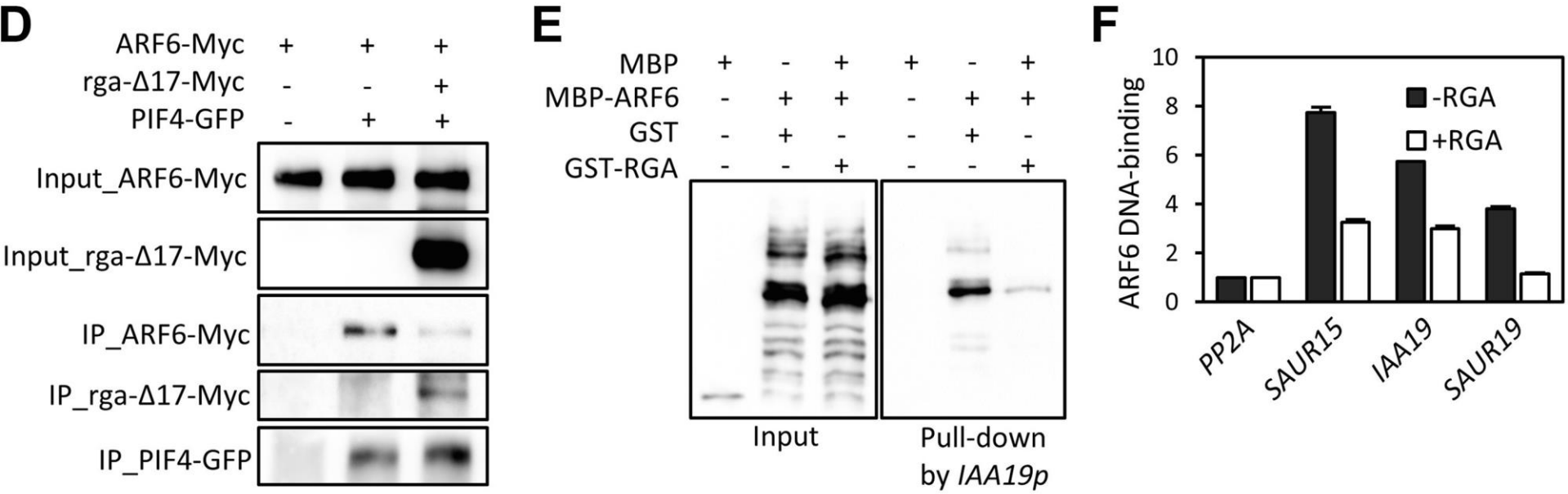
(A)Box diagram of various fragments of ARF6 used in Y2-H. (B) RGA interacts strongly with a middle domain of ARF6 (ARF6 M) and interacts weakly with a DNA binding domain of ARF6 (ARF6 D).

These results demonstrate that RGA directly interacts with ARF6

The middle domain of ARF6 also mediates the ARF6-PIF4/BZR1 interactions

RGA is likely to compete with PIF4/BZR1 for interaction with ARF6

RGA disrupts the ARF6–PIF4 interaction



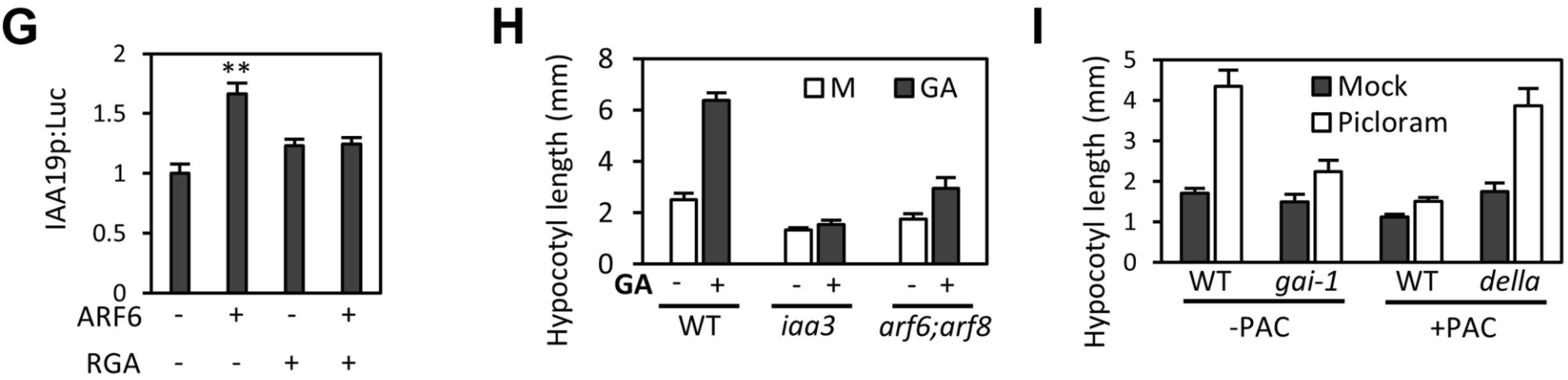
(D) Protoplasts transfected with ARF6-Myc or with PIF4-GFP and *rga*-Δ17-Myc, and proteins immunoprecipitated with anti-GFP ab. Probed with anti-Myc or anti-GFP ab.

(E) RGA inhibits ARF6 binding to the IAA19 promoter in DNA pull-down assay.

(F) RGA inhibits ARF6 DNA-binding ability in vivo. Protoplasts transfected with GFP-Myc or ARF6-Myc with or without RGA-GFP used for ChIP assay.

Error bars indicate the s.d. of two technical repeats. Similar results were obtained in two independent experiments.

RGA inhibits ARF6 transcriptional activity

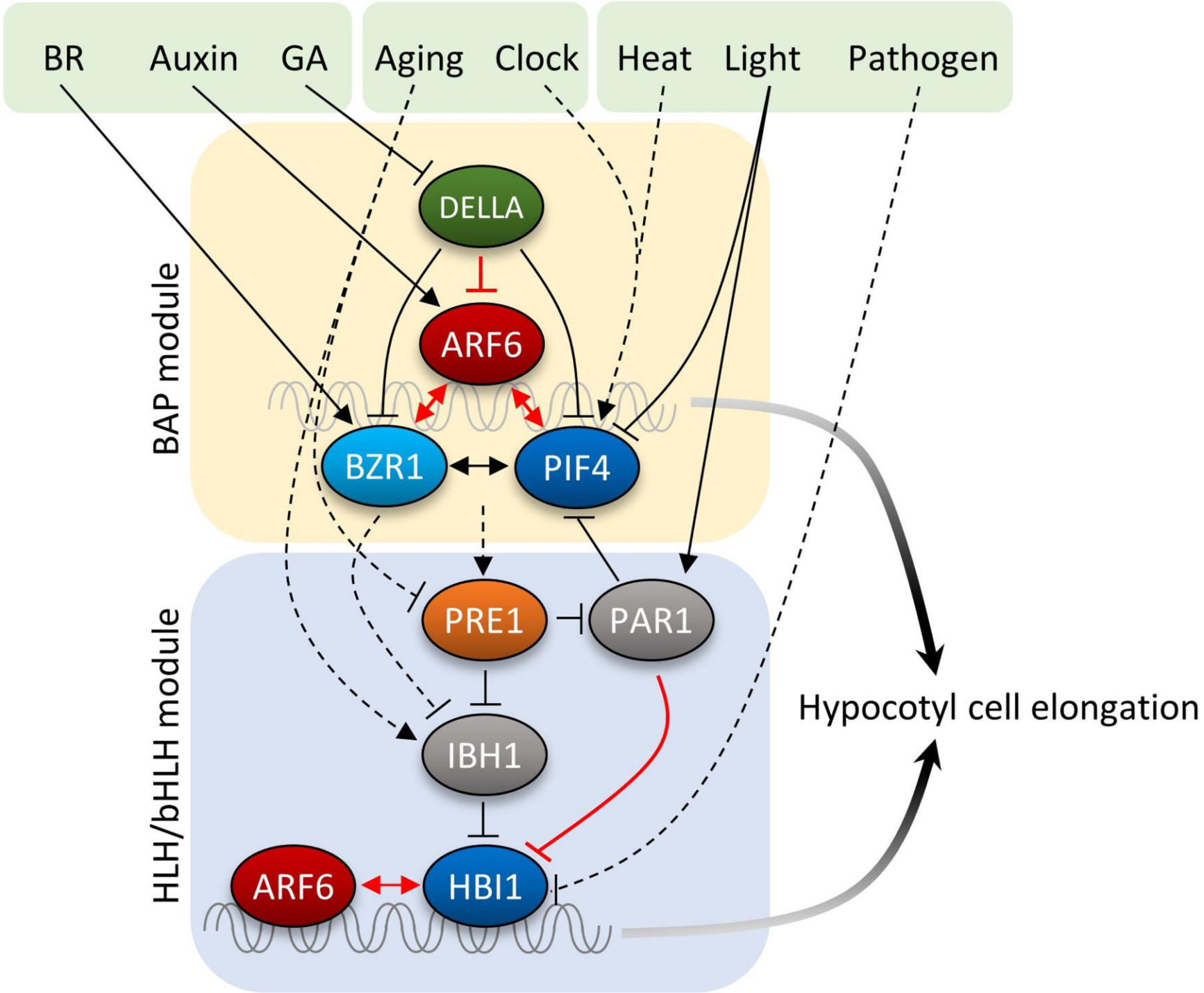


(G) IAA19p::Luc co-transfected with ARF6-GFP, RGA-GFP, or both, into protoplasts. IAA19p::Luc activities normalized by the 35S::renilla luciferase.

Error bars indicate the s.e. of 10 biological repeats (n = 10) and **p<0.01.

(H) Auxin signaling mutants are less sensitive to GA. Seedlings were grown on the 10 µM PAC with or without 1 µM GA in the dark. Error bars indicate SD (n = 10 plants).

(I) DELLA inhibits the auxin promotion of hypocotyl elongation. Seedlings were grown on MS medium for 3 days and then transferred to the medium containing mock or 5 µM picloram, with or without 10 µM paclobutrazol (PAC), and incubated for 4 days. Error bars indicate SD (n = 10 plants).


BR
Auxin
GA
Aging
Clock
Heat
Light
Pathogen
DELLA
ARF6
BZR1
PIF4
BAP module
PRE1
PAR1
IBH1
ARF6
HBI1
HLH/bHLH module
Hypocotyl cell elongation

Auxin Signaling

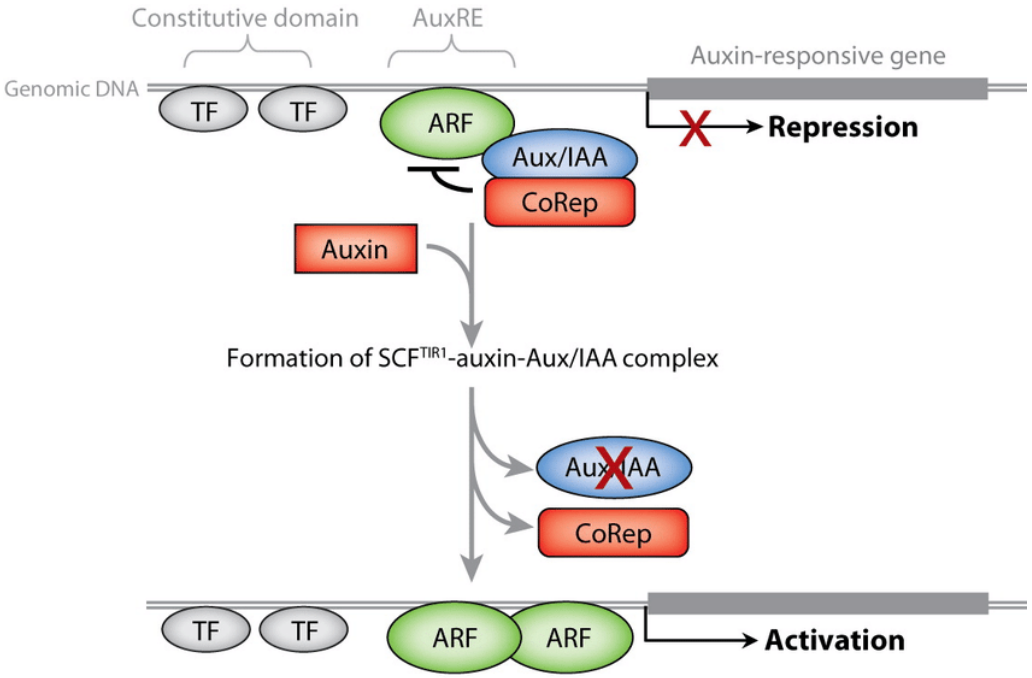
AREs: Auxin-Responsive Elements in the promoters of auxin-response genes.

ARFs: Auxin Response Factors, TFs binding AREs on auxin-responsive genes

Aux/IAA: Repressors of auxin-mediated responses

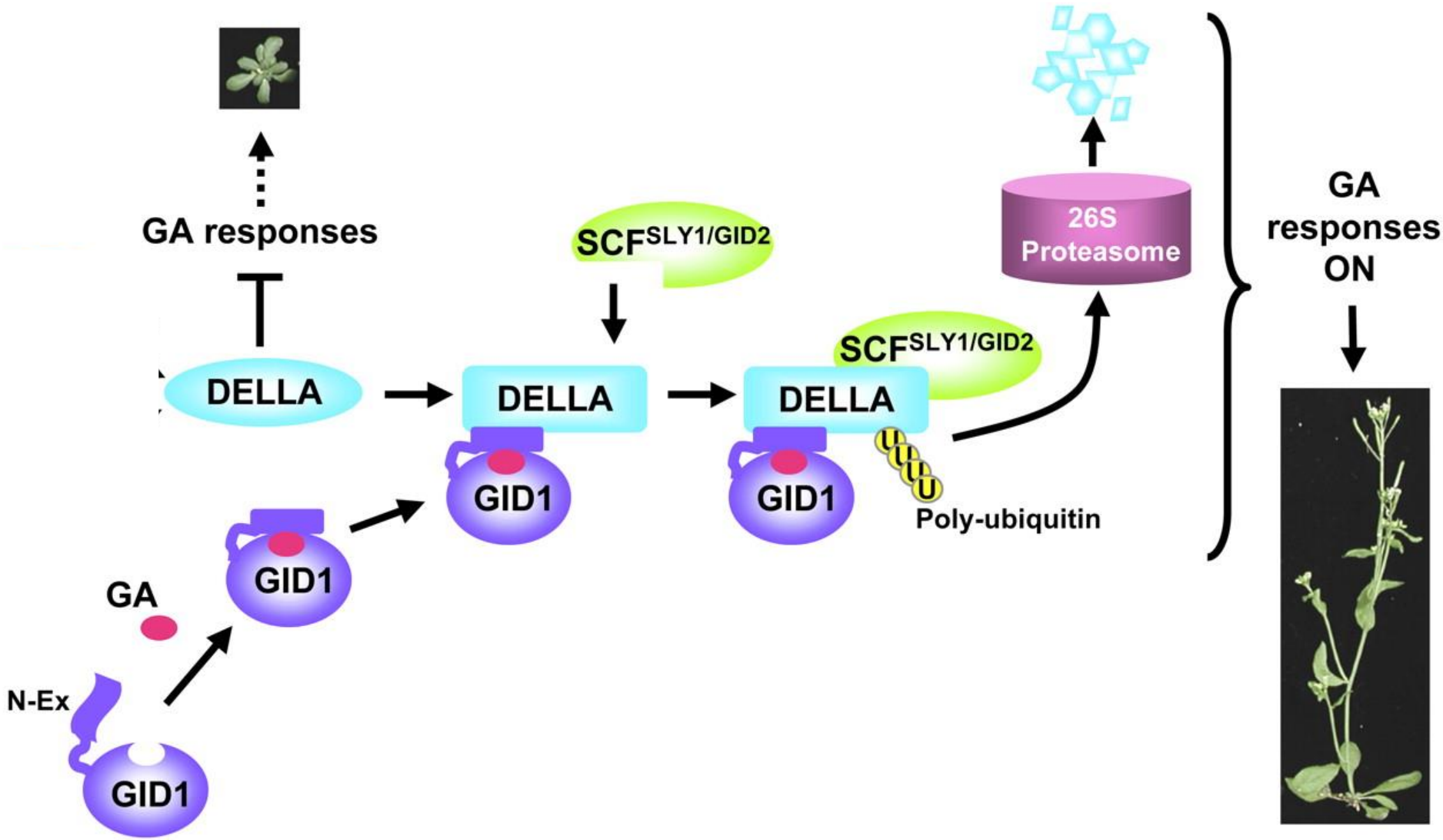
Aux/IAAs are marked for proteolysis by the action of the SCF^{TIR1} E3 Ub ligase. The link TIR1-Aux/IAAs is enhanced by the direct binding of IAA to TIR1. TIR1 is an F-box protein, which mediate auxin response and represent the first confirmed auxin receptor.

BR acts through a receptor kinase pathway to inhibit BIN2/ GSK3-mediated phosphorylation of the brassinazole resistant



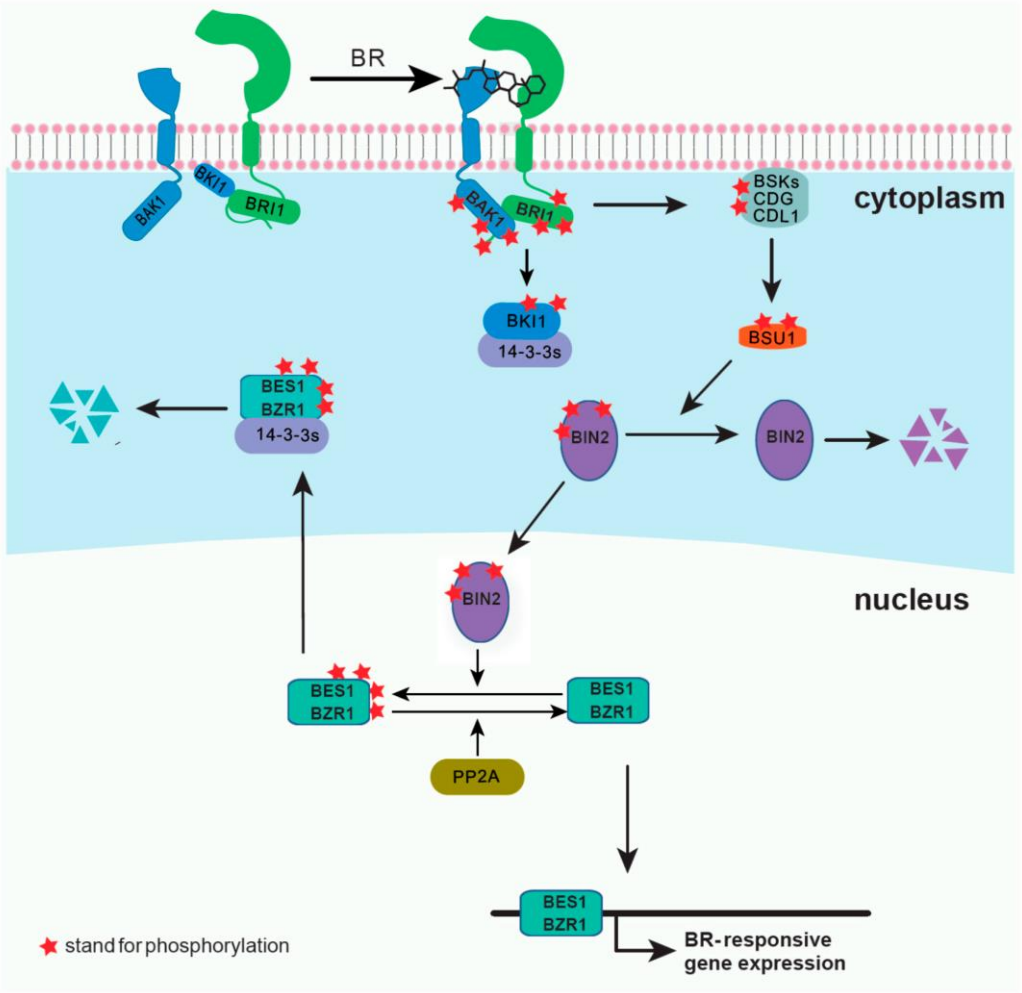
Chapman EJ, Estelle M. 2009.
Annu. Rev. Genet. 43:265–85

GAs Signaling



BRs Signaling

BR acts through a receptor kinase pathway to inhibit BIN2/GSK3-mediated phosphorylation of the brassinazole resistant (BZR) family of transcription factors, leading to their accumulation in the nucleus and regulation of thousands of target genes



Plant growth is dependent on cell expansion or cell division. Plant growth is carefully controlled, but it must be able to respond to changes in the plant's environment.

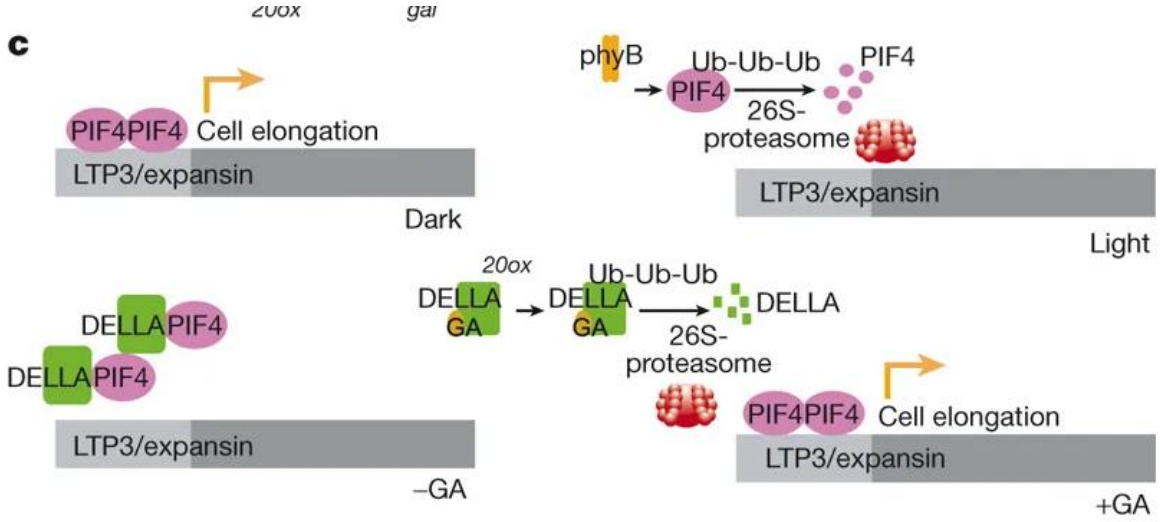
Many different plant hormones and various signals from the environment—such as light and temperature—influence how and when a plant grows. The different signals that affect cell growth typically act via distinct pathways that change which genes are switched on or off inside the cells. However, how these different signals are coordinated is not fully understood.

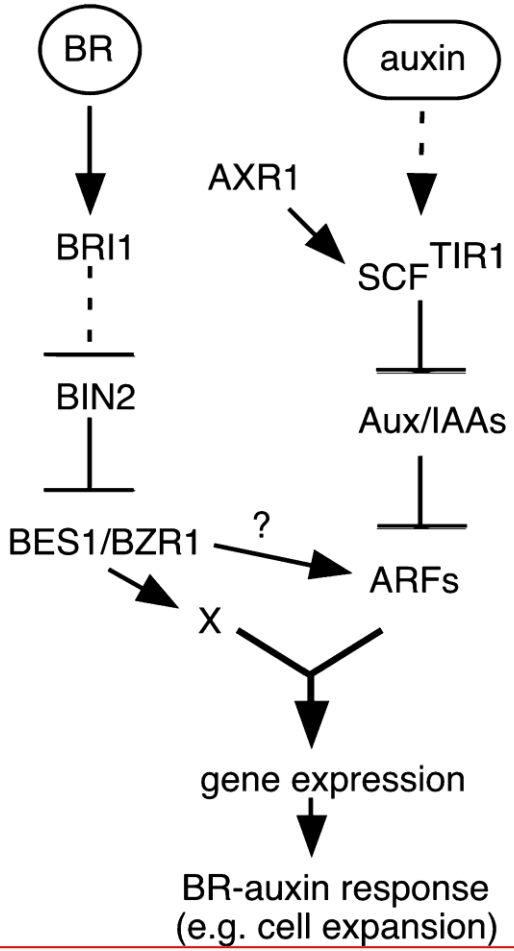
Oh et al. have **looked at the genes that are switched on and off in response to the major signals regulating growth of the embryonic stem** of *Arabidopsis:* the **hypocotyl**

Oh et al. found that the **proteins that change gene expression in response to hormones or the environment bind to each other**. These transcription factors were also revealed to cooperate to regulate the expression of hundreds of genes.

The mechanisms for regulation of auxin levels and distribution through metabolism and polar auxin transport have been studied extensively, however**, little is known about direct interaction between the signal transduction pathways of auxin and other signals at the molecular level.**

Previous data





Model for PIF4 integration of light and GA signals. In the light, phyB induces PIF4 destabilization. DELLAs interact with PIF4 and repress its DNA-binding ability. GAs trigger proteasome degradation of DELLAs thus allowing PIF4-activated gene expression.

IAA & BR signals are likely integrated on promoters of shared target genes. The node(s) of intersection between both pathways must be downstream of BZR1 and Aux/IAA. One likely mechanism is via regulation of transcriptional complexes, such as those containing the ARFs.

(**A**) Box diagram of various fragments of ARF6 used in the yeast-two hybrid assay. (**B**) **ARF6 middle and C-terminal domains are required for the interaction with BZR1**. Yeast clones were grown on the synthetic dropout (+HIS) or synthetic dropout without histidine (−HIS) plus 1 or 5 mM 3AT medium. (**C**) **ARF6 middle and C-terminal domains are required for the interaction with PIF4.**

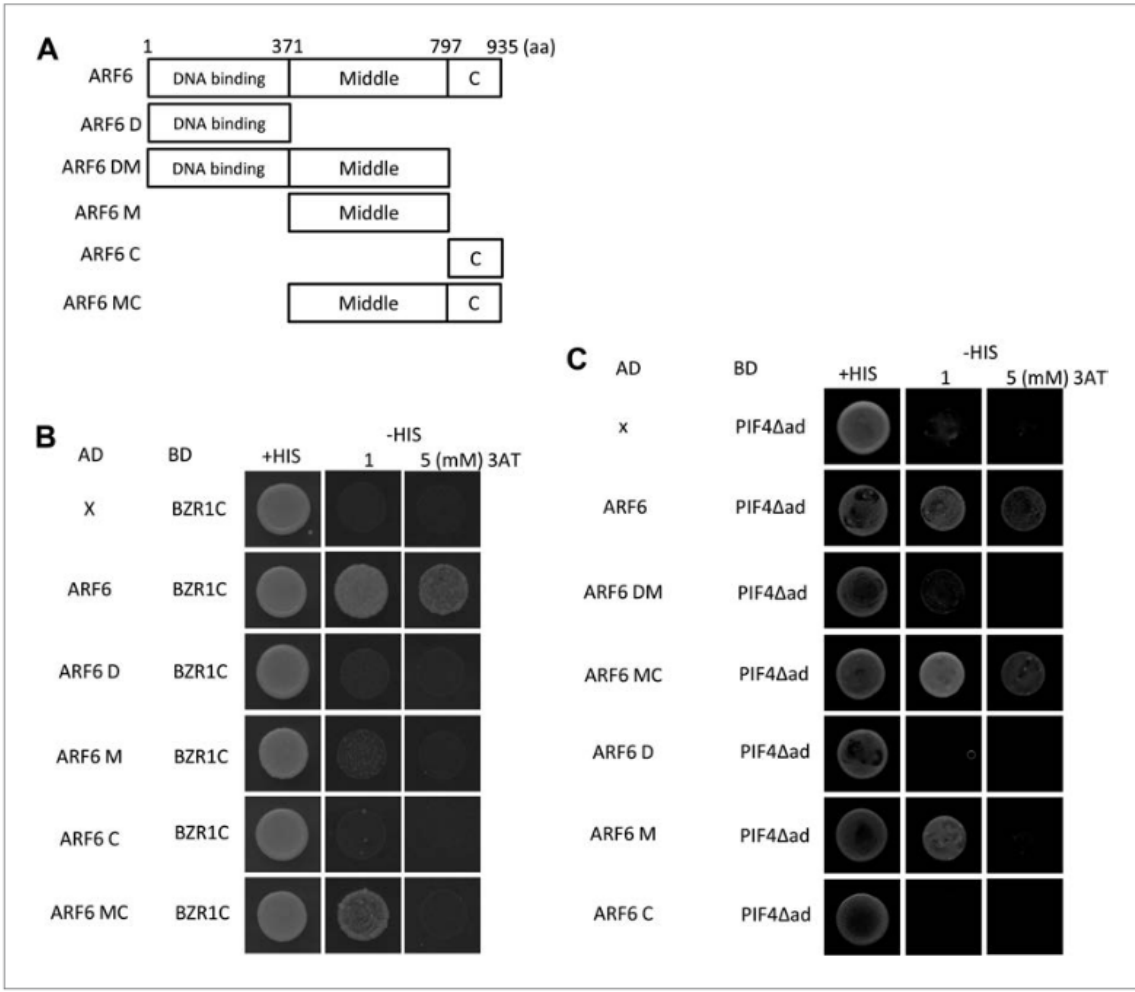


Figure 2—figure supplement 1.