Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in Arabidopsis

Ming-Yi Bai1,4, Jian-Xiu Shang1,2,4, Eunkyoo Oh1, Min Fan1, Yang Bai1, Rodolfo Zentella1, Tai-ping Sun3 and Zhi-Yong Wang1,5

Brassinosteroid and gibberellin promote many similar developmental responses in plants; however, their relationship remains unclear. Here we show that BR and GA act interdependently through a direct interaction between the BR-activated BZR1 and GA-inactivated DELLA transcription regulators. GA promotion of cell elongation required BR signalling, whereas BR or active BZR1 suppressed the GA-deficient dwarf phenotype. DELLA proteins directly interacted with BZR1 and inhibited BZR1–DNA binding both in vitro and in vivo. Genome-wide analysis defined a BZR1-dependent GA-regulated transcriptome, which is enriched with light-regulated genes and genes involved in cell wall synthesis and photosynthesis/chloroplast function. GA promotion of hypocotyl elongation requires both BZR1 and the phytochrome-interacting factors (PIFs), as well as their common downstream targets encoding the PRE-family helix–loop–helix factors. The results demonstrate that GA releases DELLA-mediated inhibition of BZR1, and that the DELLA–BZR1–PIF4 interaction defines a core transcription module that mediates coordinated growth regulation by GA, BR and light signals.

The remarkable plasticity of plant development is believed to rely on networks of interconnected signal transduction pathways that integrate multiple hormonal and environmental signals coordinately regulating common cellular activities and developmental processes1. However, direct crosstalk between hormone pathways has rarely been observed in plants, although many signalling pathways have been characterized in detail2. BR and GA are two major growth-promoting hormones that have similar effects on a wide range of developmental processes1. Mutants deficient in either BR or GA show various degrees of similar phenotypes, including dwarfism, reduced seed germination, de-etiolation in the dark and delayed flowering2–4. Despite the physiological evidence for overlapping actions on development2, the relationship between the BR and GA signalling pathways has remained unclear.

Both BR and GA signalling pathways have been studied extensively2,4. BR is perceived by the receptor kinase Brassinosteroid-Insensitive 1 (BRI1), and downstream signal transduction leads to activation of the Brassinoazole-Resistant 1 (BZR1) family transcription factors, which control BR-responsive gene expression5. When BR levels are low, BZR1 is phosphorylated by the GSK3-like kinase Brassinosteroid-Insensitive-Insensitive 2 (BIN2), and as a result, loses its DNA-binding activity and becomes retained in the cytoplasm6. When BR levels are high, activation of BRI1 leads to sequential phosphorylation and activation of the BR-signalling kinases (BSKs and CDG1) and members of the BRI1 Suppressor 1 (BSU1) family phosphatases7,8, which inactivates the GSK3s through tyrosine dephosphorylation9, allowing BZR1 activation by PP2A-mediated dephosphorylation10. Dephosphorylated BZR1 translocates into the nucleus to regulate over a thousand target genes9,11.

GA promotes plant growth by removing the DELLA proteins. Binding of GA to its receptor GA INSENSITIVE DWARF 1 (GID1) induces GID1–DELLA interaction and association with the E3 ubiquitin ligase SCF22, leading to polyubiquitylation and degradation of DELLA proteins11,12. When GA levels are low, DELLA proteins accumulate and directly inactivate a number of transcription factors13, including the bHLH factor PIF4, which promotes cell elongation when plants are in the dark, shade or high temperature22. Despite their overlapping physiological functions and the extensive knowledge about each signalling pathway, little is known about how BR and GA interact at the molecular level. Previous genome expression analysis of BR- and GA-responsive genes identified largely non-overlapping transcriptional responses23; however, such meta-analysis might have biased the results1. Therefore, it is unclear whether and how the actions of BR and GA are coordinated in regulating common developmental processes1,2. Here we demonstrate an interdependent
To understand the relationship between BR and GA, we examined these results provide evidence for a central growth-regulating transcription module that integrates BR, GA and environmental signals for regulating cell elongation and seedling etiolation.

RESULTS
BR or active BZR1/BZR2 is required for GA promotion of cell elongation

To understand the relationship between BR and GA, we examined how defects in one hormone pathway influence the sensitivity to the other in hypocotyl elongation. We found that GA increased hypocotyl length in wild-type plants but not in the BR-deficient mutant det2-1 or BR-insensitive mutant bri1-5 and bri1-119 (Fig. 1a,b). BR restored GA response to det2-1 (Fig. 1a,b), and increased the GA sensitivity in a dose-dependent manner (Supplementary Fig. S1a). However, BR cannot restore the GA responsiveness of the BR-insensitive mutants bri1-5 and bri1-119 (Fig. 1a,b), indicating that BR signalling is required for GA-induced hypocotyl elongation. The GA-insensitive phenotypes of bri1-119 and bri1-116 were suppressed by the dominant gain-of-function mutant bsr1-1D (Fig. 1b and Supplementary Fig. S1b,c), in which active BZR1 accumulates as a result of increased interaction with PP2A phosphatase. The bsr1-1D mutation also partially suppressed the short-hypocotyl phenotype of the GA-deficient ga1-3 mutant and wild-type plants treated with a GA biosynthesis inhibitor paclobutrazol (PAC; Fig. 1c and Supplementary Fig. S1d–f). BZR2 (also known as BES1) is a homologue of BZR1 and they have 88% protein identity; its gain-of-function mutant bes1-D showed resistance to PAC and partially suppressed the GA insensitivity of BR-deficient plants (Supplementary Fig. S1g–i). These results suggest that BR or BR-activated BZR1/BZR2 is required for GA promotion of hypocotyl elongation.

GA-induced DELLA degradation enhances BR response

In contrast to the GA insensitivity of BR mutants, the GA-deficient mutant ga1-3, GA-insensitive mutant sly1-10 and wild-type plants treated with PAC were responsive to BR and partly rescued by a high concentration of BR (Fig. 1d and Supplementary Fig. S1j). GA and PAC had no effect on hypocotyl elongation of det2-1 and bri1-5 mutants, but enhanced and reduced, respectively, the BR-induced hypocotyl elongation in det2-1 (Fig. 1e and Supplementary Fig. S1k,l), suggesting that GA promotes cell elongation by enhancing the BR-induced response. Such an essential role for BR and an enhancing role for GA are consistent with the stronger dwarf/de-etiolation phenotypes of BR-deficient than GA-deficient mutants.

GA is known to promote growth by degradation of the growth-repressor proteins DELLLAs (ref. 9). The della pentuple mutant lacking all five members of the DELLA family genes showed a markedly enhanced BR response, whereas the GA-insensitive mutant gai-1, which accumulates high levels of GAI (one of the five DELLA proteins in Arabidopsis; refs 9,15), showed a slightly reduced BR response.
Figure 2 RGA interacts with BZR1 and inhibits the DNA-binding activity of BZR1 in vitro and in vivo. (a) Wild-type and dominant mutant forms of BZR1 and RGA interact in yeast. (b) A diagram of the structure of RGA. (c) The LHR1 domain is necessary for both RGA homodimerization and the interaction with BZR1; the SAW domain is also required for interaction with BZR1. (d,e) MBP and MBP fusions with the BZR1 protein were incubated with GST–RGA bound to glutathione–agarose beads and then eluted and analysed by anti-MBP immunoblotting. (f,g) BZR1 and RGA interact in plants. (f) The seedlings of Col and pBZR1:BZR1-CFP grown in medium containing 100 nM PAC under light for seven days were treated with 100 nM BL for 1 h, and then co-immunoprecipitation was performed using anti-YFP antibody and immunoblotted using anti-RGA and anti-YFP antibodies. (g) Immunoprecipitation was performed using anti-YFP antibody on transgenic Arabidopsis plants expressing 35S::BZR1-Myc only or co-expressing 35S::BZR1-Myc and 35S::RGA-YFP, and immunoblotted using anti-Myc or anti-YFP antibodies. (h) RGA inhibits BZR1 DNA binding in vitro. MBP–BZR1 pre-incubated with MBP or MBP–RGA was incubated with biotinylated DNA fragments from the IAA19 and Saur-AC1 promoters immobilized on streptavidin beads. The DNA-bound proteins were immunoblotted using anti-MBP antibody. (i) GA increases the level of BZR1–DNA binding in vivo. ChIP was performed using anti-YFP antibodies followed by qPCR analysis. The level of BZR1 binding was calculated as the ratio between BZR1–CFP and the 35S::YFP control, normalized to that of the control gene CNX5. Error bars, s.d. of three biological repeats. Significant differences between GA and mock treatment are marked by asterisks (P < 0.01). (j,k) Transient reporter gene assays show RGA inhibition of BZR1 transcription activity. Arabidopsis protoplasts were transformed with the dual luciferase reporter construct containing pPRE5::LUC (luciferase) and 35S::REN (renilla luciferase), and constructs overexpressing the indicated effectors. The LUC activity was normalized to REN. Error bars, s.d. of three biological repeats. #: No significant difference between BZR1 and BZR1 + RGA samples (P > 0.05). *: Significant difference between BZR1 and BZR1 + GA samples (P < 0.01). (l) Transient reporter gene assays show RGA inhibition of BZR1 transcription activity. Arabidopsis protoplasts were transfected with the dual luciferase reporter construct containing pPRE5::LUC (luciferase) and 35S::REN (renilla luciferase), and constructs overexpressing the indicated effectors. The LUC activity was normalized to REN. Error bars, s.d. of three biological repeats. #: No significant difference between BZR1 and BZR1 + RGA samples (P > 0.05). *: Significant difference between BZR1 and BZR1 + GA samples (P < 0.01). (m) Uncropped images of blots are shown in Supplementary Fig. S5.

RGA interacts with BZR1 and inhibits BZR1–DNA binding ability

DELLAs are known to inhibit several transcription factors through protein–protein interactions16,17,19,21. The requirement of BR-activated BZR1 for GA/DELLA regulation of hypocotyl elongation raises a possibility that DELLAs may directly repress BZR1. Indeed yeast two-hybrid assays showed direct interaction between BZR1 and the DELLA protein RGA. Further yeast two-hybrid assays showed that both BZR1 and bzr1-1D interacted with RGA (Fig. 2a). RGA contains the amino-terminal DELLA domain, which is required for GA-induced degradation24 and possesses transactivation activity24, and the carboxy-terminal GRAS domain, which is important for its repressor function25–27 (Fig. 2b). Deletion of the DELLA domain had no effect on the BZR1 interaction, indicating that BZR1 interacts with the GRAS domain, but not the DELLA domain, of RGA (Fig. 2a,c).

The GRAS domain can be subdivided into five distinct sequence motifs: leucine heptad repeat I (LHRI), the VHIID motif, leucine...
heptad repeat II (LHRII), the PFYRE motif and the SAW motif\(^5\) (Fig. 2b). Deletion of either the LHRI or SAW motif abolished the interaction with BZR1 (Fig. 2c). The LHRI domain is required for RGA homodimerization (Fig. 2c), and both the LHRI and SAW domains are required for growth-suppression function of DELLAS (refs 24, 26,27). RGA can also heterodimerize with other DELLA proteins (Supplementary Fig. S3a,b). These results thus support a possibility that both dimerization and the SAW domain are required for RGA binding to BZR1 and suppressing plant growth. The GRAS domain is highly conserved in all members of the DELLA family, and both BZR1 and BZR2 interacted with RGA, GAI, RGL1 and RGL3, but not RGL2, in yeast (Supplementary Fig. S2a).

**In vitro** pulldown assays showed that GST–RGA interacted strongly with MBP-tagged full-length BZR1 and the N-terminal part of BZR1 (BZR1N), and weakly with the C-terminal part of BZR1 (BZR1C), but not MBP alone (Fig. 2d), suggesting that the N-terminal DNA-binding domain of BZR1 has a high affinity for RGA. Interestingly, RGA binds only to unphosphorylated BZR1 but not the BIN2-phosphorylated MBP–BZR1 (Fig. 2e). Consistent with the **in vitro** data, co-immunoprecipitation assays and bimolecular fluorescence complementation assays showed that BZR1 interacts with RGA in **vivo** and BR-induced BZR1 dephosphorylation increased the interaction (Fig. 2f,g and Supplementary Fig. S3b). These results demonstrate that RGA binds specifically to the BR-activated form of BZR1.

DELLAs are known to inhibit the DNA-binding of transcription factors\(^6,16,17,19\). We thus investigated whether DELLA blocks BZR1–DNA binding. MBP–BZR1 can be pulled down by biotinylated DNA fragments of the promoters of the BZR1 target genes IAA19 or SAUR-AC1, but not by the non-target CNDX5 promoter\(^9\) (Fig. 2h and Supplementary Fig. S3c), confirming the specific interaction between BZR1 and target promoters. Incubation of MBP–BZR1 with MBP–RGA markedly reduced the level of BZR1 binding to DNA, whereas incubation with MBP alone had no effect (Fig. 2h and Supplementary Fig. S3c,d), indicating that RGA inhibits BZR1–DNA binding in **vivo**.

To determine whether DELLA proteins inhibit BZR1–DNA binding in **vivo**, we performed chromatin immunoprecipitation followed by quantitative real-time PCR (ChIP–qPCR) assays. The ChIP–qPCR results show that BZR1 binding to the promoters of five BZR1 target genes (PRE1, PRE5, IAA19, SAUR-AC1 and DWF4; ref. 13) was enhanced by GA treatment, presumably owing to GA-induced degradation of the DELLA proteins (Fig. 2i). GA treatment and GA-signalling mutants (rga-24/gai-16 and spy-3) did not affect the abundance or phosphorylation status of BZR1 protein (Supplementary Fig. S2c,d), consistent with DELLA directly blocking DNA binding. In protoplast transient assays, the expression level of luciferase driven by the BR-responsive PRE5 promoter was increased by BZR1 and bbr1-1D, but this increase was abolished by co-expression of RGA, GAI and rga-A17, but not by RGL2 and RGAASAW (Fig. 2j,k). These results indicate that RGA specifically interacts with BZR1 to inhibit its abilities to bind DNA and regulate transcription.

**GA and BR regulate overlapping genomic targets involved in photomorphogenesis and cell elongation**

If DELLA inhibits BZR1 activity in **vivo** and GA releases DELLA inhibition, GA should affect the expression of BZR1 target genes in similar manners as BR. Indeed, the previously identified microarray data sets of genes affected in the BR-insensitive mutant brr1-116 and GA-deficient mutant ga-3 overlap significantly\(^{13,28}\) (Fig. 3a). Of the 1,194 genes differentially expressed in ga-1 at 3 when compared with the wild type, 419 genes (35%) were also affected in the brr1-116 mutant, of which 296 were also affected by light (Fig. 3a and Supplementary Table S1). Among these co-regulated genes, 387 genes (92.3%) were affected in the same way by brr1-116 and ga-1, with a correlation coefficient \(R = 0.76\) (Fig. 3b). The effects of brr1 and ga-1 are also similar to that of light on these genes, consistent with BR and GA repressing light responses (Fig. 3b). For 276 (71%) of these genes, the effects of brr1-116 were reversed by the brr1-1D mutation (compare brr1-1D/brr1-116 and brr1-116) and the effects of ga-1 were reversed by loss of DELLA proteins (compare dellla/ga-1 and ga-1) (Fig. 3c). These results show that GA and BR exert similar effects on a large number of common genes through DELLA and BZR1 activities.

To further define the BR/BZR1-dependent genomic targets of GA signalling, we analysed the effects of BR-deficiency and brr1-1D mutation on GA-induced gene expression changes using RNA-sequencing (RNA-Seq). RNA-Seq analysis identified 3,570 genes affected \(>1.5\)-fold by GA treatment in wild-type plants grown without propiconazole (PPZ, a specific inhibitor of BR biosynthesis\(^29\)), 1,629 genes affected by GA in wild-type plants grown on PPZ medium (BR-deficient plants), and 4,306 genes affected in brr1-1D when compared with wild-type plants when grown on the PAC+PPZ medium (deficient for both GA and BR; Fig. 3d). Of the 3,570 genes regulated by GA in BR-sufficient plants, only 1,187 genes (33%) were affected by GA in the BR-deficient plants, suggesting that GA-responsive expression of most genes requires BR. About half (549 genes) of the BR-independent GA-regulated genes were not affected by brr1-1D in any significant amount or pattern (Class A, Fig. 3d,e), whereas the other half (638 genes) were affected by brr1-1D, mostly in similar manners as GA treatment (Class B, Fig. 3d,f). It is likely that Class A genes are regulated by other DELLA-interacting transcription regulators, such as JAZ1 and EIN3 (refs 18,19), independently of BR/BZR1, and the Class B genes are regulated through both BR/BZR1-dependent and -independent mechanisms. Of the BR-dependent GA-responsive genes, 1,027 were affected by brr1-1D (Class C, Fig. 3d), mostly in similar manners as GA treatment (Fig. 3g), and their GA responses were quantitatively reduced in the BR-deficient plants (Fig. 3g); these genes apparently are regulated by GA through a BR/BZR1-dependent mechanism.

Gene Ontology analyses showed that cell-wall-related genes are markedly enriched in the GA-induced Class B (10%) and Class C genes (10%) when compared with the random control (3%; Fig. 3h), suggesting that GA promotes cell elongation preferentially through the BR1-dependent mechanism but also through BR-independent mechanisms. In contrast, photosynthesis/chloroplast genes are markedly enriched in the GA-repressed Class B (44%) and C (62%) but not in Class A or D (13–17%) when compared with the control (15%; Fig. 3h), indicating that GA represses photosynthetic genes mainly through a BZR1-dependent mechanism. These genomic data thus provide direct evidence for the important role of BZR1 in GA regulation of genome expression, particularly the transcriptomes that promote cell elongation and repress photosynthetic development.
GA and BR co-regulate a large number of genes through DELLAs and BZR1. (a) Venn diagram showing the overlap between sets of genes differentially expressed in dark-grown bri1-116 versus wild type (bri1-116 D versus WT D), ga1-3 versus wild type (ga1-3 D versus WT D), and light-grown versus dark-grown wild type (WT L versus D). (b) Scatter plot of log2 fold change values for 419 genes differentially expressed in bri1-116 D versus WT D and ga1-3 D versus WT D. Red and blue colours indicate light-activated and light-repressed genes, respectively; black colour indicates the genes are not regulated by light. (c) Hierarchical clusters analysis of the genes differentially expressed in bri1-1D/bri1-116 versus bri1-116 (bri1-1D/bri1-116), bri1-116 versus WT (bri1-116), ga1-3 versus Ler (ga1-3), and del1a/ga1-3 versus ga1-3 (del1a/ga1-3). The gradient bar represents the log2 of the ratio. Genes are listed in Supplementary Table S1. (d–h) RNA-Seq analyses of genes affected by GA treatments or by bri1-1D in BR-deficient plants (grown on 2μM PPZ medium). (d) Venn diagram showing overlaps between sets of genes affected by GA treatment in wild-type (Col) plants grown on medium containing PAC and PPZ (Col_PPZ GA+/–), and genes affected by bri1-1D in the presence of PAC and PPZ (bzr1-1D_PPZ versus Col_PPZ). (e–g) Hierarchical cluster analysis of the expression data of the genes in each gene Class (A–D) shown in (d). (h) Gene Ontology analysis of cellular functions represented by GA-upregulated and -downregulated genes in each gene Class (A–D) shown in (d). All genes detected in RNA-Seq samples were used as control (random).

GA-promotion of hypocotyl elongation requires BZR1, PIF4 and their common downstream targets paclobutrazol resistance factors (PREs)

Both DELLAs and BZR1 also interact with PIF4 (refs 16,17,30), and PIF4 and BZR1 together bind to a large number of common promoters in the genome18. To determine whether the common targets of both BZR1 and PIF4 were preferentially regulated by GA, we grouped genes on the basis of the ChIP data of BZR1 and PIF4 and the microarray expression profiling data of pifq and bzr1-1D/bri1 mutants15,30,31, and we calculated the percentage of GA-regulated genes (on the basis of expression data for the ga1-3 mutant5) of each group. The genes regulated by PIFs and/or BZR1 included higher percentages of GA-regulated genes than genes unregulated by BZR1 and PIFs, and the genes that are common targets co-regulated by BZR1 and PIF4...
Figure 4 BZR1 and PIF4 are required for the GA promotion of hypocotyl elongation. (a) Venn diagram showing the percentage of GA-regulated genes among the gene sets that BZR1 and PIF4 bind to and/or regulate. PIF4 and BZR1 targets were identified by PIF4 ChIP-Seq and BZR1 ChIP-chip, respectively; PIFs- and BZR1-regulated genes were differentially expressed in pif4 versus the wild type and in bzip1-1D/bir1-116 versus bir1-116 grown in the dark. GA-regulated genes were differentially expressed in gai-1 versus the wild type in the dark. The numbers show the percentages of each gene set that are GA-regulated genes. (b,c) PIFs are required for BZR1-mediated GA promotion of hypocotyl elongation. Seedlings were grown in the dark for five days on medium containing 0.5 μM PAC and 10 μM PPZ, with or without 1 μM GA3. Error bars, s.d. (n = 10 plants). The asterisks mark significant differences between GA and mock treatments (P < 0.01). (d,e) Both BZR1 and PIFs are required for GA promotion of hypocotyl elongation in light. Seedlings were grown under red light for five days on medium containing 0.1 μM PAC and 2 μM PPZ, with 0 (M) or 1 μM GA3 (GA). Error bars, s.d. (n = 10 plants). The asterisks mark significant differences between GA and mock treatments (P < 0.01).

DISCUSSION

How plant growth is controlled by the wide range of environmental signals and endogenous cues is a fundamental question in plant biology. This study demonstrates a major mechanism of crosstalk between two hormonal signals and illustrates a central network that integrates signalling and growth regulation in plants. Whereas previous studies suggested that BR and GA act independently on highly overlapping developmental responses, this study reveals a much closer relationship between these two hormones. Strong evidence from genetic, biochemical and genomic analyses support a model in which GA and BR crosstalk through direct interaction between the key components of each pathway, DELLAs and BZR1 (Fig. 5g). BR signalling through the BRI1 receptor kinase pathway leads to dephosphorylation and subsequent nuclear accumulation of BZR1 (ref. 8). However, the activity of BZR1 is attenuated by DELLAs when GA levels are low. GA-induced

including the highest percentage of GA-regulated genes (Fig. 4a and Supplementary Fig. S4), indicating that the genome targets shared by BZR1 and PIF4 tend to be regulated by GA.

We then examined whether GA-induced cell elongation also requires both BZR1 and PIFs. The dominant bzip1-1D mutation rescued the GA response in the wild-type background but not in the pif4 background when seedlings were grown on the BR biosynthesis inhibitor PPZ in the dark (Fig. 4b,c), indicating that PIFs are required for the BZR1-mediated GA response. When grown on medium containing PPZ under light (which causes degradation of PIFs), only bzip1-1D/PIF4-Ox plants showed a robust GA response, whereas PIF4-Ox, bzip1-1D and wild-type plants were all insensitive to GA (Fig. 4d,e). These results indicate that GA promotion of hypocotyl elongation requires both BZR1 and PIF4, and is thus probably mediated by the BZR1–PIF4 heterodimer.

Previous studies have shown that GA, BR and auxin induce the expression of the PRE family helix–loop–helix factors32–34, which promote cell elongation by antagonizing several inhibitory HLH factors35–37. Several PRE family members, including PRE1, PRE5 and PRE6/KIDARI, are direct targets of both BZR1 and PIF4 (ref. 30). Consistent with GA acting through the DELLA–BZR1–PIF4 module, the expression of PRE1, PRE2, PRE5 and PRE6 was induced by GA treatment in the wild type, but their GA induction was decreased in the brip1-119 mutant and recovered in the brip1-119 bzip1-1D double mutant, indicating that GA induction of these genes requires active BZR1 (Fig. 5a). Similarly, the BR-induction of PRE1, PRE5 and PRE6 was reduced in the dominant gain-of-function gai-1 mutant when compared with the wild type (Fig. 5b), indicating that BR induction was negated by accumulation of GAI. Two genes, EXP1 and EXP8, encoding expansins, cell wall proteins that loosen the cell wall38, are affected similarly to PREs by brip1-119 and gai-1 (Fig. 5a,b), suggesting that these expansins might mediate the downstream response in cell elongation. The role of PREs in the GA response was confirmed using the pre-amiR transgenic line, in which four PRE members are suppressed by artificial microRNA39, and a transgenic line that overexpresses IBH1, the antagonistic partner of PRE1 (ref. 33). Both pre-amiR and IBH1-overexpression lines had GA-insensitive hypocotyls when compared with the wild type (Fig. 5c). Consistent with the GA-insensitivity of pre-amiR, PRE1 overexpression reduced plants’ sensitivity to the biosynthesis inhibitors of GA and BR (Fig. 5d,e). CHIP–reChIP with transgenic Arabidopsis expressing both BZR1–myc and PIF4–YFP showed that BZR1 and PIF4 co-occupy the promoter of PRE1, PRE6, EXP1 and EXP8 (Fig. 5f). These results demonstrate that PREs are essential positive regulators for GA-promoted hypocotyl elongation, acting downstream of the DELLA–BZR1–PIF4 module.
 degradation of DELLAs frees BZR1 for DNA binding and transcriptional regulation. BR is required for GA promotion of cell elongation, because when BZR1 is inactivated by phosphorylation and unable to interact with DELLAs in the absence of BR, GA-induced DELLA degradation cannot significantly increase the level of nuclear BZR1 activity. As such, BR seems to be essential for GA-induced hypocotyl growth, whereas GA quantitatively enhances BR-potentiated growth.

The DELLA–BZR1 interaction is a critical link in the photomorphogenic regulation system. Previous studies have shown similar DELLA interaction with members of the PIF family of phytochrome-interacting bHLH factors, and our accompanying study showed that BZR1 also interacts with PIFs to co-regulate large numbers of common target genes, and they also each regulate unique target genes. Thus, DELLAs can potentially inhibit BZR1 and PIF4 individually and modulate their actions of unique targets, and/or inhibit the BZR1–PIF4 heterodimer to modulate their common targets. The higher percentage of GA-responsive genes in the BZR1–PIF4 co-regulated than uniquely regulated genes suggests that DELLAs preferentially target the BZR1–PIF4 heterodimer. Interestingly, the downstream genes controlled by the interdependent actions of BR/BZR1, light/PIF4 and GA/DELLAs are enriched with cell-wall- and photosynthesis/chloroplast-related genes, which are affected in ways that are consistent with the actions of these pathways on cell elongation and photomorphogenesis. These observations demonstrated that the interdependent interactions among DELLAs, BZR1 and PIFs regulate a core transcription module that mainly controls cell elongation and chloroplast development.

Considering that the levels of PIFs are controlled by light, the circadian clock and temperature, and the level of DELLAs is affected not only by GA but also by auxin, abscisic acid, ethylene, jasmonate and abiotic stresses, the interdependent interactions of BZR1 with PIFs and DELLAs would allow BR to modulate the growth responses to all of these other signals, consistent with an ancient and central role of steroid hormones in regulating growth.

The DELLA–BZR1 interaction is a critical link in the photomorphogenic system. Previous studies have shown similar DELLA interaction with members of the PIF family of phytochrome-interacting bHLH factors, and our accompanying study showed that BZR1 also interacts with PIFs to co-regulate large numbers of common target genes, and they also each regulate unique target genes. Thus, DELLAs can potentially inhibit BZR1 and PIF4 individually and modulate their actions of unique targets, and/or inhibit the BZR1–PIF4 heterodimer to modulate their common targets. The higher percentage of GA-responsive genes in the BZR1–PIF4 co-regulated than uniquely regulated genes suggests that DELLAs preferentially target the BZR1–PIF4 heterodimer. Interestingly, the downstream genes controlled by the interdependent actions of BR/BZR1, light/PIF4 and GA/DELLAs are enriched with cell-wall- and photosynthesis/chloroplast-related genes, which are affected in ways that are consistent with the actions of these pathways on cell elongation and photomorphogenesis. These observations demonstrated that the interdependent interactions among DELLAs, BZR1 and PIFs regulate a core transcription module that mainly controls cell elongation and chloroplast development.

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AUTHOR CONTRIBUTIONS
M-Y.B. and Z-Y.W. together designed the experiments. M-Y.B. performed statistical analysis of plant growth, ChiP-qPCR, DNA-binding assay, RNA-Seq, RT-qPCR analysis together with E.O. and Y.B. analysed microarray and RNA-Seq data. J-X.S. performed the yeast two-hybrid, co-immunoprecipitation, protein–protein pulldown, transient expression assays and generated bsr1-1/del5-3. E.O. analysed bsr1-1/DPI4-Ox and bsr1-1/Dpi/+. M.F. analysed the GA responses of bhi1-1. R.Z. performed RGA protein degradation studies. T-p.S. provided sly1-10 seeds and helped with critical discussion on the work. M-Y.B. performed all other experiments. M-Y.B. and Z-Y.W. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS
Methods and any associated references are available in the online version of the paper.

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METHODS

Plant materials and growth conditions. The det2-1, bsr1-1D, br1L-116, bsr1-1D/br1L-116, bsr1-1D/pif4, bsr1-1D/pif4-YYF, 35S::Pif4-YYF, bsr1-1D, 35S::pre-anrR and 35S::IBH1-myc are in the Arabidopsis thaliana Columbia ecotype background; bsr1-1D, bsr1-119 and bsr1-1D/br1L-119 are in the En2 ecotype. br1L-5 is in the Ws ecotype, and gai-3, del1, gai-1 and rpo-24/gai-1/rpo-24 are in the Landsberg erecta ecotype4,5,27,34,47. The gai-3 mutant was backcrossed with the wild type (Col) for four generations and then crossed with bsr1-1D to generate the gai-3/bsr1-1D double mutant. Plants were grown on medium containing 0.5x Murashige and Skoog salt, 1% sucrose and 0.68% phytohormone agar. For hypocotyl length measurement, seedlings were grown at 22°C on vertical agar plates, supplemented, or not, with hormones in the dark for six days or under constant light for seven days. Seedlings were photographed and their hypocotyl length was measured using ImageJ software (http://rsbweb.nih.gov/ij/).

In vitro pulldown assays. RGA fused to GST was purified using glutathione beads (GE Healthcare). BZR1, BZR1N and BZR1C fused to maltose-binding protein (MBP) were purified using amylose resin (NEB). Glutathione beads containing 1µg of GST–RGA were incubated with 1µg MBP, MBP–BZR1, MBP–BZR1N, MBP–BZR1C or phosphorylated MBP–BZR1 (phosphorylated by tag-free BIN2) in pulldown buffer (15mM HEPES–NaOH at pH 7.9, 50mM potassium glutamate, 5mM magnesium chloride, 5% glycerol, 0.1% NP40, 1µg µl−1 BSA and 1mM dithiothreitol). The mixture was rotated at 4°C for 1h, and the beads were washed five times with wash buffer (15mM HEPES–NaOH at pH 7.9, 30mM potassium glutamate, 5mM magnesium chloride and 0.1% NP40). The proteins were eluted from the beads by boiling in 50µl 2x SDS sample buffer and loaded onto a SDS–PAGE gel. Gel blots were analysed using anti-MBP antibody (NEB, 1:5,000 dilution).

Co-immunoprecipitation assays. Plant materials were ground in liquid nitrogen and then extracted with NEB buffer (20mM HEPES–KOH, at pH 7.5, 40mM KCl, 1mM EDTA, 0.3% Triton X-100 and 1x protease inhibitors, Roche) at a ratio of 3ml buffer per gram of tissue. After centrifugation at 20,000g for 10 min, the supernatant was incubated with anti-YFP antibody bound to Protein A Sepharose beads (GE Healthcare) for 1 h, and the beads were washed four times with wash buffer and eluted by boiling with 2x SDS sample buffer for 5 min. Samples were analysed by SDS–PAGE and immunoblotted with anti-YFP (home-made, 1:3,000 dilution), anti-Myc antibodies (Cell Signaling, 1:5,000 dilution) or affinity-purified anti-RGA antibodies (Cell Signaling, 1:5,000 dilution)60.

DNA-binding assay. The recombinant proteins MBP–BZR1 and MBP–RGA were expressed and affinity-purified from Escherichia coli using amylose resin (NEB). The A1a19, Saur-AC1 and CNX5 promoter fragments were amplified by PCR using the biotin-labelled primers (Supplementary Table S3). The DNA and proteins were incubated, and then DNA-binding proteins were pulled down using streptavidin–agarose beads and analysed by immunoblotting, as described previously90.

ChiP. The ChiP experiments were performed following the procedure described previously with some modifications90. Arabidopsis plants transformed with pbZR1::BZR1-CFP and 35S::YFP were grown in liquid 0.5x Murashige and Skoog medium containing 1% sucrose with or without hormones under constant light for seven days. The seedlings were crosslinked for 10 min in 1% formaldehyde and quenched by 0.25M glicerine. Chromatin extracts were sonicated with a Branson sonifier 450 (VWR) to achieve an average fragment size of 250 base pairs. Immuno precipitation was performed using anti-YFP antibody bound to Protein A agarose/salmon sperm DNA beads. The immunoprecipitated protein and DNA were eluted with 1% SDS and 0.1M NaHCO3, and the crosslink was reversed by incubation at 65°C overnight in the presence of 250mM NaCl. DNA was extracted by a PCR purification kit (Fermentas) and analysed by qPCR using the oligonucleotide primers listed in Supplementary Table S3.

Protoplast transient reporter gene assays. The protoplast transient assays were performed following the procedure described previously93,94. Approximately 3 x 104 protoplasts in 0.2 ml of MMG solution (0.4 M mannitol, 15mM MgCl2 and 4mM MES, at pH 5.7) were transformed with 20µg of a mixture of plasmid DNA using the PEG method. The protoplasts were resuspended in 250 µl of W1 (0.5 M mannitol, 20mM KCl and 4mM MES at pH 5.7) and were incubated in 24-well plates coated with 5% BSA in a growth chamber for 16 h in light, and the LUC and REN activities were measured using the Dual–Luciferase reporter kit (Promega)93.

Quantitative reverse transcription PCR analysis. Total RNA was extracted from seven-day-old Arabidopsis seedlings grown with or without hormone treatment using the Spectrum Plant Total RNA kit (Sigma). The first-strand complementary DNA was synthesized using RevertAid Reverse Transcriptase (Fermentas) and used as RT–PCR templates. qPCR analyses were performed on a plate-based LightCycler 480 (Roche) using a SYBR Green reagent (Bio-Rad) with gene-specific primers (Supplementary Table S3).

RNA-Seq. Wild-type Arabidopsis and bsr1-1D were grown in media containing 1µM PAC and 0 or 2µM PP3 for 4.5 days in the dark, and then treated with 10µM GA3 or mock solution for 12 h. Total RNA was extracted with Spectrum Plant Total RNA Kit (Sigma) and the mRNA sequencing libraries were constructed with barcodes using the TrueSeq RNA Sample Preparation Kit (Illumina). Six barcoded libraries were pooled together and sequenced by Illumina HiSeq2000. The sequence reads were mapped to the Arabidopsis genome using TopHat software95, and differential gene expression was analysed using DEseq software96. Differentially expressed genes were defined by a 1.3-fold expression difference with a P value <0.05. The accession number for the RNA-Seq data in the Gene Expression Omnibus system for transient gene expression analysis.

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Figure S1 Active BZR1/BES1 are required for GA promotion of cell elongation. (a) BR signaling is required for GA promotion of cell elongation. The seedlings of wild type (Col and WS), det2-1, and bri1-5 were grown on ½ MS medium with or without 1 μM GA3 and different concentrations of BL for 7 days under constant light. GA sensitivity was calculated as the average ratio of hypocotyl length of GA treated plants to GA untreated plants (ratio of 1 indicate no response to GA). (b, c) Active BZR1 is required for GA promotion of cell elongation. The seedlings of wild type (Col), bri1-116, bri1-1D and bri1-1D/bri1-116 were grown in the dark for 6 days on ½ MS medium containing 1 μM PAC with or without 1 μM GA3. Error bars mean s.d. (n=22 plants). *: Significant difference between with or without GA treatment. (p < 0.01). (h, i) bes1-D suppresses the GA-insensitivity phenotype of BR-deficient plants. The seedlings of En2 and bri1-1D were grown in the dark on ½ MS medium containing 1 μM PAC, 2 μM PPZ and with or without 1 μM GA3 for 6 days. Error bars mean s.d. (n=23 plants). *: Significant difference between with or without PAC treatment. (p < 0.01). (b, c) Active BZR1/BES1 are required for GA promotion of cell elongation. The seedlings of wild type (Col and En2), bri1-1D, and bes1-D were grown in the dark on ½ MS medium with or without different concentrations of PAC for 6 days. Error bars mean s.d. (n=23 plants) *. Significant difference between with or without PAC treatment. (p < 0.01). (h, i) bes1-D suppresses the GA-insensitivity phenotype of BR-deficient plants. The seedlings of En2 and bri1-1D were grown in the dark on ½ MS medium containing 1 μM PAC, 2 μM PPZ and with or without 1 μM GA3 for 6 days. Error bars mean s.d. (n=23 plants) *. Significant difference between with or without PAC treatment. (p < 0.01). (j, l) GA increases, but PAC decreases the BR sensitivity of det2-1. The seedlings of det2-1 and bri1-5 were grown on ½ MS medium (M), or medium containing 1 μM PAC or 1 μM GA3, and different concentrations of BL under constant light for 7 days. Average hypocotyl lengths (k) and relative hypocotyl lengths to BL untreated seedlings (l) were measured from at least 30 seedlings. Error bars mean s.d. (n=45 plants).
**Figure S2** BR and GA regulate BZR1 and RGA independently. (a) Eight-day old seedlings of wild type (Col), *det2-1* and *bri1-116* were treated with mock solution (-GA) or 10 μM GA$_3$ (+GA) for 1 hr. The immuno-blot was analyzed by anti-RGA antibody. (b) Eight-day old Col seedlings were treated with 10 μM GA$_3$, 100 nM BL, or both, for 1 hr, and analyzed by anti-RGA and anti-BZR1 immunoblot. Ponceau S staining of the blots showed equal loading of all samples. (c) The *pBZR1:BZR1-CFP* transgenic plants were grown on medium containing 1 μM PAC for 7 days, treated with 100 μM GA$_3$ or mock solution for 3 hr, then with 100 nM BL or mock solution for 1 hr. The blot was analyzed by anti-GFP antibody. (d) Wild type (Ler and Col), *rga-24/gai-6*, and *spy-3* plants were grown on ½ MS medium for 7 days, then treated with 100 nM BL or mock solution for 1 hr. The immuno-blot was probed with anti-BZR1 antibody, and the phosphorylated BZR1 (pBZR1) and unphosphorylated BZR1 (BZR1) were detected as two distinct bands. A non-specific band is shown as loading control.
Figure S3  RGA interacts with BZR1 and inhibits BZR1 DNA binding.  
(a) Yeast two hybrid assays of the interaction between BZR1, BES1 and 
DELLAs in yeast.  (b) Bimolecular fluorescence complementation (BiFC) 
assays of the in vivo protein interaction.  Leaf epidermal cells of N.  
benthamiana were cotransformed with the indicated pairs of constructs.  
(c, d) MBP-RGA inhibits BZR1 DNA binding in vitro.  (c) MBP-BZR1 
pre-incubated with MBP or MBP-RGA was incubated with biotinylated 
DNA fragments from the CNX5 and IAA19 promoters immobilized on 
streptavidin beads.  (d) MBP-BZR1 pre-incubated with biotinylated DNA 
fragments from IAA19 promoters immobilized on streptavidin beads was 
icubated with MBP or MBP-RGA.  The DNA-bound proteins were immuno-
blotted using anti-MBP antibody.
Figure S4 Venn diagrams show GA regulated genes among the gene sets that BZR1 and PIF4 bind to and/or regulate. The PIF4 and BZR1 targets were identified by PIF4 ChIP-Seq and BZR1 ChIP-chip, respectively; PIF- and BZR1-regulated genes were differentially expressed in *pifq* versus WT and in *bzz1-1D/bri1-116* versus *bri1-116* grown in the dark. GA-regulated genes were differentially expressed in *ga1-3* versus WT in the dark. Numbers show the percentages of each gene set that are GA-regulated genes.
Figure S5 Full scan data of immunoblots. Red asterisks indicate the bands shown in the figures.