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Accessibility
RD26 mediates crosstalk between drought and brassinosteroid signalling pathways

Brassinosteroids (BRs) regulate plant growth and stress responses via the BES1/BZR1 family of transcription factors, which regulate the expression of thousands of downstream genes. BRs are involved in the response to drought, however the mechanistic understanding of interactions between BR signalling and drought response remains to be established. Here we show that transcription factor RD26 mediates crosstalk between drought and BR signalling. When overexpressed, BES1 target gene RD26 can inhibit BR-regulated growth. Global gene expression studies suggest that RD26 can act antagonistically to BR to regulate the expression of a subset of BES1-regulated genes, thereby inhibiting BR function. We show that RD26 can interact with BES1 protein and antagonize BES1 transcriptional activity on BR-regulated genes and that BR signalling can also repress expression of RD26 and its homologues and inhibit drought responses. Our results thus reveal a mechanism coordinating plant growth and drought tolerance.
Branzosteroïdes (BRs) sont un groupe de stéroïdes végétaux régulant la croissance végétale, le développement et les réponses biotiques et abiotiques1–2. Au cours des deux dernières décennies, les principaux composants du chemin de signalisation BR ont été identifiés et caractérisés13–22. Le signal BR conduit à la formation de BR5/BRASSINAZOLE RESISTANT1 (BRASSR1) et de ses homologues, qui sont des facteurs de transcription cible des montrants dans le noyau pour contrôler l’expression de gènes cibles pour les réponses BR13–28.

Plusieurs études ont indiqué que le traitement avec des BR exogènes pouvait augmenter la tolérance des plantes au stress hydrique1,29,30. Cependant, des BR déficientes ont été rapportées pour avoir une tolérance d’augmentation d’une tolérance de diminution au stress hydrique31–33, suggérant un effet inhibiteur de BRs sur la tolérance hydrique. Ces études récentes impliquent des relations complexes entre le contrôle BR-contrôlé de croissance et de réponses à la tolérance au stress hydrique. Ces récentes études impliquent un contrôle important des relations entre le contrôle BR-contrôlé de croissance et de réponses à la tolérance au stress hydrique.

RD26 appartient au NAC (No apical meristem, Arabidopsis transcription activation factor) et à la famille de facteurs de transcription qui sont contrôlés par les BR, l’acide abscisique, NaCl et l’acide jasmonique34–37. Les expériences d’expression de gènes révélèrent que RD26 est exprimé de manière constitutive dans les deux shoots et les racines sous conditions d’endo- ou stress salé38,39. RD26 et ses homologues ont pour fonction de promouvoir la réponse à la croissance des BR et de contrôler la tolérance au stress hydrique33. Les études récentes ont révélé que RD26 et ses homologues, ANAC019 et ANAC055, sont impliqués dans la réponse au pathogène bactérien, l’acide jasmonique-médiane défense et tolérance thermique37–42.

Dans cette étude, nous avons confirmé que RD26 est un gène cible de BES1 et négativement régulé le chemin de signalisation BR. RD26 affecte la croissance de manière régulée de gene expression lorsque sur-exprimée globalement par la liaison et l’antagonisme de BES1. Les mutations de gain ont pour effet le BR-contrôlé de croissance et de réponses à la tolérance hydrique, qui ont exercé une influence plus faible dans les shoots ou les racines sous conditions d’endo- ou stress salé38,39. Les résultats suggèrent que RD26 inhibe la croissance des BR et que le chemin de signalisation BR négativement régulé la croissance, établissant un mécanisme de transcription croisée entre ces deux voies importantes pour la croissance végétale et les stress hydriques.

**Résultats**

**RD26 est un régulateur négatif du chemin de signalisation BR.**

Les précédents études d’expression par ensuite indiquées que RD26 était un gène cible de BES1 et BZR1, et son expression était réprimée par BR (brassinolide, le plus actif BR), BES1 et BZR1 (refs 23,24). Depuis les BES1 et BZR1 peuvent s’interagir et antagoniser BES1, nous avons examiné le chemin de signalisation BR. Les expériences de signalisation BR ont été examinées par des techniques de sequencing (RNA-seq), nous avons utilisé des plantes de 4 semaines. Ces expériences de sequencing (RNA-seq) ont révélé que 22,000 gènes ont été analysés (Fig. 2 et les données supplémentaires 1 à 6). Consistant avec les résultats précédents, nous avons observé que RD26 et ses homologues ont pour fonction de contrôler la tolérance hydrique, ce qui a pour effet de réprimer la croissance des BR et la croissance des BR négativement régulée la croissance, établissant un mécanisme de croissance croisée entre ces deux voies importantes pour la croissance végétale et les stress hydriques.

RD26 négativement régulé exprime BR-responsive genes. Afin de déterminer la relation entre le fonctionnementembryonnaire de RD26OX plants is indeed related to BR response, we examined several known BR-induced genes by quantitative PCR (qPCR; Supplementary Fig. 2a). In general, many BR-induced genes we tested are downregulated in RD26OX, including genes involved in BR-regulated cell elongation (TCH4 and EXPL2), supporting a role of RD26 in modulating BR-regulated gene expression and plant growth. To fully understand how RD26 negatively regulates BR responses, we performed global gene expression studies with RD26 mutants in the absence or presence of BRs by high-throughput RNA-sequencing (RNA-seq). We used 4-week-old adult plants for gene expression studies because RD26OX plants display the most obvious growth phenotype at this stage. In WT, 2,678 genes were induced and 2,376 genes were repressed by BL, among ~22,000 genes analysed (Fig. 2 and Supplementary Data 1 and 2), as we previously reported44. The BR-regulated genes from our RNA-seq analysis in adult plants have significant overlap (~43%) with previous microarray analyses of BR-regulated genes in either seedlings or adult plants (Supplementary Data 3 and 4 and Supplementary Fig. 2b)44,45–49. Consistent with the strong phenotype of RD26OX plants, 3,246 genes are upregulated and 5,479 genes are downregulated in the transgenic plants, respectively (Fig. 2 and Supplementary Data 5 and 6).

Pour explorer l’effet de RD26 sur la réponse à la croissance régulée par gene expression, nous avons examiné les overlaps entre le contrôle BR-contrôlé de croissance et les genes affected in RD26OX plants by performing clustering analysis with specific gene groups. RD26 modulates BR-responsive genes in complex ways (Fig. 2 and Supplementary Fig. 3). Consistant avec le contrôle de la tolérance au stress hydrique, 43% (1,141, Group 1) of BR-induced genes were downregulated in RD26OX plants and their induction by BRs was reduced, but not abolished (Fig. 2a,b). In contrast, only 20% (539, Group 3) of BR-induced genes were upregulated in RD26OX (Fig. 2a and Supplementary Fig. 3a). These results suggest that RD26 negatively modulates a significant portion of BR-induced genes.

On the other hand, among 2,376 BR-repressed genes, 595 (25%, Group 2) were upregulated and 823 (35%, Group 4) were
downregulated in RD26OX plants (Fig. 2c,d and Supplementary Fig. 3b). While Group 3 and Group 4 genes suggest a positive role for RD26 in BR response (that is, BR-induced genes are upregulated and BR-repressed genes are downregulated in RD26OX), Group 1 and Group 2 genes demonstrated a negative role of RD26 in BR response (BR-induced genes are downregulated and BR-repressed genes are upregulated in RD26OX). In this study, we focus on the Group 1 and Group 2 genes to determine the mechanisms by which RD26 negatively regulates BR responses.

Consistent with the relatively weak BR-response phenotype of the rd26 anac019 anac055 anac102 mutant, only 405 genes are upregulated and 378 are downregulated in rd26 anac019 anac055 anac102 quadruple mutant (Supplementary Fig. 4 and Supplementary Data 7 and 8). We further compared BR-regulated genes and genes affected in RD26OX and the rd26 anac019 anac055 anac102 mutant (Supplementary Fig. 5a,b). Four subgroups are subjected to further clustering analysis: BR-induced genes that are downregulated in RD26OX and upregulated in the quadruple mutant (36, Supplementary Fig. 5c); BR-induced genes that are upregulated in RD26OX and downregulated in the quadruple mutant (15, Supplementary Fig. 5e); BR-repressed genes that are upregulated in RD26OX and downregulated in the quadruple mutant (44, Supplementary Fig. 5d); and BR-repressed genes that are downregulated in RD26OX and upregulated in the quadruple mutant (19, Supplementary Fig. 5f). Most of these genes are affected in opposite ways in the rd26 anac019 anac055 anac102 mutant and RD26OX. These results support the conclusion that RD26 and its homologues function in a complex way to modulate BR-regulated gene expression.

**RD26 and BES1 differentially control BR-regulated genes.** Previous studies indicated that both BES1 and BZR1 can bind to the BRRE site or E-boxes to inhibit or activate gene expression, respectively. We examined the Group 1 and Group 2 gene promoters and found that BRRE elements are especially enriched in Group 2 gene promoters (Supplementary Fig. 6a and Supplementary Table 1), within 500 base pairs (bp) relative to the transcriptional start sites. The differential enrichments within −500 bp promoter regions are significant as most BES1- and BZR1-binding sites are located in the region as revealed by genome-wide ChiP–chip studies. We selected several gene promoters from Group 1 and Group 2 and fused with luciferase (LUC) gene to generate reporter constructs. BES1, RD26 or BES1 plus RD26 were co-expressed with the reporter constructs and the reporter gene expression was determined. While BES1 repressed and RD26 activated the expression of Group 2 genes, the reporter gene expression level was in between when BES1 and RD26 were
co-expressed (Fig. 3a–c). In contrast, BES1 activated and RD26 repressed Group 1 reporter genes, and the expression level fell in the middle when RD26 and BES1 were co-expressed (Fig. 3d–f). These results indicated that RD26 acts to antagonize BES1 actions on these BR-regulated genes.

To reveal the mechanisms by which RD26 inhibits the large number of BR-induced genes (Group 1, Fig. 2b) and upregulates many BR-repressed genes (Group 2, Fig. 2d), we chose one gene representative of each group for further mechanistic studies. A BR-repressed gene, At4g18010, was chosen to represent Group 2 genes because it is upregulated in RD26OX and its promoter contains a BRRE site at −405 bp relative to the transcription start site (Supplementary Fig. 7a). Likewise, a BR-induced gene At4g00360 was chosen to represent Group 1 genes as its promoter contains a well-established BES1-binding site, CATGTG E-box, at nucleotide −470 (Supplementary Fig. 7b).

To confirm the antagonistic effect of RD26 on BES1-mediated gene expression observed by LUC reporter gene assays, we examined the expression of these two genes in bes1-D, RD26OX and bes1-D RD26OX plants, in which BES1, RD26 or both are increased. As shown in Fig. 3g, the expression of At4g18010 was downregulated in bes1-D and upregulated in RD26OX, but the expression level was in between in bes1-D RD26OX double mutant. In contrast, the expression of At4g00360 was much higher in bes1-D compared with bes1-D RD26OX, while its expression was significantly repressed in RD26OX (Fig. 3h).

**RD26 and BES1 bind to promoters simultaneously.** Previous DNA-binding experiments showed that NAC transcription factors including RD26 (ANAC072) and ANC019 could bind to DNA sequences with two motifs—CATGT(G) and a CACG core spaced by varying numbers of nucleotides. The NAC-binding sites are very similar to E-box (CANNTG) or conserved core sequence of the BRRE site (CGTGT/CG), well-established binding sites for BES1/BZR1. These results suggest that RD26 and BES1 could potentially bind to the same site to modulate BR-regulated gene expression.

We first used yeast one-hybrid assays to test whether BES1 and RD26 can target to the same promoter fragments (Fig. 4). We fused several fragments of the At4g18010 promoter (-P1, -P2 and -P3, with BRRE located in P3) and At4g00360 promoter (-P1, -P2 and -P3, with CACGTG E-box located in P3) to pLacZi reporter (Clontech Inc.) and integrated them into the yeast genome (Fig. 4a). Mutants were also generated in which At4g18010-P3 BRRE and At4g00360-P3 E-box were mutated to unrelated sequences (see Fig. 5a). BES1 (with pGBK7 vector), RD26 (with pGADT7 vector) or both BES1 and RD26 were expressed in each of the reporter yeast strain and the LacZ expression was determined. As shown in Fig. 4b, while neither BES1 nor RD26 significantly changed the gene expression from At4g18010-P3, co-expression of BES1 and RD26 activated the reporter gene expression. It is worth noting that the fusion of the GAL4 activation domain in pGADT7 to RD26 apparently changed RD26 property in yeast to become an activator in combination with BES1 (compared with the result from plants in Fig. 3), which is necessary to detect BES1/RD26 interaction in yeast. Moreover, mutation of the BRRE in At4g18010-P3 completely abolished the activation (Fig. 4b). The results demonstrated that BES1 and RD26 act through the BRRE site in the At4g18010-P3 promoter.
fragment. Similarly, co-expression of BES1 and RD26 activated At4g00360-P3 reporter, which is much reduced when the CATGTG E-box is mutated, indicating that BES1 and RD26 act through the CATGTG E-box in At4g00360-P3 (Fig. 4c) to regulate gene expression.

We also performed ChIP assays with WT and RD26OX transgenic plants, with BES1 antibody or RD26 antibodies we generated (Supplementary Fig. 8). While BES1 itself binds to the At4g18010 promoter (P3) in WT plants, such binding is enhanced in RD26OX plants (Fig. 4d, columns 3 and 4), suggesting that BES1 and RD26 together enhance binding to the promoter region. Consistent with the result that RD26 antibody detects RD26 in RD26OX but not in WT plants (Supplementary Fig. 8a,b), RD26 binding to the At4g18010
promoter (P3) in RD26OX was strongly apparent but barely detectable in WT (Fig. 4d, columns 5 and 6). In contrast, such cooperative binding is not detected in the more upstream promoter region (Fig. 4d, columns 9–12).

To confirm that BES1 and RD26 can bind to the same promoter regions at the same time, we also performed ChIP–reChIP with chromatin prepared from RD26OX, rd26 anac019 anac055 anac102 (rdQ) or BES1 RNAi plants in which the BES1 level is reduced 27 (Supplementary Fig. 9). When the first ChIP was performed with anti-BES1 antibody and eluted chromatin samples were then immunoprecipitated with anti-RD26 or IgG control, significant enrichment of BES1/RD26 binding was detected in RD26OX plants, which is clearly reduced in rdQ mutant, and moderately reduced in BES1RNAi plants with two

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**Figure 4 | BES1 and RD26 act together through E-box or BRRE sites in target gene promoters.** (a) At4g18010 and At4g00360 promoters were divided into three fragments based on known BRRE site and CATGTG E-box present in P3 fragments. Mutant P3 fragments (P3m) in which BRRE and CATGTG E-box was mutated (see Fig. 5a) were also generated. Each fragment was cloned into in yeast one-hybrid vector pLacZi (Clontech Inc.) and integrated into yeast strain YM4271. (b) BES1 (in pGBKT7, TRP marker), RD26 (in pGADT7, LEU marker), BES1 + RD26 were transformed into yeast reporter strains described in a with control plasmids and selected in media lacking LEU and TRP. The yeast colonies were grown on filter paper for LacZ assays. BES1 and RD26 seem to be able to function through At4g18010-P2, although there are no known BES1 or RD26-binding sites in this fragment (Supplementary Fig. 7). (c) At4g00360-P3 reporter was activated when both BES1 and RD26 are expressed in yeast, but not activated when either BES1 or RD26 are expressed. (d) BES1 binding to At4g18010 promoter is enhanced in RD26OX plants as revealed by ChIP assays. WT and RD26-MYC overexpression plants (RD26OX) were used to prepare chromatin and ChIP with antibodies (Ab) against BES1, RD26 or IgG control. The ChIP products were used to detect At4g18010 using primers for qPCR1 (within P3 fragment, see a) and qPCR2 (about −4,000 bp upstream of the transcriptional start site). Error bars represent s.e.m. from four biological replicates. The significance of enrichment was determined by Student’s t-test (*P<0.05).
pairs of independent qPCR primers (Supplementary Fig. 9a,b). Similar results were obtained when the first ChIP was performed with anti-RD26 antibody and reChIP with anti-BES1 (Supplementary Fig. 9c). These results suggest that BES1 and RD26 can simultaneously bind to the At4g18010 gene promoter in vivo.

To further reveal the biochemical mechanisms by which RD26 antagonizes plant drought response. Previous data showed that the BR pathway affects plant drought response. Previous data showed that the BR pathway interacts with BES1/BZR1 to repress the expression of Group 1 and Group 2 genes. Other through corresponding DNA-binding/dimerization domains these results indicated that BES1 and RD26 can interact with each other through corresponding DNA-binding/dimerization domains.

We further confirmed that BES1 and RD26 interaction in vivo by co-immunoprecipitation and by BiFC experiments. GFP antibody (tagged to BES1) can specifically pull down RD26 and truncations with Glutathione S-transferase tag, respectively (Fig. 6a). Glutathione S-transferase pull-down assays indicated that full-length RD26 could interact with full-length BES1 protein (Fig. 6b). The domains involved in DNA binding/dimerization of BES1 (aa 1–89) and RD26 (aa 1–140) are sufficient for the interaction (Fig. 6c). Split Luciferase (Luc) assay was used to test whether RD26 and BES1 interact in plants. RD26 was fused with the amino part of Luc (NLuc) and BES1 was fused with carboxyl-part of Luc (CLuc), respectively (Fig. 6d). Co-expression of RD26-NLuc and CLuc-BES1 in tobacco leaves led to increased Luc activity, while co-expression of controls (RD26-NLuc with CLuc or CLuc-BES1 with NLuc) only produced background-level activities (Fig. 6e).

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The BR signalling pathway inhibits drought response. Since BRs function through BES1/BZR1 to repress the expression of RD26 and its homologues, we tested whether the BR pathway affects plant drought response. Previous data showed that the
expression of RD26 was induced by drought. Drought induces 2,503 and represses 2,862 genes (combination of 2- and 3-day drought treatment data, Supplementary Data 9 and 10). Analysis of gene expression affected in RD26OX and drought-regulated genes revealed that RD26 upregulated 38% (963) of drought-induced genes, but only 12% (346) of drought-repressed genes; similarly, RD26 downregulated 45% (1299) of drought-repressed genes, but only 19% (488) of drought-induced genes (Supplementary Fig. 11). The results suggest that RD26 plays a major role in plant drought responses.

We also compared BR-regulated genes and drought-regulated genes and found that ~38% of BR-regulated genes are modulated by drought (Supplementary Fig. 12). If BR signalling indeed inhibits drought response, we expect that loss-of-function BR mutants exhibit increased drought tolerance. The results suggest that RD26 plays a major role in plant drought responses.

Figure 6 | RD26 interacts with BES1 in vitro and in vivo. (a) Schematic representation of BES1 and RD26 proteins. Full-length (FL) or domains involved DNA binding/dimerization for BES1 (aa 1-89) or RD26 (aa 1-140) are shown. (b) BES1 interacts with RD26 in glutathione S-transferase (GST) pull-down assays. GST-RD26, but not GST, pulled down BES1. MBP-BES1 was detected by anti-MBP antibody. MBP-BES1 (20%) input is shown. (c) The DNA binding and dimerization domains of BES1 (1-89) and RD26 (1-140) interacts with each other. (d) Schematic representation of constructs used for split-LUC assays. Amino or carboxyl parts of Luciferase (NLuc and CLuc) were fused with RD26 or BES1, respectively. (e) RD26 and BES1 interact with each other in vitro. RD26-NLuc and CLuc-BES1 as well as indicated controls were co-expressed in tobacco leaves and Luc activities were measured and normalized against total protein. The averages and s.d. of relative luciferase activities were derived from six independent biological replicates. All the experiments were repeated three times with similar results. (f) BES1 interacts with BES1 through co-immunoprecipitation assay. BES1-GFP and RD26-MYC were co-expressed in tobacco leaves and protein extract was immunoprecipitated with anti-GFP antibody and detected with anti-BES1 (top panel) or anti-MYC (bottom panel) antibodies. (g-l) BES1 interacts with RD26 in BiFC assays. Co-expression of 35S:BES1-YFPN with 35S:RD26-YFPC in tobacco leaves led to reconstitution of YFP signal in the nucleus. No positive signal was observed in control samples co-expressing 35S:BES1-YFPN and 35S:YFPN or 35S:YFPN and 35S:RD26-YFPC. For each panel YFP as well as YFP and bright field (BF) merged images (YFP + BF) from confocal microscopy are shown. Scale bars, 20 µm. The experiments were repeated three times with similar results. Significant differences were based on Student’s t-test (**P<0.01).
mutants have increased, and gain-of-function mutants have decreased, drought tolerance. BR loss-of-function mutant, bri1-5, a weak BR receptor mutant, was exposed to drought stress. After drought stress and recovery, 50% of bri1-5 mutant plants survived, compared with 16% for WT (Fig. 7a, top panel). On the other hand, a gain-of-function mutant in the BR pathway, bes1-D, showed less drought tolerance. Only 22% of bes1-D mutants survived, but all of WT controls survived in the drought stress experiment (Fig. 7a, bottom panel). The drought response phenotypes were also confirmed in bes1-D in Col-0 background with the same trend (Supplementary Fig. 13).

To test our hypothesis that the BR signalling pathway inhibits drought response by repressing RD26 and its homologues, the expression of several drought-induced or drought-related genes were examined in bri1-5 mutant and bes1-D mutant. Transgenic plants overexpressing RD26/ANAC072, ANAC019 or ANAC055 could enhance the tolerance to drought stress, suggesting that RD26 and its homologues ANAC019 and ANAC055 are involved in drought response. Reverse transcriptase qPCR (RT–qPCR) results showed that the expression of all three genes plus ANAC102 are increased in bri1-5 mutant and decreased in bes1-D mutant (Fig. 7b). We also examined five other genes involved in drought tolerance. All five genes are upregulated in bri1-5 and downregulated in bes1-D (Fig. 7b). The results demonstrated that drought response genes are constitutively expressed in loss-of-function BR mutants and repressed in gain-of-function BR mutants, confirming that the BR signalling pathway inhibits drought response, likely by repressing the expression of RD26 and its homologues.

We examined the double-mutant bes1-D RD26OX and found that RD26 overexpression can clearly rescue the bes1-D phenotype in drought response (Supplementary Fig. 14a). Consistent with the facts that RD26OX suppress bes1-D phenotypes, several bes1-D-induced genes are downregulated in RD26OX plants (Supplementary Fig. 14b). The expression of these genes is also reduced in bes1-D RD26OX double-mutant compared with bes1-D (Supplementary Fig. 14b). The gene expression studies support the idea that RD26 suppresses bes1-D phenotypes.

To further understand the relationships among BES1 and RD26/its close homologues, we constructed a Gene Regulatory

Figure 7 | The BR signalling pathway inhibits drought response. (a) BR loss-of-function mutant plants (bri1-5) have increased and gain-of-function BR mutants (bes1-D) have decreased drought tolerance. Survival rates of WS (WT), bri1-5 mutant, EN2 (WT) and bes1-D mutant plants after withholding water for 14-20 days (drought stress) and rehydration for 7 days (rehydration). The survival rate is indicated in the picture. Scale bars, 3 cm. This experiment was repeated three times with similar results. (b) Drought-responsive genes are upregulated in bri1-5 and downregulated in bes1-D mutants. The expression levels of drought-induced genes were examined by qPCR using RNA prepared from bri1 and bes1-D mutants. Error bars indicate s.d. (n = 3). The difference was significant based on Student’s t-test (*P<0.05, **P<0.01). (c) RD26-BES1 GRN. A 103-gene subnetwork extracted from the Arabidopsis whole-genome network using the subnetwork analysis tool, GeNA. Seed genes (ANAC019, RD26, ANAC055, ANAC102 and BES1) are shown in green. The network topology is displayed using Cytoscape.
The GRN showed that BOS1, BES1 cluster and BES1 cluster are connected through only connections to other genes; in addition, the RD26/homo-genes (Fig. 7c). Interestingly, have extensive expression correlations, directly or through other drought, high salinity and fungal pathogens 54,56.

Drought stress induces the expression of pathways. Figure 8 | A model of crosstalk between BR and drought response pathways. Drought stress induces the expression of RD26 to mediate the response of plants to drought. Upon the increased expression, RD26 not only inhibits the expression of BES1 at the mRNA level, but also binds to E-box and BRRE site to inhibit BES1’s functions in mediating BR-regulated gene expression (Group I and II genes), which results in the inhibition of BR-regulated growth. On the other hand, BR signalling represses the expression of RD26 through BES1 and also directly inhibits the expression of other drought-related genes to inhibit drought response.

Network (GRN) based on gene expression correlations using BES1, RD26, ANAC019, ANAC055 and ANAC102 as seed genes55. The GRN showed that RD26 and three of its close homologues have extensive expression correlations, directly or through other genes (Fig. 7c). Interestingly, BES1 has relatively fewer connections to other genes; in addition, the ‘RD26/homo-logue cluster’ and ‘BES1 cluster’ are connected through only one gene, BOS1, which was implicated in plant responses to drought, high salinity and fungal pathogens54,56.

To validate the GRN, we compared the genes in the network with genes affected in RD26OX, as well as drought- and BR-regulated genes (Supplementary Fig. 15). Interestingly, 82% of the 103 genes in the GRN are affected in RD26OX, although only about one-third of total detected genes are affected in RD26OX plants. Similarly, 72 and 52% of the genes in the GRN are either regulated by drought or BRs, despite the fact that only about one-fourth of total genes are regulated by drought or BRs. The computationally generated GRN and its validation by RNA-seq data support the conclusions that (1) there are close interactions between the BES1-mediated BR pathway and the drought pathway represented by RD26 and its homologues; (2) although the interactions between BES1 and RD26 can happen at a transcriptional level (that is, through BOS1), post-transcriptional regulations such as protein–protein interaction between RD26 and BES1 likely play a major role.

Discussion
In this study, we found that the drought-responsive transcription factor RD26 is a target of BES1 and functions to inhibit BR responses. Gene expression studies revealed that RD26 and BES1 act antagonistically in the regulation of many BR-regulated genes. The antagonistic interactions happen at multiple levels. While BES1/BZR1 functions to repress the expression of RD26 at a transcription level, the RD26 protein interacts with BES1 and inhibits its transcriptional activity. Our results thus establish a molecular link and mechanism of interaction between BR and drought response pathways (Fig. 8).

Our genetic, genomic, molecular and biochemical results demonstrated that RD26 functions to inhibit the BR pathway (Fig. 8). RD26 is induced by drought, promotes drought-regulated gene expression and confers drought tolerance when overexpressed55,36. Our genetic studies demonstrate that RD26 is a negative regulator of the BR pathway as overexpression of RD26 leads to reduced plant growth and BR response and knockout of RD26 and three of its homologues lead to increased BR response. The relatively weak growth phenotype of rd26 anac019 anac055 anac102 mutant may be explained by additional family members, which possibly function redundantly in the inhibition of BR response. The fact that a smaller number of genes affected in rd26 anac019 anac055 anac102 mutant compared with RD26OX transgenic plants is consistent this hypothesis. RD26 and its homologues appear to function as part of a highly redundant and complex network to confer drought tolerance and to inhibit plant growth during drought stress.

Global gene expression studies revealed that RD26 functions to modulate BR-responsive gene expression in a complex manner, that is, RD26 can either activate or repress both BR-induced and BR-repressed genes. However, a large number of BR-induced genes (1,141 or 43% of BR-induced genes identified in this study) are significantly downregulated in RD26OX (Group 1, Fig. 2). Our molecular and biochemical studies suggest that RD26 affects Group 1 gene expression by binding to the BES1 target site (E-box) and neutralizing BES1 activation activity, potentially by forming an inactive heterodimer (Figs 3–5). Likewise, 595 (or 25%) BR-repressed genes are upregulated in RD26OX, suggesting that BR and RD26 have opposite function on these genes (Group 2, Fig. 2). Indeed, the molecular and biochemical evidence suggests that, while BES1 binds to BRRE to repress gene expression, RD26 can antagonize BES1-mediated gene repression (Fig. 3). We also provided evidence that BES1 and RD26 protein can interact with each other in vitro and in vivo (Fig. 6). While many protein–protein interactions between transcription factors synergistically activate or repress transcription, our results suggest that BES1 and RD26 interact and antagonize each other’s transcriptional activities on Group 1 and Group 2 gene promoters. Our findings thus reveal a previously unknown mechanism that two signalling pathways converge on the same promoter element through two interacting transcription factors to coordinate plant growth and stress responses. Consistent with our conclusion, recent ChIP-seq studies showed that RD26 target gene promoters under abscisic acid treatment are enriched in G-box sequence (CAGCGT, a specialized E-box)57, very similar to BES1 target sites derived from ChIP–chip study23.

We also observed an inhibitory effect of the BR pathway on drought response as a loss-of-function BR mutant is resistant to drought and a gain-of-function mutant of the BR pathway had compromised drought response. The transcriptional repression of RD26 and its homologue genes by BRs likely play a major role in the observed inhibition of drought response by the BR pathway as the expression of RD26 and its homologues (including ANAC019, ANAC 055 and ANAC102) are significantly increased in bri1 and decreased in bes1-D (Fig. 7b). While we have provided experimental evidence that RD26 antagonizes BES1-mediated gene expression on the BES1 target sites, it remains to be determined whether BES1 inhibits RD26-mediated gene expression on RD26-related drought target genes.

We propose that the antagonistic interaction between BES1 and RD26 likely ensures that plant growth is reduced when plants are under drought stress, under which RD26 and its homologues are upregulated to inhibit BR-induced growth, thus allowing
more resources to deal with the drought stress. On the other hand, under normal growth conditions, i.e., in the absence of drought stress, BR signaling represses the drought pathway by repressing the expression of RD26 and its homologues.

It is worth noting that RD26 and BES1 do not seem to act antagonistically at all times. For example, 539 BR-induced genes (20%, Group 3) are upregulated and 823 BR-repressed genes (35%, Group 4) are downregulated in RD26OX (Supplementary Fig. 3), indicating that RD26 and BES1 act in a similar manner on these two groups of genes. It is possible that RD26 and BES1 target different promoters to achieve the positive interactions between RD26 and BES1. It has been suggested that a least under some conditions, exogenously applied BR can improve plant drought tolerance.94. It is possible that under these circumstances, the Group 2 and Group 4 genes play more dominant roles than Group 1 and Group 2 genes, which can potentially allow BR to activate some drought-induced genes and repress BR-repressed genes and thus promote drought tolerance. More investigation is needed to better understand the interaction between RD26 and BES1 on Group 3 and Group 4 genes.

In summary, we have identified RD26 as a molecular link that coordinates BR and drought responses. We further found that, while BES1 functions to repress RD26 gene expression, RD26 interacts with BES1 and inhibits BES1 transcriptional activity. This reciprocal inhibitory mechanism not only ensures that BR-induced growth is inhibited under drought conditions, but also prevents unnecessary activation of drought response when plants undergo BR-induced growth.

Methods

Plant materials and growth condition. T-DNA insertion mutants, rd26 (At4g27410, SALK_063576), aux10 (At1g52890, SALK_096295), aux55 (At1g15500, SALK_014331) and aux102 (At1g63790, SALK_030702) were obtained from ABRC (Arabidopsis Biological Resource Center). All plants were grown on 1/2MS plates and/or in soil under long day conditions (16 h light/8 h dark) at 22 °C. BRZ and BL response experiments were carried out as previously described.95 Briefly, seeds were sterilized with 70% ethanol and 0.1% Triton X-100 for 15 min and washed with 100% ethanol three times and dried in filter papers in a sterile hood. The seeds were sprinkled on half Linsmaier and Skoog medium (Caiong Lab) with 0.7% Phytobland agar (Caiong Lab) and various concentrations of BRZ (provided by Professor Tadasi Asami) or BL (Wako Biochemical). Both BRZ and BL (1 mM stock in dimethylsulphoxide) were added to medium after autoclave and the plates with seeds were placed at 4 °C for 3 days. After exposing to light for 8 h, the plates were wrapped with three layers of aluminium foil and incubated in the dark at 25 °C for 5 days for BRZ response and in the constant light for 7 days for BL response experiments. Hypocotyls were scanned and measured using Image J (https://image.nih.gov/ij/). Ten to fifteen hypocotyls were measured, and averages and s.d. were calculated and plotted.

Plasmid constructs. For MYC-tagged transgenic plants, RD26 genomic sequence including its 5' UTR was cloned from WT and fused with MYC tag and CaMV 35S promoter in pZIP vector.96 For recombinant protein purification, full-length or fragments of RD26 and BES1 coding regions were cloned into the pETMALc- H vector97 or pET-42a (Novagen). All primers used in this study are provided in Supplementary Table 2.

Generation and analysis of transgenic plants. The construct of RD26-MYC driven by 35S promoter was transformed into Agrobacterium tumefaciens (strain GV3101), which were used to transform plants by the floral dip method.98 Transgenic lines were selected on 1/2 MS medium plus 60 g ml−1 kanamycin. The transgenic lines were grown on 1/2MS plates and/or in soil under long day conditions (16 h light/8 h dark) at 22 °C. BRZ and BL (1 mM stock in dimethylsulphoxide) were added to medium after autoclave and the plates with seeds were placed at 4 °C for 3 days. After exposing to light for 8 h, the plates were wrapped with three layers of aluminium foil and incubated in the dark at 25 °C for 5 days for BRZ response and in the constant light for 7 days for BL response experiments. Hypocotyls were scanned and measured using Image J (https://image.nih.gov/ij/). Ten to fifteen hypocotyls were measured, and averages and s.d. were calculated and plotted.

Gene expression analysis. For RD26, At1g0360 and At1g18010 gene expression, total RNA was extracted and purified from 2-week-old plants of different genotypes using the RNeasy Mini Kit (Qiagen). The Mx4000 multiplex Quantitative PCR System (Stratagene) and SYBR GREEN PCR Master Mix (Applied Biosystems) were used in quantitative real-time PCR analysis. For transient expression, promoters for At1g188010 (1,552 bp) and At1g18010 (1,515 bp), At1g22400 (1,922 bp), At5g17860 (1,119 bp), At4g14365 (430 bp) and At3g19720 (411 bp) were cloned and used to drive luciferase reporter gene expression. The BES1-coding region driven by CaMV 35S promoter was cloned into pZP211 vector, while RD26-MYC construct used in transgenic plants' generation was also co-transformed with the RD26 promoter targeting vector.99

For transgenic plants' generation, the bombardment conditions were optimized to examine the effect of RD26 and BES1 on reporter gene expression either with or without combination of BES1 and RD26. Equal amount of Agrobacterium cells (measured by OD600 adjusted to the same with vector-containing strain) were co-infiltrated into leaves of tobacco. The activities of the luciferase, which were measured in total protein extracts from triplicate samples (collected with a 5 mm leaf puncher with same number of leaf discs in each sample) using Berthold Centro LB960 luminometer with the luciferase assay system following the manufacturer's instruction (Promega). The relative level of luciferase activity was normalized by the total amount protein for each sample.

For global gene expression, total RNA was extracted and purified from 4-week-old plants of different genotypes using the RNeasy Mini Kit (Qiagen). Duplicate RNA samples were subjected to RNA-seq using HiSeq2000 50 bp single-end sequencing in the DNA facility at Iowa State University. Raw RNA-seq reads were subjected to quality-checking and trimming and then aligned to the Arabidopsis reference genome (TAIR10) using an in-tron aware aligner, Genomic Short-read Nucleotide Alignment Program.100 The alignment coordinates of uniquely aligned reads for each sample were used to independently calculate the read depth of each annotated gene. Genes with an average of at least one uniquely mapped read across samples were tested for differential expression using QuaSeq (http://cran.r-project.org/web/packages/QuasiSeq). The generalized linear model Quasi-likelihood spline method assuming negative binomial distribution of read counts implemented in the QuaSeq package was used to compute a P value for each gene. The 0.75 quantile of reads from each sample was used as the normalization factor101. A multiple test-controlling approach was used to convert P values to q-values for controlling false-detection rate.102 For most of the comparisons, q-values no larger than 0.05 were considered to be differentially expressed. Owing to the strong growth phenotype of RD26OX transgenic lines, more stringent (q < 0.003) condition was used to determine differentially expressed genes. Clustering was performed using the ‘hclustmap’ function of the NMF package in R (https://cran.r-project.org/web/packages/NMF/index.html). Log2 reads per million mapped read values were used for clustering analysis and values were normalized for each gene by centring and scaling each row of the heatmap. The overlapped genes were identified and displayed using Venny 2.0 programme (http://bioinfo.cnb.csic.es/tools/venny/).

Chromatin immunoprecipitation. ChIP was performed as previously described103 with modifications.94 Briefly, 5 g of 4-week-old plants were fixed in 1% formaldehyde and used to isolate nuclei and chromatin. The chromatin was sheared with Diagenode Bioruptor Sonication System with 30 cycles of 30 s on and 30 s off in icy water bath. Twenty micrograms of affinity-purified BES1 (ref. 23), RD26 antibodies (see ‘Generation and analysis of transgenic plants’ section) or IgG (Santa Cruz Biotechnology) were used to immunoprecipitate chromatin, which were collected with 20 μl Dynabeads protein A (Invitrogen). Three qPCR technical repeats were used to calculate enrichment folds compared to ubiquitin control (UBQ5). The enrichment of specific transcription factors was examined by qPCR with primers from indicated regions. The averages and s.e.s were derived from four biological repeats.

For ChIP–reChIP, chromatin was prepared from 15 g RD26OX, BES1RNAi or rdQ mutant plants with a modified protocol in which the crosslinking with formaldehyde was performed after tissue grinding in liquid nitrogen, and all the buffer volumes were scaled up by 15-folds compared with the published protocol.104 The sonication and immunoprecipitation were performed as described above with BES1 or RD26 antibody. Each first immunoprecipitated chromatin sample was eluted with 75 μl 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2% SDS and 15 mM dithiothreitol and diluted 20-folds for second immunoprecipitation with corresponding antibody (RD26 or BES1) or IgG control. The enrichment at specific region was determined by qPCR with indicated primers as described above. The averages and s.e.s were derived from three biological repeats.

Other bioinformatics analysis. For promoter motif analysis, we downloaded Group 1 and 2 genes upstream 3 kb sequence from TAIR database (https://www.Arabidopsis.org/tools/bulk/sequences/index.jsp). On the basis of this sequence information, we coded in-house Perl scripts to match possible E-box and BRRE motif in upstream 3,000 bp region by searching conserved sequence ‘CANNTG’ for general E-box or ‘CATGTCG’ for specific E-box and conserved sequence ‘CGTCG(TAG)’ for the BRRE site. All the statistical analyses was done by R language (http://www.R-project.org/). We fitted a negative binomial model for fitting the frequency of E-box and BRRE domain in ‘glm.nb’ function and then calculated P value for each comparison. The density plots were generated by R language’s ‘plot’ function. For re-analysis of previously published microarray data,24,45–49, we downloaded the microarray raw CEL data from Riken and analysed the arrays using the ‘Robust
Multi-array Average method to obtain gene expression data. To analyse gene expression and compare the expression between the WT and hormone treatments, we used the linear model for microarray (limma) package from the Bioconductor project (http://www.bioconductor.org). When estimating statistical significance for log2-transformed fold-change replicates were combined analogous to the classical pooled two-sample t-test. To account for multiple testing, we used the Benjamini-Hochberg method, and significance level for detection is at 5%. The differential expressed genes were combined with published gene lists to obtain the BR-regulated genes by microarrays were and listed in Supplementary Data 3 and 4.

Protein-protein interaction experiments. The Split Luciferase Complementation Assays were performed as described. The coding region of RD26 and BES1 were cloned into the pCAMBIA1300-nLUC and pCAMBIA1300-cLUC constructs, respectively. Tobacco leaf transient assay was used to examine luciferase activity in the presence or absence of RD26 and/or BES1. Equal amounts of Agrobacterium cells (measured by OD600, adjusted to same with vector-containing strain) were injected to tobacco leaves. The luciferase activities were measured from protein extracts from triplicate samples as described above. For the immunoprecipitation (IP) experiments, tobacco leaves were homogenized in protein lysis buffer (1 mM EDTA, 10% glycerol, 75 mM NaCl, 0.05% SDS, 100 mM Tris-HCl pH 7.4, 0.1% Triton X-100 and 1 × complete cocktail protease inhibitors). After protein extraction, anti-GFP antibody (10 μL, Life Technologies-Molecular Probes, A21311) was added to total proteins. After incubation with gentle mixing for 1 h at 4 °C, 200 μL fresh 50% slurry of protein A beads (Trisacryl Immobilized Protein A-20338, Thermofisher) were added, and incubated for 2 h at 4 °C. Protein A beads were pelleted by centrifugation at 20,000 rpm for 1 min, and the supernatant was removed. The precipitated beads were washed at least four times with the protein extraction buffer and then eluted by 2 × SDS protein-loading buffer with boiling for 5 min. The IP products were used for western blotting with 2 μg of anti-BES1 antibody or MYC antibody (Sigma, C9356). BIFC experiments were performed as recently described. The coding region of RD26 and BDE2 DNA were cloned into the N- or C terminus of EYFP vectors. Sequence-confirmed constructs were transformed into Agrobacterium tumefaciens strain GV3101. Agrobacteria were grown in LB medium containing 0.2 M acetylsyringone and washed with infiltration medium (10 mM MgCl2, 10 mM MES, pH 5.7, 0.2 M acetylsyringone) and resuspended to OD600 0.5 with infiltration medium. Combinations of Agrobacterium were infiltrated into Nicotiana benthamiana leaves and examined for YFP signals 2 days after infiltration. A Leica SPS X MP confocal microscope equipped with an HCS PL APO CS 20.0 0.70 oil objective was used to detect reconstituted YFP. YFP was excited with a 514-nm laser line and detected from 530 to 560 nm. The LAS AF software (Leica Microsystems) was used to obtain images with same settings.

EMSA experiments. EMSA experiments were carried out as described previously. After annealing, oligonucleotide probes were labelled with P32-γ-ATP using T4 polynucleotide kinase. About 0.2 ng probe and indicated amount of proteins purified from Escherichia coli were mixed in 20 μL binding buffer (25 mM HEPEs-KOH (pH 8.0), 1 mM DTT, 50 mM KCl and 10% glycerol). After 40 min incubation on ice, the reactions were resolved by 5% native polyacrylamide gels with 1 × TGE buffer (6.6 g L−1 Tris, 28.6 g L−1 glycerine and 0.78 g L−1 EDTA (pH 8.7)).

Drought stress tolerance of BR signalling mutants. Drought stress tolerance experiments were carried out as described previously with minor modifications: different genotype plants were grown on 1/2 MS medium for 2 weeks, and then transferred to soil and grown for one more week in growth chamber (22 °C, 60% relative humidity, long day conditions) before exposure to drought stress. Drought stress was imposed by withholding water until the lethal effect of dehydration was observed on WT control or bes1-D plants. The numbers of plants that survived and continued to grow were counted after watering for 7 days.

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Author contributions

H.Y. and Y.Y. originally conceived the project. H.Y., B.T., J.C., Z.X. and H.J. performed genetic, physiological, biochemical and gene expression studies. T.M.N. performed BiFC and gene-clustering analyses. S.L.-Y., L.L., Y.W., M.Z., Z.L. and P.S.S. conducted the RNA-seq and bioinformatics analyses. H.T. and C.C. are involved in yeast one-hybrid assays. H.G. and Y.Y. performed ChIP and reChIP experiments. M.A. and S.A. performed computational modelling. H.Y. and Y.Y. wrote the paper with contributions from most co-authors.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: S.L. and P.S.S. are shareholders of Data2Bio LLC, Ames, IA, USA. The remaining authors declare no competing financial interests.

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