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## TOPLESS mediates brassinosteroid-induced transcriptional repression through interaction with BZR1

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### Abstract

Brassinosteroid (BR) regulates plant development by activating the transcription factor BRASSINAZOLE RESISTANT1 (BZR1), which activates and represses different target genes to switch cellular programs. The mechanisms that determine BZR1's transcriptional activities remain largely unknown. Here we show that BZR1 represses target genes by recruiting the Groucho/TUP1-like transcriptional corepressor TOPLESS (TPL). Specific deletion or mutation of an evolutionarily conserved ERF-ASSOCIATED AMPHIPHILIC REPRESSION (EAR) motif at the C-terminus abolishes BZR1's abilities to regulate gene expression and cell elongation, but these defects are rescued by TPL fusion to the EAR motif-mutated BZR1. The EAR motif in BZR1 mediates recruitment of TPL to BZR1-repressed promoters. A triple *tpl* mutant (*tpl; tpr1; tpr4*) shows reduced BR sensitivity and suppresses the gain-of-function *bzr1-1D* mutant phenotype. BR repression of gene expression also requires histone deacetylases that interact with TPL. Our study demonstrates key roles of the EAR motif and TPL in BR regulation of gene expression and plant growth.

### Introduction

Steroids are important hormones for regulation of gene expression and development in both animals and plants. Unlike animal steroid hormones, which directly bind to nuclear receptor transcription factors, the plant steroid hormone brassinosteroid (BR) binds to a trans-

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#### Contributions

E.O., J.-Y.Z., Z.-Y.W. designed the research; E.O. and J.-Y.Z. conducted all the experiments, except that H.R. and I.H. performed the experiments of Figure 5e and 5f; and E.O. and Z.-Y.W. wrote the manuscript.

#### Competing Financial Interests

The authors declare no competing financial interests.

#### Supplementary information

Supplementary Figures 1– 4 and Supplementary Table 1

membrane receptor kinase, BRASSINOSTEROID INSENSITIVE1 (BRI1) (ref. 1), which has a leucine-rich repeat (LRR) extracellular domain similar to the Toll receptors in metazoans<sup>2</sup>. BRI1 signaling activates the members of the BRASSINAZOLE RESISTANT1 (BZR1) family of transcription factors through a phosphorylation-mediated signal transduction pathway<sup>3, 4</sup>. BZR1 activates and represses different target genes to program genome expression and cell growth. The mechanisms that specify BZR1's function in transcription activation and repression are not understood.

BZR1 is a plant-specific transcription factor with a DNA binding domain that recognizes BR response element (BRRE, CGTG(T/C)G) (ref. 5). Both subcellular localization and DNA-binding activity of BZR1 are tightly regulated by phosphorylation. In the absence of BR, a GSK3-like kinase named BIN2 interacts with BZR1 via a docking motif at the C-terminus of BZR1 and phosphorylates BZR1 (ref. 6–8). The phosphorylated BZR1 is unable to bind DNA and also is retained in the cytosol due to binding to the 14-3-3 proteins<sup>9, 10</sup>. BR binding to BRI1 triggers a signaling cascade that leads to inactivation of BIN2 (ref. 11), resulting in dephosphorylation of BZR1 catalyzed by PROTEIN PHOSPHATASE 2A (PP2A) (ref. 12). Dephosphorylated BZR1 enters nucleus and binds to target promoters to mediate BR-responsive gene expression<sup>3</sup>. BZR2 (also named BES1), the close homolog of BZR1, is regulated through similar mechanisms<sup>6, 12–14</sup>.

Like many plant transcription factors, BZR1 functions as a transcriptional repressor for some promoters but an activator for others. Previous genome-wide identification of direct binding sites of BZR1 coupled with transcriptome profiling showed that BZR1 binds to promoters of both BR repressed and induced genes<sup>5, 15</sup>. Apparently, BZR1 transcriptional activity is partly determined by the context of promoter *cis*-elements and *trans*-factors. Indeed the genome-wide data showed that BRRE is enriched in the promoters of BR-repressed gene, whereas the E-box motif (CANNTG), a binding site for bHLH factors, is more enriched in the promoters of BR-activated genes<sup>15</sup>. BZR1 and its homologous BZR2/BES1 interact with the bHLH factor PHYTOCHROME INTERACTING FACTOR4 (PIF4) and BZR1 shares with PIF4 over 50% of their target genes in the genome. BZR2/BES1 also interacts with BES1 INTERACTING MYC-LIKE1 (BIM1). The BZR1-PIF4 and BZR2-BIM1 heterodimers bind to the E-box motif in the promoters of target genes and activate gene expression<sup>13, 16</sup>. Several other transcription factors and histone modifying enzymes have been reported to interact with BZR2/BES1 and participate in BZR2/BES1 activation of gene expression<sup>17–19</sup>. In addition, recent studies have suggested that MYELOBLASTOSIS TRANSCRIPTION FACTOR LIKE2 (MYBL2) and HOMEODOMAIN-LEUCINE ZIPPER PROTEIN1 (HAT1) interact with BZR2/BES1 and repress several BR target genes<sup>20, 21</sup>. However, it remains unclear how BZR1 represses gene expression, and specifically how its gene repression and activation activities are mediated by interactions with general transcriptional regulators.

In this study, a genetic screen for the intragenic suppressors of the gain-of-function mutant *bzr1-ID* reveals an important function of an evolutionarily conserved ERF-ASSOCIATED AMPHIPHILIC REPRESSION (EAR) motif in BZR1 regulation of target gene expression. We further show that BZR1 EAR motif mediates interaction with the Groucho/TUP1-like corepressor TOPLESS (TPL), selectively recruiting TPL to the promoters of BZR1-

repressed target genes. BZR1 repression of gene expression requires both TPL and also activity of histone deacetylase (HDA) that is known to interact with TPL. Our study unveils the molecular mechanism of BR repression of gene expression through BZR1-TPL interaction.

## Results

### EAR motif is crucial for BZR1 functions

The gain-of-function mutant BZR1 protein, *bzr1-1D*, is constitutively dephosphorylated and active even in the BR deficiency condition due to a strong interaction with PP2A (ref. 12). Thus, the hypocotyl elongation of *bzr1-1D* seedling is resistant to BR biosynthesis inhibitors, brassinazole (BRZ) and propiconazole (PPZ) (ref. 12). We identified suppressors of the *bzr1-1D* mutant through an EMS mutagenesis screen. One of the mutants, *bzs285*, carried a mutation that creates a stop codon after amino acid 284, resulting in deletion of 52 amino acids at the C-terminus of *bzr1-1D* protein (Fig. 1a–b). The truncated region includes a BIN2 docking motif and an LxLxL type of EAR motif. Since the BIN2 docking motif mediates inactivation of BZR1 through BIN2-mediated phosphorylation<sup>8</sup>, the suppression of the *bzr1-1D* phenotype by the *bzs285* mutation was most likely due to loss of the EAR motif. The EAR motif is highly conserved in the BZR1 family of transcription factors across land plants from bryophytes to angiosperms (Supplementary Fig. 1a). In addition, all other BZR1-like proteins in *Arabidopsis* have the EAR motif at their C-terminus (Supplementary Fig. 1b), implicating an indispensable role of the EAR motif in BZR1 functions.

To functionally characterize the EAR motif of BZR1, we generated transgenic plants overexpressing the EAR motif-deleted BZR1 (*BZR1 EAR-OX*) or *bzr1-1D* (*bzr1-1D EAR-OX*). Transgenic plants overexpressing wild type BZR1 (*BZR1-OX*) were almost indistinguishable from wild-type plants (Fig. 1c) due to posttranslational regulation of BZR1 activity by BIN2, whereas overexpression of *bzr1-1D* (*bzr1-1D-OX*) caused BR-activation phenotypes similar to the *bzr1-1D* mutant (Fig. 1c), including leaf bending, organ fusion, and insensitivity to BR inhibitors<sup>22</sup>. In contrast, both *BZR1 EAR-OX* and *bzr1-1D EAR-OX* plants displayed severe dwarfism (Fig. 1c), similar to the BR-insensitive or BR-deficient mutant plants, suggesting that the EAR-deleted BZR1 proteins have dominant negative effects on BR responses. Consistent with *bzr1-1D* suppression by *bzs285*, *bzr1-1D EAR-OX* plants were sensitive to BR biosynthetic inhibitor PPZ (Fig. 1d) and showed no organ-fusion phenotypes in contrast to the fusion between cauline leaves and axillary branches often observed in the *bzr1-1D* and *bzr1-1D-OX* plants<sup>22</sup> (Fig. 1e). The *bzr1-1D EAR-OX* seedlings grown in the dark also showed shorter hypocotyls (Fig. 1d). These results confirm an essential role of the EAR motif in BZR1 function.

To determine whether the dwarfism is caused by a defect in BR signaling or BR biosynthesis, we examined BR response of the *bzr1-1D EAR-OX*. While the hypocotyls of *bzr1-1D* were more elongated by BR treatment than those of wild type, hypocotyls of *bzr1-1D EAR-OX* were insensitive to BR (Fig. 1f), indicating that BR signaling is impaired in the *bzr1-1D EAR-OX* plants. BR inhibits primary root growth mainly through reducing cell division in the root meristem<sup>23</sup>. Root growth of *bzr1-1D* was more sensitive to BR inhibition than wild type, whereas roots of the *bzr1-1D EAR-OX* were insensitive to BR

(Fig. 1g). Similar to *bzr1-1D EAR-OXs*, *BZR1 EAR-OXs* also showed BR insensitivity in hypocotyl elongation (Fig. 1h). The severe dwarfism and BR insensitivity of *BZR1 EAR-OX* and *bzr1-1D EAR-OX* are most likely due to the dominant negative effects of the EAR motif-deleted BZR1 over endogenous BZR1 and BZR1-like transcription factors. Together, these results demonstrate that the EAR motif is essential for BZR1 functions in various BR responses.

### EAR motif mediates BZR1 regulation of gene expression

We next analyzed the effects of the EAR motif deletion on BZR1 regulation of target gene expression. BZR1 is known to directly repress BR biosynthesis genes, including *DWF4* and *CPD*, to provide the feed-back regulation of BR level<sup>5</sup>. The expression levels of *DWF4* and *CPD* were dramatically reduced in *bzr1-1D* compared to wild type, but was only marginally reduced in the *bzr1-1D EAR-OX* plants (Fig. 2a), indicating that the transcriptional repression activity is impaired in *bzr1-1D EAR*. BZR1 also activates expression of many of its target genes<sup>15</sup>. While the expression levels of *SAURs*, *IAA19* and *PRE5* were highly increased by *bzr1-1D*, expression of *SAUR19*, *IAA19* and *PRE5* was not activated, and *SAUR15* expression was only slightly activated by *bzr1-1D EAR* (Fig. 2b), suggesting that the EAR motif is also required for the BZR1-mediated transcriptional activation of target genes.

In order to more directly examine the functions of the EAR motif in BZR1 regulation of gene expression, we generated transgenic plants expressing *bzr1-1D* or *bzr1-1D EAR* fused to the glucocorticoid receptor (GR), *bzr1-1D-GR* or *bzr1-1D EAR-GR*, which allows inducible nuclear localization of the fusion proteins. Transient DEX treatment (four hours) of the *bzr1-1D-GR* plants dramatically repressed *CPD* expression and activated *SAUR15* expression, confirming the inducible nuclear localization and function of *bzr1-1D-GR in planta* (Fig. 2c,d). In contrast, the DEX treatment of the *bzr1-1D EAR-GR* plants did not repress *CPD* or activate *SAUR15* (Fig. 2c,d), indicating that the EAR motif is necessary for both transcriptional repression and activation activities of BZR1. In fact, the *CPD* expression levels were slightly increased by DEX treatment in *bzr1-1D EAR-GR* plants (Fig. 2c), most likely due to the competition by the transcriptionally inactive *bzr1-1D EAR* protein with the endogenous BZR1 for target promoter occupancy. Consistent with the results of the DEX treatment assays, in transient gene expression assays using *Arabidopsis* mesophyll protoplasts, *bzr1-1D* increased *PRE5* promoter activity and decreased *DWF4* promoter activity, whereas *bzr1-1D EAR* could neither activate *PRE5* nor repress *DWF4* (Fig 2e,f). The deletion of the EAR motif did not affect the BZR1 binding to the promoters of target genes (Fig. 2g). These results indicate that the deletion of the EAR motif abolishes both gene repression and activation functions of BZR1.

To examine the possibility that the deletion of the EAR motif affects the phosphorylation of BZR1, we checked the phosphorylation status of BZR1 and BZR1 EAR before and after BR treatment. Wild type BZR1 protein was mostly phosphorylated when seedlings were grown on the medium containing PPZ and dephosphorylated after BR treatment, but BZR1 EAR was highly dephosphorylated even without BR treatment (Supplementary Fig. 2a). Such high level of dephosphorylated BZR1 EAR is likely responsible for the strong

phenotypes of *BZR1 EAR-OX* plants in contrast to the lack of phenotype of *BZR1-OX* plants (Fig. 1). Since the EAR motif is located near the BIN2-docking motif<sup>8</sup>, it is likely that the deletion of the EAR motif affects BZR1-BIN2 interaction. However, BZR1 EAR still interacted with BIN2 in a yeast two-hybrid assay (Supplementary Fig. 2b). Moreover, the endogenous BZR1 was also partially dephosphorylated before BR treatment in the *BZR1 EAR-OX* plants (Supplementary Fig. 2a), suggesting that BR signaling upstream of BZR1 is activated in the *BZR1 EAR-OX* plants, most likely due to the loss of the feedback inhibition of BR synthesis and upstream BR signaling. Indeed qRT-PCR analysis showed that the expression level of *BIN2* is decreased in *bzr1-1D EAR-OX* compared to wild type (Supplementary Fig. 2c), which likely contributes to the decreased BZR1 phosphorylation.

### SRDX fusion restores the functions of EAR motif-deleted BZR1

An EAR motif-based artificial transcriptional repression domain (LDLDLELRGFA), named SRDX, confers a dominant transcriptional repression activity to the linked transcription factor, and has been widely used in functional studies of transcription factors in plants<sup>24</sup>. To determine whether SRDX provides the same function as the EAR motif in BZR1, we generated transgenic plants overexpressing *bzr1-1D EAR* fused with SRDX at the C-terminus (*bzr1-1D EAR-SRDX-OX*) (Fig. 3a). On the medium containing PPZ, the hypocotyls of wild type and *bzr1-1D EAR-OX* were much shorter than that of *bzr1-1D*, indicating the inability of *bzr1-1D EAR* to promote hypocotyl elongation (Fig. 3b). In contrast, *bzr1-1D EAR-SRDX-OX* had much longer hypocotyl than wild type (Fig. 3b). In addition, the SRDX fusion restored the BR sensitivity of *bzr1-1D EAR-OX* in the inhibition of primary root growth (Fig. 3c), indicating that the functions of EAR motif can be replaced by SRDX.

Consistent with the established SRDX function, the SRDX fusion restored the transcriptional repression activity of *bzr1-1D EAR* as shown by down-regulation of *DWF4* expression in the *bzr1-1D EAR-SRDX-OX* plants (Fig. 3d). However, to our surprise, *bzr1-1D EAR-SRDX* also activated expression of *SUAR15* and *IAA19* (Fig. 3e), which are direct targets of BZR1, suggesting that SRDX also mediates the transcriptional activation function of BZR1.

### BZR1 interacts with TOPLESS through the EAR motif

In several transcription factors, the EAR motif has been known to mediate transcriptional repression through interaction with the Groucho/Tup1-like transcriptional corepressor TOPLESS (TPL) and the homologous TOPLESS RELATED (TPR) proteins<sup>25–28</sup>. Our recent tandem affinity purification of BZR1 complex followed by mass spectrometry analysis suggested that TPL associates with BZR1 *in vivo*<sup>29</sup>. We therefore tested whether BZR1 directly interacts with TPL and TPRs. Indeed, in the yeast two-hybrid assay, BZR1 interacted with TPL and also other TPRs (Fig. 4a,c). Deletion of the EAR motif or mutation of the conserved Leu residues in the EAR motif abolished the BZR1-TPL interaction (Fig. 4a,b), indicating that BZR1 interacts with TPL through the EAR motif. To confirm the BZR1-TPL interaction *in vivo*, we performed co-immunoprecipitation (co-IP) assays using *Arabidopsis* mesophyll protoplasts expressing *bzr1-1D-GFP* or *bzr1-1D EAR-GFP* and TPL-Myc. The TPL-Myc protein was co-immunoprecipitated by anti-YFP antibody

specifically when *bzr1-1D-GFP* was co-expressed (Fig. 4d), confirming the *in vivo* BZR1-TPL interaction. The deletion of EAR motif greatly reduced the TPL-BZR1 interaction *in vivo* (Fig. 4d), which is consistent with the result of the yeast two-hybrid assay (Fig. 4a). Thus, both yeast two-hybrid and co-IP assays demonstrate that BZR1 interacts with TPL through the EAR motif.

TPL is not a DNA-binding protein, but it is usually recruited to specific promoter DNA through interaction with DNA-binding transcription factors. To test if BZR1 recruits TPL to the promoters of BZR1 target genes, we first carried out DNA pull-down assays with a biotin-labelled *DWF4* promoter. As expected, while TPL was pulled down by the *DWF4* promoter DNA only in the presence of BZR1 but not when BZR1 was absent (Fig. 4e). Next, we carried out chromatin immunoprecipitation (ChIP) assays using the transgenic plants expressing TPL-HA driven by the native *TPL* promoter, to test the TPL binding to the promoters of BZR1 targets *in vivo*. Consistent with the results of the DNA pull-down assays, TPL is associated with the promoters of BZR1-repressed genes (*DWF4* and *CPD*) *in vivo* and the TPL binding to these promoters was enhanced by BR treatment (Fig. 4f), which is known to induce BZR1 DNA-binding<sup>5</sup>. TPL also bound to the promoters of BR-activated genes (*SAUR15* and *IAA19*), but unlike the binding to *DWF4* and *CPD* promoters, the TPL binding to *SAUR15* and *IAA19* promoters was not enhanced by BR treatment (Fig. 4f). TPL is likely also recruited to these promoters through other BR-independent transcription factors, such as the AUX/IAA proteins, which also interact with TPL and mediate auxin responsive expression of *SAUR15* and *IAA19* (ref. 27, 30). Together, these results indicate that, through its EAR motif, BZR1 recruits TPL to the promoters of BR-repressed genes.

### TOPLESS is required for BR responses

To further verify the requirement of TPL/TPRs activities in BR responses, we tested the BZR1-repressed genes in the *tpl;tpr1;tpr4* triple mutant lacking *TPL* and two *TOPLESS RELATED (TPR)* genes<sup>28</sup>. As expected, *bzr1-1D* caused a much milder repression of *CPD* and *DWF4* in *tpl;tpr1;tpr4* than in wild type background (Fig. 5a), supporting that the full TPL/TPRs activities are required for BZR1 repression of these target genes. In contrast, *bzr1-1D* activation of *IAA19* and *EXP8* was not significantly affected by the *tpl;tpr1;tpr4* mutation, and *SAUR15* activation by *bzr1-1D* was only slightly decreased in *tpl;tpr1;tpr4* (Supplementary Fig. 3). These results indicate that TPL/TPRs play a major role in BZR1 repression of target genes but not in BZR1 activation of gene expression. Consistent with the defect in the BZR1 repression of gene expression, the *tpl;tpr1;tpr4* seedlings were significantly less sensitive to BR treatment (Fig. 5b) in hypocotyl elongation compared to wild type. In addition, the *tpl;tpr1;tpr4* triple mutation partially suppressed the PPZ-insensitive phenotypes of the *bzr1-1D* mutation both in the dark and under light (Fig. 5c,d). These results demonstrate that the normal level of TPL/TPR activity is required for BZR1-mediated gene repression and hypocotyl cell elongation.

The BR-related phenotypes of the *tpl;tpr1;tpr4* triple mutant are weaker and likely also less specific than typical BR-insensitive mutants, because of genetic redundancy and broad functions of TPL/TPRs as general transcription repressors. To further confirm the essential and sufficient role of the BZR1-TPL interaction in the BR repression of gene expression, we

generated transgenic plants overexpressing *bzr1-1DmEAR* (mutation in the EAR motif) and *bzr1-1DmEAR-TPL* fusion protein. The mutation of the core Leu residues in the EAR motif (mEAR) abolished the BZR1 interaction with TPL (Fig. 4b) and the BZR1 transcriptional repression activities on *DWF4* and *CPD* (Fig. 5e). TPL fusion to the C-terminus of *bzr1-1DmEAR* restored the BZR1 transcriptional repression activities (Fig. 5e), as well as the hypocotyl promoting activity (Fig. 5f), indicating that the TPL recruitment through the EAR motif is necessary and sufficient for these BZR1 functions.

### TPL/TPRs modulate light sensitivity

The BR repression of gene expression has been shown to mediate the BR inhibition of light-induced seedling morphological changes, photomorphogenesis<sup>31, 32</sup>. BR-activated BZR1 directly binds to the promoters of *GATA4* and *BZS1*, which are positive regulators of photomorphogenesis, and represses their expression levels<sup>31, 32</sup>. Since TPL/TPRs are involved in the BZR1 repression of gene expression, we tested if TPL/TPRs are also required for BZR1 to repress *GATA4* and *BZS1*. First, we showed, using ChIP assays, that TPL was recruited to the promoters of *GATA4* and *BZS1* in a BR dependent manner (Fig. 6a). Consistently, BZR1 repression of *GATA4* and *BZS1* expression was diminished in the *tpl1; tpr1; tpr4* mutant compared to wild type (Fig. 6b). Next, we tested if loss of TPL/TPRs affects light response of seedlings. Figure 6c shows that *tpl1; tpr1; tpr4* is hypersensitive to the red light inhibition of hypocotyl elongation, which is consistent with the high expression levels of *GATA4* and *BZS1*. Together, these results suggest that BZR1-TPL/TPRs interactions modulate the light sensitivity of seedlings through the transcriptional regulation of *GATA4* and *BZS1*.

### Histone deacetylase mediates BR repression of gene expression

The Groucho/TLE-mediated transcription repression has been known to engage histone deacetylase (HDA) (ref. 33). In addition, previous studies have shown that TPL interacts with HDA6 and HDA19 (ref. 25, 28), and our proteomic study has identified HDA19 in the BZR1 complex<sup>29</sup>. We confirmed that BZR1 associates with both HDA19 and TPL *in vivo* (Fig. 7a). These results suggested that the histone deacetylase activity may be required for the BR repression of gene expression, and thus we tested this hypothesis using a HDA inhibitor Trichostatin A (TSA). While BR treatment dramatically repressed expression levels of *DWF4* and *CPD*, their expression levels were elevated by TSA treatment and much less affected by BR in the presence of TSA (Fig. 7b), indicating that the histone deacetylase activity is required for BR repression of gene expression. Consistent with the gene expression pattern, the long-hypocotyl phenotype of *bzr1-ID* was largely suppressed by the TSA treatment, whereas the wild type seedlings were much less affected under this condition (Fig. 7c). These results support the notion that the BZR1-TPL interaction exploits HDA for the BR repression of gene expression and promotion of cell elongation.

### BZR1 directly represses the expression of TPL and TPRs

The genome-wide analyses of BZR1 direct targets showed that *TPL* and *TPRs* are direct targets of BZR1 (Fig. 8a) (ref. 15). We performed independent ChIP assays using transgenic plants expressing *bzr1-ID-CFP* under the control of native *BZR1* promoter

(*BZR1p::bZR1-1D-CFP*). Consistent with the ChIP-Seq result, the promoter regions of *TPL*, *TPR1*, *TPR2* and *TPR3* were significantly enriched by the BZR1 ChIP compared to the negative control *PP2A* and *UBC30* promoter regions (Fig. 8b), confirming that BZR1 binds to these *TPL/TPR* promoters. BR treatment reduced the expression level of *TPR3* by two fold, *TPL* by about 40%, and *TPR1* slightly, but had no significant effect on *TPR2* expression (Fig. 8c), which correlate with their relative binding by BZR1 detected by ChIP-qPCR (Fig. 8b). These results suggest that BZR1 mediates repression of *TPL* and at least two *TPRs*. In addition, BZR1 repression of *TPR3* also requires *TPL/TPRs* activities, as the expression level of *TPR3* was elevated and much less repressed by *bZR1-1D* in the *tpl;tpr1;tpr4* mutant background (Fig. 8d), suggesting that BZR1-*TPL/TPRs* interactions mediate a feedback repression of many members of the *TPL/TPR* gene family.

## Discussion

BZR1 is a master transcription factor in BR signaling pathway and regulates expression of thousands of genes to elicit various BR responses<sup>15</sup>. While BR signaling pathway upstream of BZR1 has been relatively well characterized, the molecular mechanism underlying BZR1 regulation of gene expression is still poorly understood. Here, we show that the EAR motif at the C-terminus of BZR1 is essential for BZR1 regulation of transcription and plant growth, and the EAR motif mediates BR/BZR1 repression of gene expression by recruiting the co-repressor TPL and histone deacetylases.

EAR motifs of several plant transcription factors have been shown to act as a transcription repression domain by recruiting the Groucho/TLE-like transcriptional corepressor TPL (ref. 34). We found the EAR motif is highly conserved in the BZR1 family of transcription factors across land plants, and it plays a central role in BZR1's transcriptional functions. Specific deletion or mutations of the EAR motif abolished BZR1's abilities to regulate gene expression and a wide range of developmental processes, demonstrating an essential role of the EAR motif in BZR1 function.

The series *in vitro* and *in vivo* experiments demonstrated that the EAR motif of BZR1 mediates interaction with TPL. The BZR1-TPL interaction was detected in yeast two-hybrid, co-immunoprecipitation, and *in vitro* pull-down assays. TPL was also identified as a component of the BZR1 complex by affinity purification followed by mass spectrometry analyses<sup>29</sup>. Deletion of the EAR motif abolished the interaction in both yeast two-hybrid and co-immunoprecipitation assays. Both *in vitro* DNA-binding assays and ChIP assays demonstrated that BZR1 recruits TPL to the promoters of BR-repressed genes. The essential role for *TPL/TPRs* in BR repression of gene expression is supported by several lines of evidence. First, BZR1 showed significantly reduced ability to repress several BR-repressed genes in the triple *tpl;tpr1;tpr4* mutant compared to wild type plants. The weak phenotypes of the *tpl;tpr1;tpr4* mutant are likely due to redundant functions of other members of the *TPL/TPR* family, as *TPR3* expression is increased in the *tpl;tpr1;tpr4* mutant and yeast two-hybrid assays indicated that BZR1 interacts with all four *TPRs*. Second, the TPL fusion to the *bZR1-1DmEAR* restored the defect of *bZR1-1DmEAR* in repressing gene expression and promoting hypocotyl elongation, demonstrating that BZR1 interaction with TPL is essential and sufficient for repressing gene expression and promoting cell elongation. Furthermore,



*TPL* and three *TPR* genes are direct targets of BZR1, and three of them are repressed by BR treatment under our conditions, supporting complex roles of TPL/TPRs in BR responses.

Given that TPL/TPRs are positive regulators in BR responses, the transcriptional repression of *TPL/TPRs* by BZR1 would provide a negative feedback loop that enables fine-tuning the BR responses. Furthermore, since the BZR1-mediated repression requires TPL/TPRs proteins, this creates another feedback loop that helps maintain the cellular level of TPL/TPRs. As TPL/TPRs are exploited by multiple hormonal and developmental pathways (auxin, jasmonic acid, abscisic acid, and ethylene) to repress gene expression<sup>26–28, 35–37</sup>, regulation of *TPL/TPRs* expression levels by BZR1 potentially allows BR/BZR1 to modulate other signalling pathways. Therefore, TPL/TPRs play important roles in BR responses not only as the transcription repressors of specific BR-target genes but also as BR target genes themselves that potentially mediate feedback regulation of BZR1 function and modulate broader secondary gene expression responses.

The TPL-induced transcriptional repression has been reported to be at least partly mediated by HDA (ref. 34). A role of HDA in the BZR1 repression of gene expression is supported by the effect of HDA inhibitor TSA on *bzr1-1D* repression of *CPD* and *DWF4* expression and BZR1 association with both TPL and HDA19 *in vivo*. Together these results demonstrate that BZR1 recruits TPL and HDA to the BR-repressed promoters to mediate transcriptional repression through chromatin modification.

The transcriptional repression activity of BZR1 is important not only for feedback regulation of BR homeostasis but also for BR promotion of plant growth<sup>5</sup>. As essential components for BZR1-mediated transcriptional repression, TPL/TPRs play a key role in the biological functions of BR. In addition to the BR biosynthetic genes, two BR-repressed genes encoding positive regulators of photomorphogenesis, *GATA4* and *BZS1* (ref. 31, 32), showed BR-induced TPL-promoter binding and TPL/TPR-dependent transcriptional repression. Consistent with TPL/TPR's function in BZR1-mediated repression of *GATA4* and *BZS1*, the triple *tpl; tpr1; tpr4* mutant showed not only reduced sensitivity to BR treatment but also increased sensitivity to red light treatment in hypocotyl elongation, which indicate that TPL/TPRs play an important role in BR regulation of growth and photomorphogenesis. The function of TPL in BR promotion of growth is further confirmed by the recovery of growth promoting activity of *bzr1-1DmEAR* with TPL fusion. Together these results provide strong evidence supporting a central role for the TPL-mediated transcriptional repression activity of BZR1 in BR regulation of cell elongation and photomorphogenesis, as well as in the feedback inhibition of BR biosynthesis.

Although the EAR motif has been shown to mediate transcription repression in several plant transcription factors as well as in BZR1, our study provides evidence that the EAR motif of BZR1 may also mediate transcriptional activation. First, the expression of BZR1 direct target genes that are activated by BR and *bzr1-1D* were not activated by *bzr1-1D EAR* in transgenic plants. Second, when *bzr1-1D EAR-GR* was induced to translocate to the nucleus, the expression level of *SAUR15* was not increased, in contrast to *bzr1-1D-GR* which significantly activated *SAUR15* expression. Third, *bzr1-1D EAR* did not activate the *PRE5* promoter activity in the transient gene expression assay. Last, the artificial SRDX

domain recovered the defect in transcriptional activation of *bzr1-1D* EAR. SRDX contains a different EAR motif from that of BZR1 (LDLDLELRGFA instead of LELTL), and thus the results suggest that the loss of activation function in *bzr1-1D* EAR is due to the loss of the EAR motif rather than another overlapping domain. The EAR motif exists in a large number of transcription factors and whether the EAR motif can mediate transcription activation in other factors is an important question to be answered in future studies. Our observation also cautions the interpretation of results obtained with SRDX fusion transcription factors.

How the transcriptional activation and repression activities of BZR1 are determined? Apparently the *cis*-elements of the promoter specify BZR1's transcriptional activity by probably recruiting different co-factors. A recent study showed that a MYB transcription factor MYBL2 is a transcription repressor and interacts with BZR2/BES1 (ref. 20). Interestingly, MYBL2 has an internal LxLxL motif<sup>35</sup> but instead uses a C-terminal TLLFR motif for transcription repression<sup>38</sup>. Nevertheless, MYBL2 was identified as a TPL interacting protein in the yeast two-hybrid screen<sup>35</sup> and thus it is possible that MYBL2 stabilizes BZR1/BZR2 interactions with TPL and thereby enhances transcriptional repression. Another BZR2/BES1-interacting transcription factor HAT1 also represses gene expression, but loss of function *hat1;hat2* mutant showed normal fold change of BR-responsive gene expression<sup>21</sup>, and thus HAT1 appears not essential for BZR2/BES1 transcriptional repression. Alternatively, transcriptional repression might be the default activity of BZR1 in the absence of co-activator that blocks the EAR motif, as suggested by the repression of artificial promoter by the BZR1 fusion to the LexA DNA-binding domain<sup>5</sup>. It is conceivable that the EAR motif in BZR1 must be blocked and inaccessible to TPL at the promoter to be activated by BZR1. Consistent with this notion, BR treatment increased TPL association with BR-repressed promoters but not BR activated promoters. It is possible that the EAR motif in BZR1 is involved in interaction with co-activators at the BR-activated promoters, to both mediate gene activation and prevent recruitment of TPL repressor. BZR1 activation of transcription appears to involve interaction with bHLH factors. Both BZR1 and BZR2/BES1 interact with bHLH factors of the phytochrome-interacting factor (PIF) family<sup>16</sup>, and their shared target genes are significantly more likely to be activated than repressed by BR. BZR2/BES1 has also been shown to interact with another bHLH factor BIM1 and histone modifying enzymes to mediate the transcriptional activation<sup>13, 17, 19</sup>. Whether the EAR motif of BZR1 is involved in the interactions with these co-transcription activators remains to be determined in future studies.

Transcription factors with dual functions of activation and repression are common in both plants and animals. For example, WUSCHEL activates *AGAMOUS* expression, but represses the expression of *ARR6* and *ARR7* (ref. 39). In metazoan, the T-cell factor (TCF) transcription factors mediate both repression and activation of target gene expression. When the Wnt signaling pathway is off, TCF interacts with Groucho/TLE corepressor to repress its target genes. Wnt signaling inactivates GSK3, leading to accumulation of  $\beta$ -catenin in the nucleus;  $\beta$ -catenin displaces Groucho/TLE from TCF and re-assembles a transcription activator complex, switching from repression to activation<sup>40</sup>. In this case, TCF bound to the same promoter is switched from a repressor to an activator by exchange of interacting

proteins. The mode of action of BZR1 appears more similar to that of dorsal, which represses some target genes by recruiting Groucho/TLE but interacts with the bHLH factor twist to activate target genes that contain twist-binding sites<sup>41–43</sup>. Unlike BZR1's EAR motif, which is required for both repression and activation, the Groucho/TLE-interaction domain at the C-terminus of dorsal is required for transcription repression but not activation. It seems that BZR1 shares features of repressor displacement with TCF, *cis*-element-dependent co-activator interaction with dorsal, and inactivation by GSK3 with  $\beta$ -catenin. Furthermore, both BZR1 and dorsal are targets of LRR receptor-mediated signaling pathways<sup>2, 3</sup>. Therefore, the BR signaling pathway shares striking similarities with the Wnt and Toll pathways of metazoan, while mechanistic details seem to have evolved to provide appropriate efficiency required for the different levels of developmental plasticity of the organisms.

## Methods

### Plant materials and growth conditions

*Arabidopsis thaliana* plants were grown in a greenhouse with a 16-h light/8-h dark cycle at 22–24°C for general growth and seed harvesting. All the plants used in this study are in Col-0 ecotype background. The *tpl; tpr1; tpr3* triple mutant was provided by Yuelin Zhang<sup>28</sup>. The *TPLp::TPL-HA* plants used for CHIP assay was provided by David E. Somers<sup>25</sup>.

### Constructs

The full-length cDNA of *bzr1-1D* (also *bzr1-1D EAR*) was fused to glucocorticoid receptor (GR) by overlapping PCR. The resulting *bzr1-1D-GR* and *bzr1-1DdEAR-GR* were inserted into pENTR/SD/D-TOPO vector (Invitrogen) and subcloned into gateway compatible pEarleyGate101 vector for transformation into *Arabidopsis*.

For the *Arabidopsis* mesophyll protoplast transfections, the full length cDNA of *TPL* and *HDA19* (without stop codon) was cloned into the gateway compatible *pGWB17* plasmid, and the cDNA (without stop codon) of *bzr1-1D* and *bzr1-1D EAR* was cloned into the gateway compatible pEarleyGate101 plasmid.

The full-length cDNA of *bzr1-1D (BZR1P234L)* and *bzr1-1DmEAR (bzr1-1D<sup>L328/330A</sup>)* were cloned into plant expression vectors under the control of the *35S C4PPDK* promoter as previously reported<sup>10, 44</sup>. All mutants of *BZR1* were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. To generate *bzr1-1DmEAR-SRDX* and *bzr1-1DmEAR -TPL* overexpressing transgenic lines, the *SRDX* sequence and full-length cDNA of *TPL* were tagged to *bzr1-1DmEAR* at the C-terminus region and sub-cloned into a plant expression binary vector, *pCB302ES* (ref. 44).

### Hypocotyl and root length measurements

Sterilized seeds by 70% (v/v) ethanol and 0.01% (v/v) Triton X-100 were plated on MS medium (PhytoTechnology Laboratories) supplemented with 0.75% phytoagar. After three days of incubation at 4°C, seedlings were irradiated by white light for 6 hours to promote germination and then incubated in specific conditions for 5–7 days. Seedlings were

photocopied and hypocotyl and root lengths were measured by using ImageJ software (<http://rsb.info.nih.gov/ij>).

### Arabidopsis protoplast isolation and transfection

Leaves from 4-week-old plants grown in the short photoperiod were cut to 0.5-1mm leaf strips and digested by enzyme solution (1.5% cellulase R10; 0.4% macerozyme R10; 0.4 M mannitol; 20 mM KCl; 20 mM MES at pH 5.7; 10 mM CaCl<sub>2</sub>; 0.1% BSA) for 3 hr at room temperature after being vacuum infiltrated for 30 min in the dark. The enzyme/protoplast solution was diluted by an equal volume of W5 buffer (154 mM NaCl; 125 mM CaCl<sub>2</sub>; 5 mM KCl ; 5 mM Glucose ; 2 mM MES at pH 5.7). The enzyme solution containing protoplast was filtered through 75- $\mu$ m nylon mesh and the protoplasts were collected by centrifugation at 100 g for 1 min. After washing with W5 solution, the protoplasts were resuspended with W5 solution and incubated on ice for 30 min. After removing W5 by centrifugation at 100g for 30 sec, the protoplasts were resuspended with MMG solution (0.4 M mannitol; 15 mM MgCl<sub>2</sub>; 4 mM MES at pH 5.7). For DNA transfection, an equal volume of PEG buffer (40% PEG4000; 0.2M Mannitol; 0.1M Ca(NO<sub>3</sub>)<sub>2</sub>) was mixed with the protoplasts and DNA, and 4 volumes of W5 buffer were added to stop the transfection after 15 min. The resulting solution was mixed well by gently inverting the tube, and the protoplasts were pelleted by centrifugation at 100 g for 1 min. The transfected protoplasts were resuspended gently in 0.5 mL of WI solution (0.5 M mannitol; 20 mM KCl; 4 mM MES at pH 5.7) and cultured under weak light at room temperature for overnight.

### Co-immunoprecipitation (co-IP) assays

About  $2 \times 10^4$  isolated *Arabidopsis* mesophyll protoplast were transfected with a total 20  $\mu$ g of DNA and incubated overnight. Total proteins were extracted from the protoplasts using the IP buffer (50 mM Tris-Cl pH7.5, 1 mM EDTA, 75 mM NaCl, 0.1% Triton X-100, 5% Glycerol, 1 mM PMSF, 1 $\times$  Protease Inhibitor). After centrifugation at 20,000g for 10 min, supernatants were incubated for 1 hr with 5  $\mu$ g of polyclonal anti-YFP antibody (custom made) immobilized on protein A/G agarose beads. The beads were then washed for three times with 1 ml of IP buffer and eluted samples were analyzed by immunoblot using Anti-Myc (Cell Signaling Technology, mouse mAb, 1:5000 dilution) and Anti-GFP (Clontech JL-8, mouse mAb, 1:5000 dilution) antibodies.

### Transient gene expression assays

Isolated *Arabidopsis* mesophyll protoplasts ( $2 \times 10^4$ ) were transfected with a total 20  $\mu$ g of DNA and incubated overnight. Protoplasts were harvested by centrifugation and lysed in 50  $\mu$ l of passive lysis buffer (Promega). Firefly and Renilla (as an internal standard) luciferase activities were measured by using a dual-luciferase reporter kit (Promega). The reporter promoters (1kb of *CPD* promoter and 1.5 kb of *PRE5* promoter) were cloned upstream of the firefly luciferase gene in *pGreen-0800II-luc* vector, which also contains the 35S promoter.-renilla luciferase gene. Statistic significance was determined by student's *t*-test.

### qRT-PCR gene expression analysis

Total RNA was extracted by using the Spectrum Plant Total RNA kit (Sigma). M-MLV reverse transcriptase (Fermentas) was used to synthesize cDNA from the RNA. Quantitative real-time PCR (qRT-PCR) was performed in LightCycler® 480 (Roche). *PP2A* was used to as an internal control. Gene specific primers are listed in Supplementary Table 1.

### Chromatin Immunoprecipitation (ChIP) assays

The plants grown under white light for two weeks or in the dark for five days were harvested and cross-linked for 20 min in 1% formaldehyde under vacuum. The chromatin complex was isolated, resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM NaCl, 0.5% Triton X-100, 1 mM PMSF) and sheared by sonication to reduce the average DNA fragment size to around 500 bps. The sonicated chromatin complex was immunoprecipitated by affinity-purified polyclonal anti-YFP antibodies (5 µg, custom made) or anti-HA antibody (Roche; 3F10), which was incubated with protein A agarose/salmon DNA (Millipore) overnight. The beads were washed with low-salt buffer (50 mM Tris-HCl at pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100), high-salt buffer (50 mM Tris-HCl at pH 8.0, 2 mM EDTA, 500 mM NaCl, 0.5% Triton X-100), LiCl buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 0.25 M LiCl, 0.5% NP-40, 0.5% deoxycholate) and TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA) and eluted with elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). After reverse cross-linking, the DNA was purified with a PCR purification kit (Thermo Scientific) and analyzed by ChIP\_qPCR. Enrichment of DNA was calculated as the ratio between transgenic plants and wild type control, normalized to that of the *PP2A* coding region as an internal control. Statistic significance was determined by student's *t*-test. Primers for qPCR are listed in Supplementary Table 1.

### DNA pull-down assays

GST-TPL-N (amino acids 1–344 of TPL cloned into *pDEST15* vector), MBP and MBP-BZR1 (*pMAL-C2*) proteins were expressed in BL21 codon plus *Escherichia coli* cells and purified either using glutathione beads (GE Healthcare) or amylose resin (NEB) according to manufacturer's protocols. The *DWF4* promoter fragment was amplified by PCR using the biotin-labelled primers (Supplementary Table 1). GST-TPL-N was incubated with the biotin-labelled DNA together with MBP or MBP-BZR1 protein and then DNA-binding proteins were pulled down by streptavidin agarose beads (Sigma) and analyzed by immunoblot.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

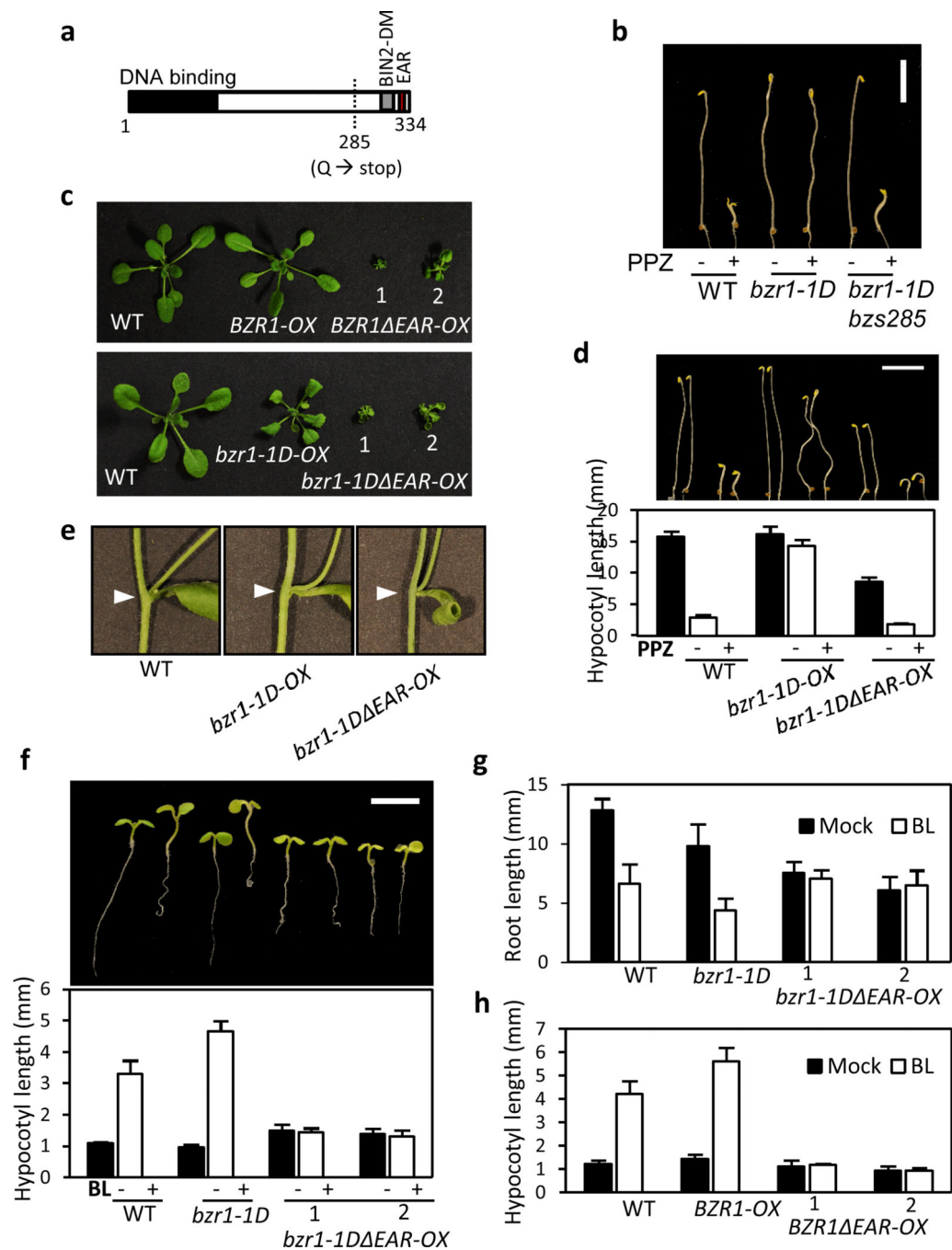
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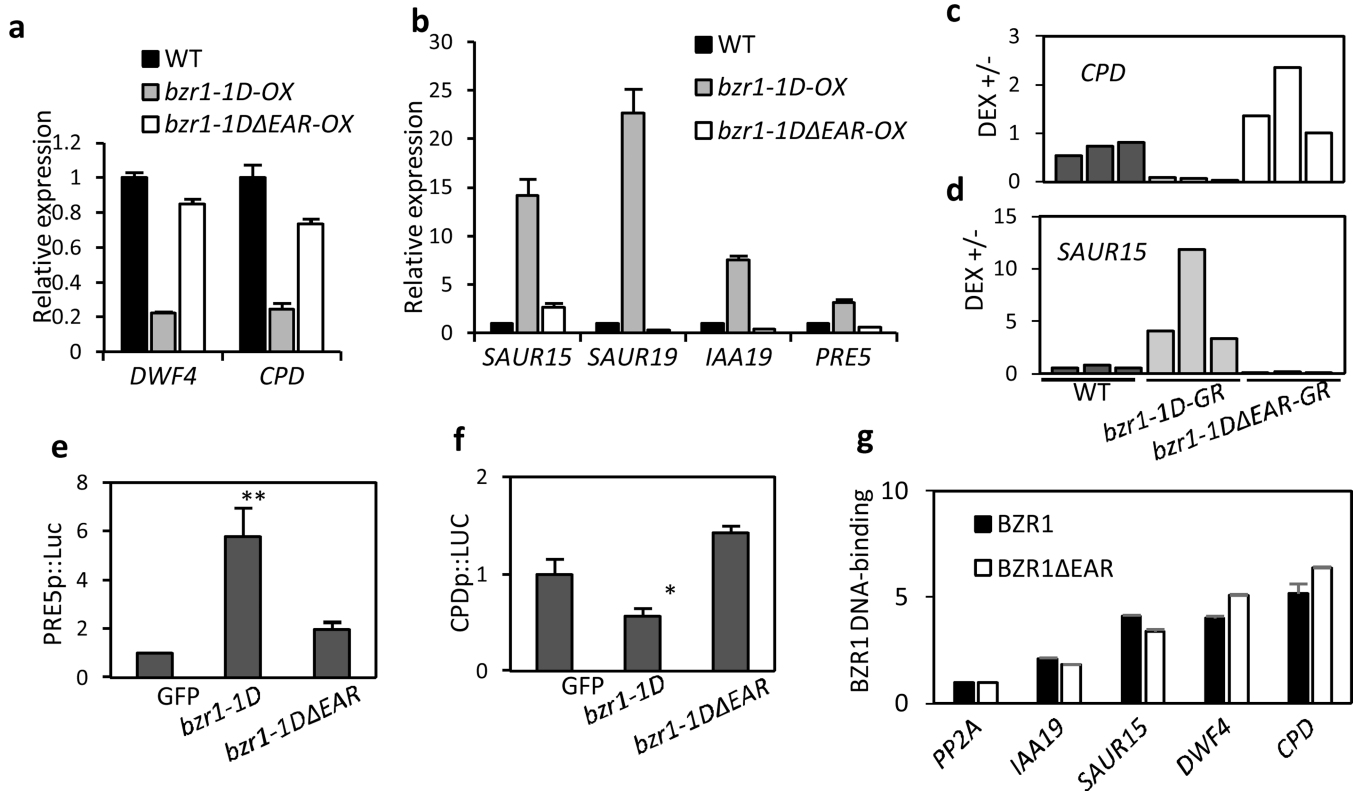
**Fig. 1. EAR motif is essential for BZR1 function in BR responses**

(a) Glutamine (Q) at position 285 in BZR1 is converted to stop codon in the *bzs285* mutant. BIN2-DM: BIN2 docking motif. EAR: EAR motif.

(b) Intragenic mutant (*bzs285*) suppresses the constitutive activation of BR response of *bzr1-1D*. Seedlings were grown in the dark on the medium containing 2  $\mu$ M propiconazole (PPZ). Scale bar: 5 mm.



- (c) Both *BZR1 EAR-OXs* and *bzr1-1D EAR-OXs* show severe dwarfism. Plants were grown in the long-day condition (16 hr of light and 8 hr of dark) for 4 weeks. Two independent lines (1 and 2) for the *BZR1 EAR-OXs* and *bzr1-1D EAR-OXs* are shown.
- (d) The hypocotyl elongation of *bzr1-1D EAR-OX* is inhibited by PPZ treatment. Seedlings were grown in the dark for 5 days on the medium containing either the mock or 2  $\mu$ M PPZ. Error bars indicate the s.d. ( $n=10$  plants). Scale bar: 5 mm.
- (e) Axillary branches and cauline leaves are not fused in *bzr1-1D EAR-OX*.
- (f,g) *bzr1-1D EAR-OXs* are insensitive to BR in the promotion of hypocotyl elongation (f) and the inhibition of primary root growth (g). Seedlings were grown under the white light for 5 days on the medium containing either mock or 1000 nM brassinolide (BL). Scale bar: 5 mm.
- (h) *BZR1 EAR-OXs* are insensitive to BR in the promotion of hypocotyl elongation. Seedlings were grown under the white light for 5 days on the medium containing either mock or 1000 nM brassinolide (BL). Error bars in (f–h) indicate the s.d. ( $n=10$  plants).



**Fig. 2. EAR motif mediates BZR1 regulation of gene expression**

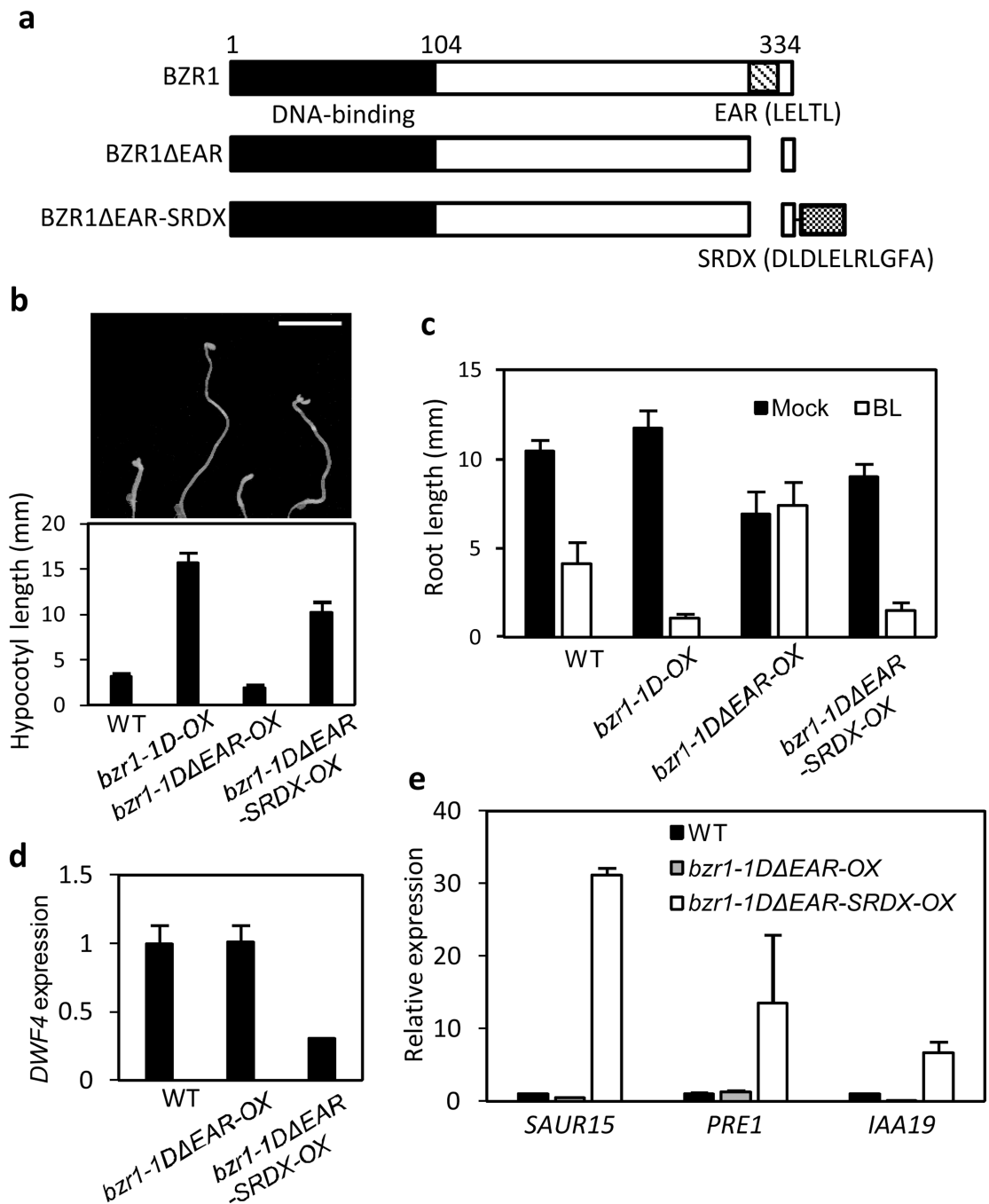
(a,b) The deletion of EAR motif abolishes BZR1 regulation of gene expression. Seedlings were grown on the medium containing 2  $\mu$ M PPZ in the dark for 5 days before harvesting for RNA extraction. Gene expression levels are normalized to that of *PP2A* and are shown relative to the expression levels in wild type (Col-0).

(c,d) The EAR motif-deleted *bzr1-1D* neither represses *CPD* expression (c) nor activates *SAUR15* expression (d). Four-week-old leaves of transgenic plants expressing *bzr1-1D-GR* or *bzr1-1D EAR-GR* were treated with mock or 40  $\mu$ M of dexamethasone (DEX) for 4 hr. Ratios of expression levels in DEX-treated to mock-treated are shown. Each bar represents independent transgenic plant.

(e,f) The EAR motif-deleted *bzr1-1D* cannot activate *PRE5* promoter activity (e) and cannot repress *CPD* promoter activity (f) in a transient gene expression assay. The 2 kb of *PRE5* promoter or 1 kb of *CPD* promoter fused to fire fly luciferase reporter gene was co-transfected with *35S::bzr1-1D* or *35S::bzr1-1D EAR* into *Arabidopsis* mesophyll protoplast. The fire fly luciferase activities were normalized by renilla luciferase as an internal control and are shown relative to that of protoplasts transfected with *35S::GFP* (GFP). \*\* :  $P < 0.01$  and \* :  $P < 0.05$  by Student's *t*-test.

(g) The deletion of EAR motif does not affect BZR1 DNA-binding. Transgenic plants overexpressing *BZR1* and *BZR1 EAR* were treated with 100 nM BL and crosslinked for the ChIP assays. In the ChIP assays, the enrichment of DNA was calculated as the ratio between transgenic plants and wild type control (Col-0), normalized to that of the *PP2A* coding region as an internal control.

Error bars in (a,b,e,f,g) indicate the s.d. ( $n=3$ ).



**Fig. 3. SRDX fusion restores the functions of EAR motif-deleted BZR1**

(a) Box diagram of BZR1, BZR1 EAR and BZR1 EAR-SRDX.

(b,c) SRDX fusion restores both BZR1 promotion of hypocotyl elongation and inhibition of primary root growth. Seedlings were grown in the dark for 5 days on the medium containing 2  $\mu$ M PPZ (b) or grown under light for 5 days on the medium containing either mock or 1000 nM BL (c). The error bars indicate the s.d. ( $n=10$  plants). Scale bar: 5 mm.

(d,e) qRT-PCR analyses of BZR1-regulated genes. RNA was extracted from seedlings grown in the dark for 5 days on the medium containing 2  $\mu$ M PPZ. Gene expression levels

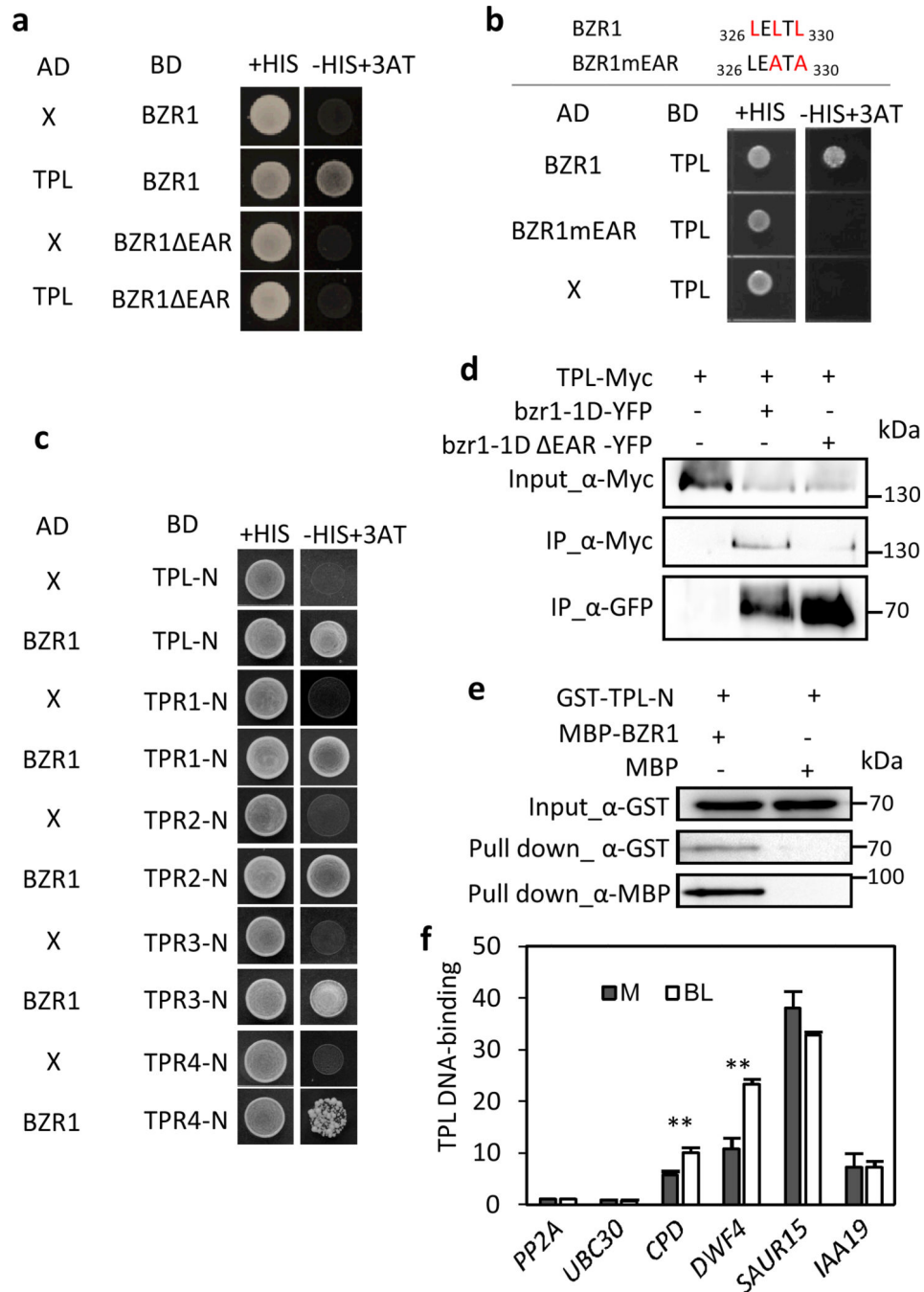
are normalized to that of *PP2A* and are shown relative to the expression levels in wild type. The error bars indicate the s.d. ( $n=3$ ).

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**Fig. 4. BZR1 interacts with TOPLESS through the EAR motif**

(a) A yeast two-hybrid assay shows the interaction between BZR1 and TPL. Yeast clones were grown on the synthetic dropout (+His) or synthetic dropout without histidine (-His) plus 50 mM 3AT medium.

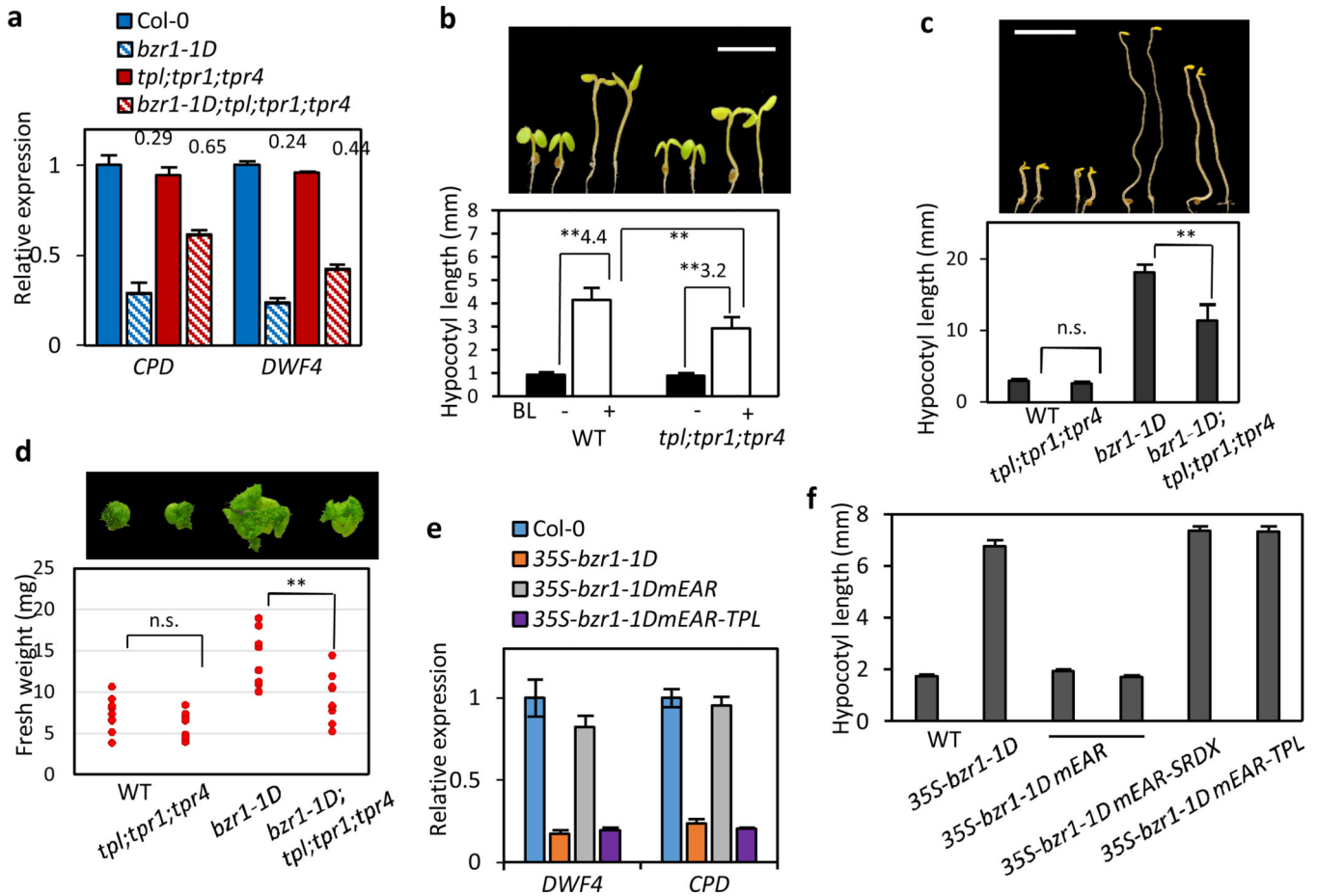
(b) Mutation in the EAR motif abolishes the BZR1-TPL interaction. Core Leu residues of the EAR motif were substituted to Ala. Yeast clones were grown on the synthetic dropout (+His) or synthetic dropout without histidine (-His) plus 1 mM 3AT medium.

(c) BZR1 interacts with TPL and other TPRs. Yeast clones were grown on the synthetic dropout (+His) or synthetic dropout without histidine (-His) plus 5 mM 3AT medium. TPL-N: N-terminal domain of TPL (1-344), TPR1-N : 1-343, TPR2-N : 1-335, TPR3-N : 1-335 and TPR4-N : 1-344.

(d) BZR1 interacts with TPL through EAR motif *in vivo*. *Arabidopsis* mesophyll protoplasts were transfected with *TPL-Myc* together with *bzr1-1D-GFP* or *bzr1-1D EAR-GFP*, and the extracted proteins were immunoprecipitated by anti-GFP antibody. Gel blots were probed with anti-Myc or anti-GFP antibody.

(e) TPL binds to *DWF4* promoter through BZR1. GST-TPL-N (amino acids 1-344) was incubated with a biotin-labelled *DWF4* promoter DNA (400 bps) together with MBP or MBP-BZR1 and pulled down with streptavidin agarose beads. Gel blots were probed with anti-GST or anti-MBP antibody. The full scans of the gel blots (d,e) are shown in Supplementary Fig. 4.

(f) The TPL DNA-binding on the promoters of *CPD* and *DWF4* are enhanced by BR treatment. TPL DNA-binding was determined by ChIP assay using *TPLp::TPL-HA* transgenic plants. One-week seedlings grown on the medium containing 2  $\mu$ M PPZ were treated with mock (M) or 100 nM BL for 4 hr before crosslinking. Error bars indicate the s.d. ( $n=3$ ) and \*\*:  $P<0.01$  by Student's *t*-test.



### Figure 5. TOPLESS is required for BR responses

(a) The BZR1 transcriptional repression activity is compromised in *tpl;tpr1;tpr4*. Total RNA were extracted from etiolated seedlings grown on the medium containing 2  $\mu$ M PPZ. The relative gene expression levels were determined by qRT-PCR. Numbers indicate the ratios of expression levels (*bzr1-1D*/Col-0 or *bzr1-1D;tpl;tpr1;tpr4*/*tpl;tpr1;tpr4*). Error bars indicate the s.d. ( $n=3$ ).

(b) The *tpl;tpr1;tpr4* triple mutant is less sensitive to BR. Seedlings were grown for 5 days on the medium containing either mock or 1000 nM BL. Numbers indicate ratio of hypocotyl lengths (BL-treated / mock-treated). Error bars indicate the s.d. ( $n=10$  plants) and \*\*:  $P<0.01$  by Student's *t*-test. Scale bar: 5 mm.

(c) The *tpl;tpr1;tpr4* triple mutation suppresses the long hypocotyl phenotype of *bzr1-1D*. Seedlings were grown on the medium containing 2  $\mu$ M PPZ for 6 days in the dark. Error bars indicate the s.d. ( $n=10$  plants). n.s.=not significant and \*\*:  $P<0.01$  by Student's *t*-test. Scale bar: 5 mm.

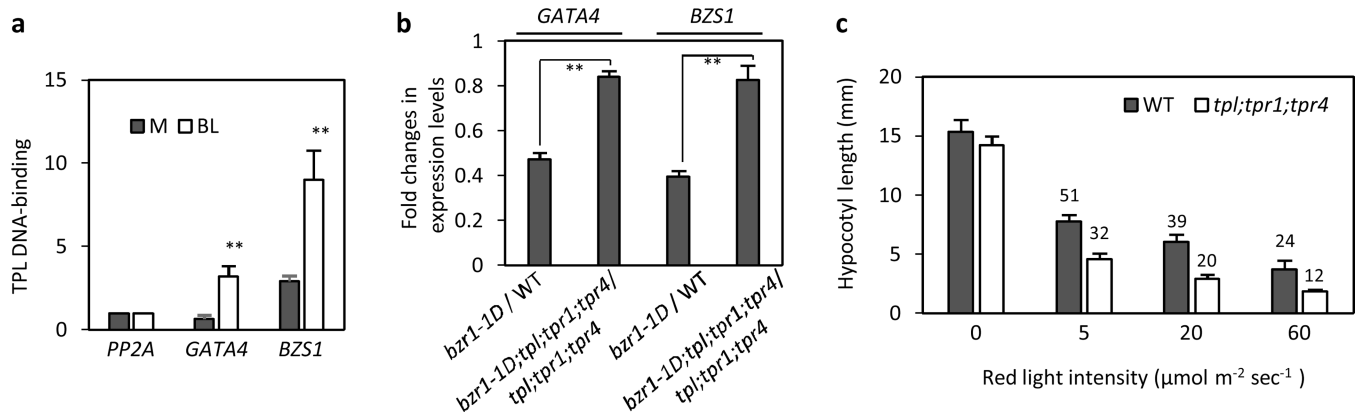
(d) Scatter plot of fresh weight measurements of 3-week-old plants grown on the medium containing 2  $\mu$ M PPZ. This experiment was repeated with similar results. n.s.=not significant and \*\*:  $P<0.01$  by Student's *t*-test ( $n=10$  plants).

(e) TPL fusion at the C-terminus restores the transcriptional repression activity of *bzr1-1DmEAR*. Relative expression fold changes of *CPD* and *DWF4* were determined by

qRT-PCR in *Arabidopsis* plants transformed with the indicated constructs, compared to wild type (Col-0). Error bars indicate the s.e. ( $n=4$ ).

(f) TPL fusion restores the activity of *bzr1-1DmEAR* in promoting hypocotyl elongation. Seedlings were grown in the dark on the medium containing 1  $\mu$ M BRZ for 5 days. Error bars indicate the s.e. ( $n=10$ )



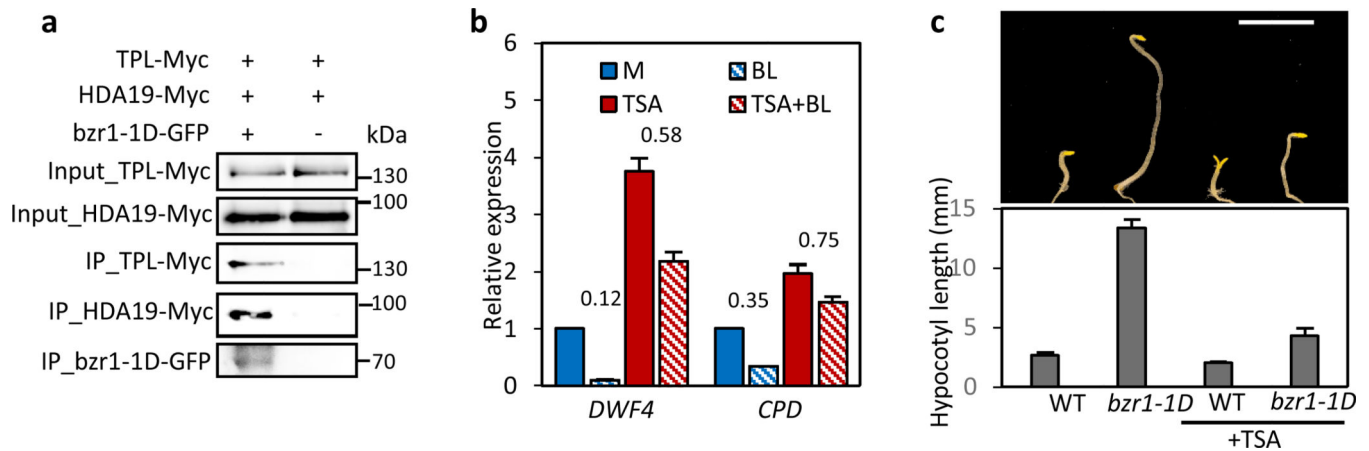


**Figure 6. TPL/TPRs modulate light sensitivity**

(a) TPL binds to the promoters of *GATA4* and *BZS1* in a BR dependent manner. The TPL-DNA binding was determined by ChIP assay using *TPLp::TPL-HA* transgenic plants. One-week old seedlings grown on the medium containing 2  $\mu\text{M}$  PPZ were treated with mock (M) or 100 nM BL for 4 hr before crosslinking. Error bars indicate the s.d. ( $n=3$ ) and \*\*:  $P<0.01$  by Student's *t*-test.

(b) The BZR1 transcriptional repression of *GATA4* and *BZS1* are compromised in the *tpl;tpr1;tpr4* triple mutant. Total RNA were extracted from etiolated seedlings grown on the medium containing 2  $\mu\text{M}$  PPZ. The relative expression fold changes of *GATA4* and *BZS1* were determined by qRT-PCR. Error bars indicate the s.d. ( $n=3$ ).

(c) The *tpl;tpr1;tpr4* seedlings are hypersensitive to red light. Seedlings were grown in various intensities of red light for 5 days. Numbers indicate the percentages of average hypocotyl length relative to that of dark-grown seedling. Error bars indicate the s.d. ( $n=10$ ).

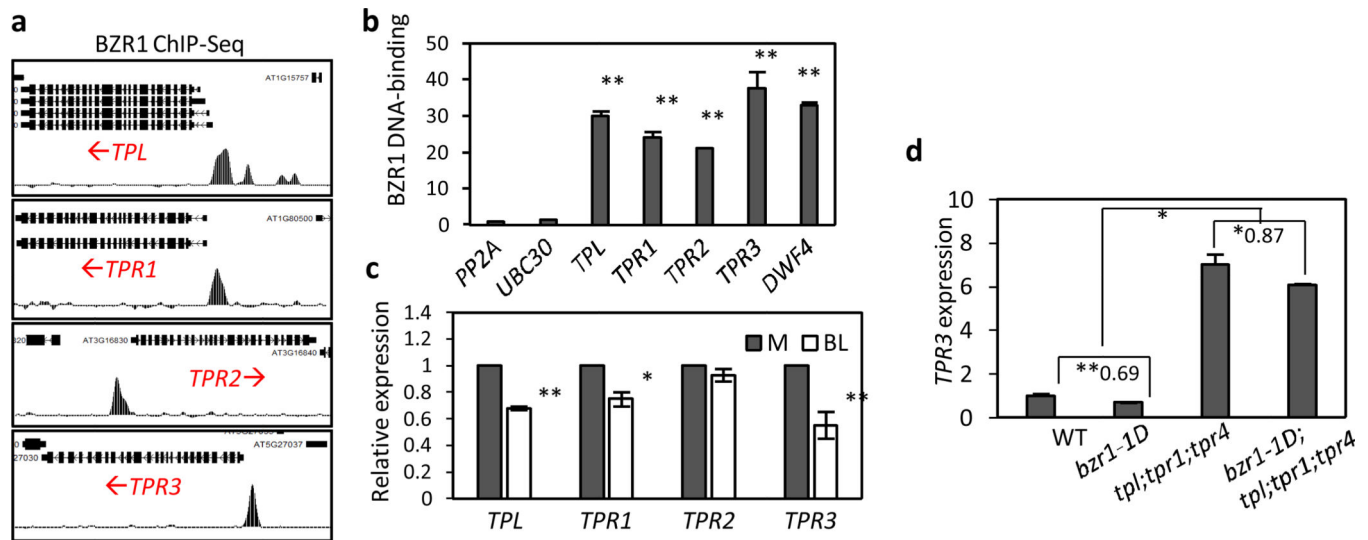


**Figure 7. BR repression of gene expression requires histone deacetylase activity**

(a) BZR1 associates with both TPL and HDA19 *in vivo*. *Arabidopsis* mesophyll protoplasts were transfected with *bzr1-1D-GFP* together with *TPL-Myc* and *HDA19-Myc*, and the extracted proteins were immunoprecipitated by anti-GFP antibody. Gel blots were probed with anti-Myc or anti-GFP antibody.

(b) Histone deacetylase (HDA) inhibitor, trichostatin A (TSA) diminishes BR repressive effect on the expression of *DWF4* and *CPD*. Total RNA was extracted from two-week-old plants treated with 100 nM BL +/- 10  $\mu$ M TSA for 1 hr. The relative gene expression levels were determined by qRT-PCR. Error bars indicate the s.d. ( $n=3$ ). Numbers indicate the ratios of expression levels (BL/M or TSA+BL/TSA).

(c) TSA treatment inhibits BZR1 promotion of hypocotyl elongation. Seedlings were grown on the medium containing 2  $\mu$ M PPZ with or without 1  $\mu$ M TSA in the dark. The error bars in the lower graph indicate the s.d. ( $n=10$  plants). Scale bar: 5 mm.



**Fig. 8. BZR1 represses the expression of *TPL* and *TPRs***

(a) ChIP-Seq analyses shows BZR1 binding on the promoters of *TPL* and *TPRs*. Arrows indicate the direction of transcription.

(b) ChIP assays confirm that BZR1 binds to promoters of *TPL* and *TPRs*. Transgenic plants expressing *bzr1-1D-CFP* from the native *BZR1* promoter were used for the ChIP assays. BZR1-bound DNA was immunoprecipitated using anti-GFP antibody and analyzed by qPCR. BZR1 DNA-binding was calculated as the ratio between *bzr1-1D-CFP* and wild type samples, normalized to that of *PP2A*.

(c) The expression levels of *TPL* and *TPR3* are reduced by BR treatment. Seedlings grown on the medium containing 2  $\mu$ M PPZ medium for 7 days were treated with mock (M) or 100 nM BL for 4 hr.

(d) *TPR3* repression by BZR1 requires *TPL/TPR* activity. Total RNA were extracted from etiolated seedlings grown on the medium containing 2  $\mu$ M PPZ. The relative gene expression levels were determined by qRT-PCR. Numbers indicate the ratios of expression levels (*bzr1-1D* / Col-0 or *bzr1-1D;tpl;tpr1;tpr4* / *tpl;tpr1;tpr4*).

The error bars in (b–d) indicate the s.d. ( $n=3$ ). \*\*:  $P<0.01$  and \*:  $P<0.05$  by Student's *t*-test.