ARTICLE

Received 20 Dec 2013 | Accepted 16 May 2014 | Published 18 Jun 2014

DOI: 10.1038/ncomms5138

Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of *ABI3*

Hojin Ryu^{1,2}, Hyunwoo Cho¹, Wonsil Bae¹ & Ildoo Hwang¹

Seed germination and young seedling establishment should be tightly regulated to maximize plant survival and thereby enable successful propagation. Plants have evolved developmental signalling networks to integrate environmental cues for proper control of these critical processes, in which brassinosteroids are known to attenuate ABA-mediated arrest of early seedling development; however, the underlying regulatory mechanism remains elusive. Here we reveal that a BES1/TPL/HDA19 repressor complex mediates the inhibitory action of brassinosteroids on ABA responses during early seedling development. BR-activated BES1 forms a transcriptional repressor complex with TPL-HDA19, which directly facilitates the histone deacetylation of *ABI3* chromatin. This event leads to the transcriptional repression of *ABI3* and consequently *ABI5*, major ABA signalling regulators in early seedling development. Our data reveal that the BR-activated BES1-TPL-HDA19 repressor complex controls epigenetic silencing of *ABI3* and thereby suppresses the ABA signalling output during early seedling development.

¹Department of Life Sciences, POSTECH Biotech Center, Pohang University of Science and Technology, Pohang 790-784, Korea. ²Division of Agricultural Microbiology, National Academy of Agricultural Science, RDA, Suwon 441-707, South Korea. Correspondence and requests for materials should be addressed to I.H. (email: ihwang@postech.ac.kr).

As sessile plants, the control of early seedling developmental processes, including seed germination and young seedling establishment, is central to the survival and successful propagation of the plant. Owing to high vulnerability to a vast array of stresses in early seedling developmental processes, multiple environmental and intrinsic signalling factors are intricately integrated to finely control these developmental processes. The signalling networks that integrate brassinosteroids (BRs), gibberellin (GA), abscisic acid (ABA) and light signals are important factors in the regulation of seed development, dormancy, germination and post-germinative developmental processes^{1–4}. ABA plays important regulatory roles in maintaining seed dormancy and early seedling developmental arrest checkpoints⁵. *ABI3* is an important regulatory factor for these ABA responses during early seedling development^{6–9}. *ABI3* loss-of-function mutations led to severe ABA insensitivity in seed germination and abnormal embryonic development^{8,10,11}. *ABI3* directly enhanced the expression of *ABI5*, encoding a major ABA signalling transcription factor, during early seedling development¹². Interestingly, *ABI3* mRNA contains a long 5'-untranslated region (UTR) and its partial truncation highly induced *pABI3-GUS* expression in seedlings¹¹. Furthermore, *ABI3* expression was highly increased in histone deacetylases *HDA6/HDA19* RNA interference seedlings or by histone deacetylase inhibitor treatment¹³. These results indicate yet unknown negative regulation on the *ABI3* promoter at the post-transcriptional level, probably by histone modification via *HDA6* and *19*.

The key roles of GA and light signalling in attenuating the ABA responses during early seedling development have long been known⁴, and recent studies have revealed the essential functions of BR signalling pathways in the regulation of GA- and light-mediated plant development^{1,3}. Exogenous BR application rescued the low germination phenotype of GA-related mutants and BR-defective mutants are hypersensitive to ABA during early seedling development and stomatal closure^{14–16}. Furthermore, the biologically active BR (brassinolide and castasterone) and BR biosynthetic gene expression were greatly increased during seed growth and germination of *Pisum sativum* (pea)¹⁷. BR signalling cascades are initiated from the plasma membrane-anchored co-receptors BRI1 and BAK1 (refs 18,19). BR signalling dynamically regulates the activity of BR-related transcriptional factors, including BES1 and BZR1, by modulating the activity of BIN2 kinase and PP2A phosphatase²⁰. Recent studies have revealed the crucial roles of BR in integrating numerous developmental signalling pathways^{20,21}. BR was reported to have antagonist effects on ABA signalling based on the findings that the BR-defective *det2*, *bri1* and *bin2-1* mutants are hypersensitive to ABA during early seedling development^{14,22}. Furthermore, *ABI2*-mediated ABA signalling pathways were shown to negatively control primary BR signalling outputs by facilitating BIN2 activity¹⁶, suggesting that the coordination of BR and ABA signalling is accomplished by the BIN2-regulated transcriptional activity of BES1 or BZR1. However, the molecular mechanisms that integrate BR signalling pathways with ABA signalling in early seedling development are largely unknown.

Here we demonstrate that BR-activated BES1 attenuates ABA sensitivities in early seedling development by recruiting the TPL-HDA19 co-repressor. The TPL-HDA19 complex enabled the nuclear-localized active BES1 to repress *ABI3*, an important regulator in ABA responses during early seedling development, by facilitating histone deacetylation. Based on these observations, we propose that BR signalling cascades repress ABA responses via BES1-TPL-HDA19 co-repressor-mediated epigenetic silencing of *ABI3*.

Results

BES1 specifically inhibits ABA responses. BR-defective *dwf4*, *bri1* and *bin2-1* mutants showed hypersensitivity to ABA, suggesting that the antagonistic roles of BR in ABA signalling pathways could be mediated by BIN2 kinase or its downstream components. As BIN2 inactivates the transcriptional activity of BES1 and BZR1, we initially analysed the ABA sensitivity of *gsk* triple knockout (*bin2 bri1 bil2* loss-of-function), *bes1-D* and *bzr1-1D* mutants^{23–25}, all of which harboured constitutively activated BES1 or BZR1, in seed germination and root growth inhibition of young seedlings. Interestingly, the *bes1-D* and *gsk* triple mutants were less sensitive to ABA during seed germination compared with their wild-type controls, En-2 and Ws-2, respectively. However, the seed germination rate of *bzr1-1D*, a constitutively activated gain-of-function BZR1 allele, was lower than that of Col-0 in the presence of ABA (Fig. 1a). Furthermore, the growth of *bes1-D* roots was barely inhibited by exogenously applied ABA, while *bzr1-1D* and BR-insensitive *bri1* mutants displayed more severe root growth inhibition than the wild-type on exogenous ABA application (Fig. 1b). We next tested the expression levels of representative ABA-responsive genes, including *RD29A*, *RD29B* and *ABI1*, in *bes1-D* and *bzr1-1D* seedlings in the absence or presence of ABA. The expression of ABA-responsive genes was greatly reduced in the *bes1-D* seedlings, but not in the *bzr1-1D* mutant. However, both *bes1-D*- and *bzr1-1D*-expressing plants exhibited lower expression levels of the BR biosynthetic genes, *CPD* and *DWF4* (Supplementary Table 1). Moreover, ABA-induced activation of the ABA-responsive genes was greatly reduced in the *bes1-D* seedlings (Supplementary Table 1). In *bzr1-1D* seedlings, however, *RD29A* and *ABI1* were expressed at similar levels as in wild-type plants, and exogenously applied ABA greatly enhanced the accumulation of *RD29B* (Supplementary Table 1). Furthermore, exogenously applied BR completely suppressed the hypersensitive ABA response of *bzr1-1D*-expressing plants in germination, similar to the wild-type control (Supplementary Fig. 1a). These results suggest that BR-mediated suppression of ABA signalling is specifically controlled by BES1, but not by BZR1, in spite of their similar roles in BR signalling pathways.

Next, we determined the ABA sensitivity of the *bes1* knockout (*bes1^{ko}*) mutant. Although the gain-of-function *bes1-D* plants had a lower sensitivity to ABA, the root growth and seed germination of the *bes1^{ko}* mutant displayed enhanced responses to ABA relative to wild-type plants (Fig. 1c–e), supporting the negative roles of BES1 in ABA signalling pathways. To verify that BES1 is required for BR-mediated suppression of ABA signalling outputs, we determined the effect of BR treatment on the germination of *bes1^{ko}* in the presence of ABA. Interestingly, although the ABA-mediated inhibition of seed germination in wild-type Col-0 was gradually abolished by exogenous BR (epi-BL) treatment in a dose-dependent manner, the effects of ABA on seed germination in *bes1^{ko}* were barely affected by exogenous BR (Fig. 1f). Furthermore, exogenous BR application gradually suppressed ABA-mediated inhibition of early seedling development of wild-type in a BR dosage-dependent manner, but *bes1^{ko}* seedlings were barely responded to exogenous BR for early development in the presence of ABA (Supplementary Fig. 1b). Taken together, we conclude that BES1-mediated BR signalling pathways specifically attenuate ABA signalling during early seedling development.

BR-activated BES1 represses *ABI3* expression. Our results showed that BR signalling pathways attenuated ABA responses through the activity of BES1, and the expression of representative ABA-responsive genes was greatly reduced in *bes1-D* seedlings, suggesting that BES1 negatively regulates the expression of ABA

Figure 1 | BES1-mediated BR signalling specifically inhibits ABA responses. (a) BRs inhibit ABA signalling specifically through BES1 functions, but not BZR1. *bes1-D bzi1-1D gsk* triple mutant seeds were germinated on 1/2 B5 agar plates containing 0, 0.5 or 1 μ M of ABA for 4 days and the germinated seeds (radicle emergence) were counted (error bars indicate s.e.; $n=3$; over 50 seeds were analysed in each independent experiment). Col-0, En-2 and Ws-2 were used as wild-type control. (b) *bes1-D* was less sensitive to ABA in terms of root growth inhibition. Four-day-old seedlings of *bes1-D*, *bzi1-1D* and *bri1* were transferred onto 1/2 B5 agar plate containing 0, 1, 2.5 or 5 μ M ABA. Root length was measured after another 5 days, and relative growth was compared with that of seedlings grown on ABA-free medium (error bars indicate s.e. $n>30$). (c,d) *bes1* knockout (*bes1^{ko}*) mutants are hypersensitive to ABA in seed germination. Seven-day-old seedlings of *bes1^{ko}* grown on 1/2 B5 medium containing 1 μ M ABA (d). *bes1^{ko}* seeds were germinated on 1/2 B5 agar plates containing 0, 0.5 or 1 μ M ABA for 3 days and the germinated seeds (radicle emergence) were counted (d); error bars indicate s.e.; $n=3$; over 50 seeds were tested in each independent experiment). (e) ABA-mediated inhibition of root growth was increased in *bes1^{ko}*. Four-day-old seedlings of *bes1^{ko}* and Col-0 were transferred onto 1/2 B5 agar plates containing 0, 1, 2.5 or 5 μ M ABA. Root length was measured after another 5 days and growth was compared with that on ABA-free medium (error bars indicate s.e.; $n>30$). (f) The inhibitory effects of BRs on ABA responses during seed germination were rarely observed in the *bes1^{ko}* mutant. Seed germination rates of the wild type and *bes1^{ko}* grown on 1 μ M of ABA with 0, 1, 10, 50 and 100 nM epi-BL 4 days after plating (error bars indicate s.e.; $n=3$; over 100 seeds were tested in each independent experiment). Asterisks indicate statistically significant differences analysed by Student's *t*-test (* $P<0.05$, ** $P<0.01$).

signalling-related genes. Recent studies also reported that BES1 represses the expression of several target genes^{26–28}. To test this idea, we determined the expression levels of ABA signalling-related genes in a gain-of-function *bes1-D* and *bes1^{ko}* plants using real-time quantitative reverse transcription-PCR (qRT-PCR). Interestingly, the expression of major transcription factors of the ABA signalling pathway, including *ABFs*, *ABI3* and *ABI5*, was greatly decreased in *bes1-D* seedlings (Fig. 2a); however, *ABF1*, *ABI3* and *ABI5* were upregulated in *bes1^{ko}* seedlings (Fig. 2b). Notably, *ABI3* and *ABI5*, central ABA signalling components in the regulation of early seedling development, were upregulated in *bes1^{ko}* seedlings (Fig. 2b). As the expression of *ABI5* is enhanced by *ABI3* (ref. 12), we propose that BR-activated BES1 regulates the *ABI3*-*ABI5*-mediated ABA signalling module. Consistently, exogenously applied BR decreased the concentration of *ABI3* transcripts in wild-type seedling in a time- and dose-dependent manner, but the expression of *ABI3* barely changed in *bes1^{ko}* in response to BR (Fig. 2c). These results suggest that BES1 has an important role in the BR-mediated repression of *ABI3*.

We next investigated how BR-activated BES1 repressed *ABI3*. Recently, EAR (ethylene-responsive element binding

factor-associated amphiphilic repression) motif-mediated transcriptional repression mechanisms have been reported, and many transcriptional repressors use this mechanism to regulate plant developmental programmes^{29,30}. In the primary structure of BES1, we identified a putative EAR motif (LxLxL) (Fig. 2d) essential for recruiting Groucho/Tup1-like transcriptional co-repressors, such as TPL, SAP18 and SIN3 (refs 31,32). To verify that BES1 interacts with transcriptional co-repressors via the EAR motif, we performed a yeast two-hybrid assay with BES1 and its EAR mutant (mEAR; Fig. 2d, upper). Interestingly, TPL interacted with *bes1-D* and BES1, as well as a positive control IAA3 (ref. 30), but mutations of the EAR motif of BES1 or a dominant negative *tpl-1* (N176H) mutation disrupted these protein interactions in yeast (Fig. 2d and Supplementary Fig. 2a,b). However, SAP18 and SIN3 were not associated with *bes1-D* or BES1 (Fig. 2d and Supplementary Fig. 2a). The *Arabidopsis* genome has four TPL-RELATED genes (*TPR1–4*). We found that *TPR2* could also interact with BES1 but not *TPR3*, and the amino-terminal domain of TPL, *TPR1*, *TPR2* and *TPR4* was enough to bind with BES1 (Supplementary Fig. 2c,d). These imply that TPL and its closely related TPRs might have

Figure 2 | BES1-TPL repressor complexes are involved in the BR-mediated repression of *ABI3*. (a,b) The expression of ABA-related transcription factors is regulated by BES1. The expression levels of *ABF1-4*, *ABI3*, *ABI4* and *ABI5* in 4-day-old *bes1-D* (a) and *bes1-ko* seedlings (b) were determined by real time qRT-PCR (error bars indicate s.e.; $n = 4$). (c) BR treatment downregulates *ABI3* expression. The expression levels of *ABI3* in 4-day-old Col-0 and *bes1-ko* seedlings treated with 1 μ M epi-BL for 1, 2 and 3 h were determined by real-time qRT-PCR (error bars indicate s.e.; $n = 4$). (d) BES1 interacts with TPL via its EAR motif, but not with SIN3 or SAP18. Core residues of the EAR motif sequence of BES1 are indicated in red (L $\times$ L $\times$ L) and the sequence is aligned with that of mutated EAR (three core Leu substitute to Ala, mEAR; upper). Yeast cells transformed with the indicated gene sets were selected in synthetic medium lacking Leu, Trp (-LT) or His (-LTH), but containing 1 mM 3AT. (e) *bes1-D-HA* proteins co-immunoprecipitated with FLAG-tagged TPL. Mesophyll protoplasts were transfected with *bes1-D-HA* and *bes1-DmEAR-HA* with TPL-FLAG, incubated for 6 h and then pulled down with anti-FLAG antibodies. *bes1-D* proteins were detected with anti-HA antibody. (f) TPL interacts with BES1 in the nucleus. BiFC (bimolecular fluorescence complementation) assay using TPL-nV (nVenus) and cV (cVenus)-tagged *bes1-D*. Scale bar, 50 μ m. Asterisks indicate statistically significant differences analysed by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$).

functional redundancy in the formation of the co-repressor complex with BES1. We next investigated the protein interactions between TPL and BES1 by *in vivo* co-immunoprecipitation assays. The wild-type BES1 proteins are highly phosphorylated and mostly localized at cytosols in plants³³, but TPL proteins are mainly localized in the nucleus³⁰. Furthermore, both phosphorylated and dephosphorylated BES1 proteins interacted with TPL-N (Supplementary Fig. 3a). Therefore, for the *in vivo* interaction assay, we used the constitutively activated *bes1-D* proteins, which are mostly dephosphorylated and located in the nucleus. Whereas *bes1-D-HA* proteins were pulled down by TPL-FLAG, *bes1-DmEAR* proteins were not (Fig. 2e). We also found that *bes1-D* and TPL interacted in the nucleus (Fig. 2f). Finally, BR enhanced the interaction of BES1 with TPL in the nucleus (Supplementary Fig. 3b). These results indicate that BES1-TPL/TPR1/2/4 could form a transcriptional co-repressor complex that plays important roles in BES1-mediated *ABI3* repression.

BES1-TPL complex regulates both BR and ABA responses. To further explore whether the BES1-TPL complex is involved in the BR-mediated suppression of ABA responses, we generated 35S-*bes1-DmEAR-HA* lines and examined ABA-related seed germination and root growth inhibition (Fig. 3a,b and Supplementary Fig. 4). We selected four independent lines in which *bes1-DmEAR-HA* proteins were highly accumulated (Supplementary Fig. 4a). Interestingly, the 35S-*bes1-DmEAR-HA* lines did not show any BR-related phenotypes, including curled rosette leaves and brassinazole resistance in hypocotyl elongation, which were generally displayed in *bes1-D*-overexpressing plants (Supplementary Fig. 4b,c). However, the BR-related phenotypes were completely recovered in *bes1-DmEAR* by its protein fusion with SRDX (the EAR repression domain), TPL or TPL-binding HDA19 at the carboxy terminus (Supplementary Fig. 5a,b). These results indicate that the formation of repressor complex with TPL/HDA19 is essential for the BES1-mediated regulation of BR-related plant growth and development. We next investigated

Figure 3 | BES1-TPL co-repressors function in the BR-mediated inhibition of ABA signalling. (a,b) The EAR motif of BES1 is required for repressing ABA responses. Four-day-old seedlings of Col-0, 35S-*bes1-D* and 35S-*bes1-D*-mEAR (lines 1 and 2) were transferred onto 1/2 B5 agar plates containing 0, 2.5 or 5 μM ABA. Scale bar, 10 mm. (a) Col-0, 35S-*bes1-D* and 35S-*bes1-D*-mEAR (1 and 2) mutant seeds were germinated on 1/2 B5 agar plates containing 0, 0.5 or 1 μM ABA for 4 days and the germinated seeds (radicle emergence) were counted (b); error bars indicate s.e.; $n = 3$; over 50 seeds were tested in each independent experiment). (c,d) A dominant negative *tpl-1* mutant was hypersensitive to ABA in terms of root growth inhibition. Four-day-old seedlings of *tpl-1* and the wild type (Ler) were transferred onto 1/2 B5 agar plates containing 0, 5 or 10 μM ABA. Scale bar, 10 mm. (c) Root length was measured after another 5 days and growth was compared with that on ABA-free medium (d), error bars indicate s.e.; $n > 60$). (e) Germination of the *tpl-1* mutant scarcely responds to BRs in the presence of ABA. Seed germination rates of the wild type and *tpl-1* mutants grown on 1 μM ABA with 0, 10, 50 and 100 nM epi-BL 4 days after plating (error bars indicate s.e.; $n = 3$; over 100 seeds were tested in each independent experiment). Asterisks indicate statistically significant differences analysed by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$).

the ABA responses in 35S-BES1-HA, 35S-*bes1-D*-mEAR, 35S-*bes1-D*-mEAR-TPL and 35S-*bes1-D*-mEAR-HDA19 lines. Interestingly, although 35S-*bes1-D* was less sensitive to ABA in terms of seed germination and root growth inhibition than wild-type or 35S-BES1-HA plants, mutation of the EAR motif of *bes1-D* disrupted ABA tolerance (Fig. 3a,b and Supplementary Fig. 4d). Consistent with activated BR responses, the 35S-*bes1-D*-mEAR-TPL and 35S-*bes1-D*-mEAR-HDA19 lines showed less sensitivity to ABA in root growth inhibition, similar to *bes1-D* mutant, than Col-0 (Supplementary Fig. 5c). Consistently, the expression of *ABI3*, *ABI5* and *AtEm6* was reduced in *bes1-D*-mEAR-HDA19-over-expressing plants but upregulated in the *bes1-D*-mEAR-over-expressing lines compared with wild-type plants. Moreover, BR biosynthesis-related *CPD* and *DWF4* were also significantly reduced in *bes1-D*- and *bes1-D*-mEAR-HDA19-overexpressing plants (Supplementary Fig. 5d). Taken together, we conclude that BES1 represses *ABI3* expression through the EAR-mediated recruitment of the TPL-HDA19 co-repressor.

To decipher the functions of the BES1-TPL complex in the BR-mediated suppression of ABA signalling outputs, we tested

the ABA responses during early seedling development in a semi-dominant negative *tpl-1* mutant. As expected, compared with wild-type plants, the root elongation of *tpl-1* seedlings was significantly reduced by exogenously applied ABA (5 and 10 μM; Fig. 3c,d). Moreover, the germination rate of *tpl-1* was significantly lower than in the wild type in the presence of 1 μM ABA (Fig. 3e), suggesting that TPL functions are negatively associated in ABA signalling pathways. To examine whether TPL functions were connected with the BR-mediated suppression of ABA signalling, we determined the effect of BR application on the germination of *tpl-1* in the presence of ABA. In the presence of 1 μM ABA, the inhibitory effect of ABA on the germination of wild-type seeds was rescued by exogenously applied BR in a dose-dependent manner. However, the BR-mediated inhibition of the effects of ABA on seed germination was barely observed in *tpl-1* (Fig. 3e). Consistently, *ABI3* and *ABI5* transcripts were highly accumulated in *tpl-1* seedlings compared with wild-type (Supplementary Fig. 6). Together, these results suggest that TPL-mediated repression of its target genes is involved in the coordination of BR and ABA signalling pathways in early seedling development.

BES1-TPL-HDA19 co-repressors directly target *ABI3* chromatin. To elucidate whether the BES1-TPL complex could directly repress *ABI3*, we carried out a chromatin immunoprecipitation (ChIP) assay using a *pBES1-bes1-D-HA* transgenic line. We found several BES1-binding E-box sequences (CAnnTG)²⁸ in a ~3-kb upstream region of the *ABI3* promoter containing the 5'-UTR region (Fig. 4a, upper green ovals and Supplementary Fig. 7). The *bes1-D-HA* proteins strongly bound to BES1-binding elements (E-box) on the 5'-UTR (6) and a promoter region (1) of the *ABI3* locus, as well as the *SAUR-AC1* promoter region used as a positive control (Fig. 4a). However, we did not observe any

differences in the DNA binding of *bes1-D* and *bzr1-1D* to the other tested promoter regions (2–5; Fig. 4a). We further confirmed the BES1 binding to 1 and 6 regions of the *ABI3* promoter by electrophoretic mobility shift assays (Supplementary Fig. 8). These results indicate that BES1 could directly repress *ABI3* transcription by binding to the 5'-UTR and a promoter region. In agreement with these results, it was previously reported that the 5'-UTR region of *ABI3* is essential for the negative regulation of its transcription¹¹. However, we could not find the direct binding of BES1 to the *ABI5* promoter (Supplementary Fig. 9). We next tested whether the BES1-binding E-box motifs in

Figure 4 | BR decreases histone H3 acetylation on the *ABI3* locus via BES1-TPL-HDA19 co-repressor complexes. (a) BES1 directly binds to the 5'-UTR and promoter of *ABI3*. ChIP assays of the *ABI3* promoter and 5'-UTR were performed with anti-HA antibody. Schematic structure of the *ABI3* promoter (3 kb). Putative E-Boxes (CAnnTG) and the 5'-UTR are marked with green ovals and yellow box, respectively (upper panel). The fold enrichments are presented as relative levels to an internal control, *35S-bzr1-1D-HA* (error bars indicate s.e.; $n = 3$). The *SAUR-AC1* promoter was used as a positive control. (b) BR treatment represses *GUS* expression driven by wild-type *ABI3* promoter, but not by mutated *ABI3* promoter. Three-day-old seedlings of *pABI3-GUS* (upper) and *pABI3m-GUS* (lower) lines incubated with (+ epi-BL) or without 1 μ M BR (-epi-BL) for 3 h were stained with *GUS* solution. Scale bar, 200 μ M. (c) BR barely suppress ABA responsiveness of *abi3-8 pABI3m-gABI3-FLAG* lines in seed germination, but that of *abi3-8 pABI3-gABI3-FLAG* lines. Seed germination (radicle emergence) rates were determined in the wild-type (Col-0), *abi3-8 pABI3-gABI3-FLAG* and *abi3-8 pABI3m-gABI3-FLAG* line grown on 0 (Mock) or 1 μ M ABA with 0 (ABA) or 100 nM epi-BL (ABA + BL) for 3 days after plating (error bars indicate s.e.; $n = 3$; over 100 seeds were tested in each independent experiment). (d) Trichostatin A (TSA) inhibits the BR-mediated repression of *ABI3*. The expression levels of *ABI3* in 4-day-old seedlings treated with 1 μ M epi-BL for 3 h after pretreatment of 5 μ M TSA for 24 h were determined by real-time qRT-PCR (error bars indicate s.e.; $n = 4$). (e) ChIP analysis of the acetylation states of histone H3 in the *ABI3* locus of wild-type and *bes1-D* plants. ChIP assays using an anti-acetyl-histone H3 antibody were performed, and the fold enrichments were normalized with the En-2 control (error bars indicate s.e.; $n = 3$). (f) BR treatment decreases the acetylation of histone H3 in the *ABI3* locus. ChIP assays using an anti-acetyl-histone H3 antibody were performed on 4-day-old wild-type seedlings treated with or without 1 μ M epi-BL for 3 h, and the fold enrichments were normalized with mock treatment control (error bars indicate s.e.; $n = 3$). Asterisks indicate statistically significant differences analysed by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$).

the *ABI3* promoter is required for BR-mediated repression of *ABI3*. A mutation of E-box motifs on either 1 or 6 regions could not block the *bes1-D*-mediated repression of *pABI3-LUC* activity⁴, but double mutations of the E-box motifs on both 1 and 6 regions (*pABI3m-LUC*) abolished the repression of the *ABI3* promoter activity by *bes1-D* (Supplementary Fig. 10a). We then generated *GUS*-expressing transgenic plants under the control of either wild-type *ABI3* (*pABI3-GUS*) or mutated *ABI3* promoter (*pABI3m-GUS*). Exogenous ABA induced *GUS* expression of both *pABI3-GUS* and *pABI3m-GUS* lines (Supplementary Fig. 10b). Interestingly, however, exogenous BR repressed *GUS* expression of the *pABI3-GUS* transgenic lines, but not of the *pABI3m-GUS* lines (Fig. 4b and Supplementary Fig. 10c,d). Conversely, the expression of endogenous *CPD* and *ABI3* was reduced by BR treatment in both *pABI3-GUS* and *pABI3m-GUS* lines (Supplementary Fig. 10c,d). To further investigate whether BES1-mediated repression of *ABI3* is functionally important for BR action on ABA signalling, we generated *pABI3-gABI3-FLAG* and *pABI3m-gABI3-FLAG* transgenic lines in the ABA-insensitive *abi3-8* mutant background³⁴. We selected two independent *abi3-8 pABI3-gABI3-FLAG* and *pABI3m-gABI3-FLAG* transgenic lines in which the ABA insensitiveness of *abi3-8* in seed germination was fully restored to wild-type level (Supplementary Fig. 11a). However, ABA inhibition of seed germination in the *abi3-8 pABI3m-gABI3-FLAG* lines was barely suppressed by exogenous BR, but in *abi3-8 pABI3-gABI3-FLAG* lines and wild-type control (Fig. 4c and Supplementary Fig. 11b). These results indicate that BR specifically targets *ABI3* expression via BES1, which is critical for determining ABA responsiveness during early seedling development.

Several prior studies suggested that RPD3-like histone deacetylase 19 (HDA19) was directly recruited to TPL to actively repress gene expression^{35,36}. To test whether the activity of HDA19 is responsible for the BES-mediated *ABI3* repression, we determined the effects of Trichostatin A, a histone deacetylase-specific inhibitor, on BR-induced *ABI3* repression. As shown in Fig. 4d, BR treatment significantly reduced *ABI3* expression, but this effect was completely abolished by Trichostatin A treatment. HDA19 activity is an important regulator of histone H3 and H4 acetylation in plants^{35,37}, indicating that BR could decrease histone acetylation states on the *ABI3* chromatin via the BES1-recruited TPL-HDA19 co-repressor complex. To test this idea, we performed a ChIP assay using an anti-acetyl-Histone H3 antibody in *bes1-D* and BR-treated wild-type and *bes1^{ko}* seedlings. The histone H3 acetylation states in the BES1-binding regions of the *ABI3* promoter (1 and 6) and the first exon region (7) were greatly reduced in the *bes1-D* and BR-treated wild-type seedlings compared with controls (Fig. 4e,f). However, BR-mediated reduction in histone H3 acetylation states of the *ABI3* promoter was mostly impaired in *bes1^{ko}* seedlings (Supplementary Fig. 11c). Together, these results imply that BR directly induces the epigenetic silencing of *ABI3* via the BES1-TPL complex.

BR signalling is directly linked to the *ABI3-ABI5* module.

To confirm the BES1-mediated epigenetic silencing of *ABI3* in plants, we overexpressed HA (haemagglutinin)-tagged *ABI3* or its downstream target, *ABI5*, in the *bes1-D* background and determined the ABA responses in seed germination and root growth inhibition. We first tested the expression levels of *ABI3-HA* and *ABI5-HA* proteins in the *bes1-D* transgenic lines using an anti-HA antibody (Fig. 5a and Supplementary Fig. 12a). We selected four and two independent *ABI3-HA* (3–6) and *ABI5-HA* (12 and 18) overexpressing lines, respectively, among several T3 homozygous plants. As expected, the overexpression of *ABI3* or *ABI5*

completely abolished the reduced ABA sensitivity phenotype of *bes1-D* plants in seed germination and root growth inhibition (Fig. 5b–d and Supplementary Fig. 12b). Especially, the ABA sensitivity of *ABI3-HA*-overexpressing *bes1-D* lines in seed germination was highly correlated with the *ABI3-HA* expression levels (Fig. 5b). Moreover, in the root growth inhibition assay, the *bes1-D 35S-ABI3-HA* transgenic seedlings (lines 5 and 6) were hyper-responsive to ABA treatment, while the ABA response was greatly reduced in *bes1-D* plants (Fig. 5b–d). However, the weak *ABI3-HA*-expressing plants (lines 3 and 4) revealed milder ABA sensitivity than the wild type, but their responses to ABA in the root growth inhibition assay were more sensitive than those of *bes1-D* seedlings (Fig. 5d). These results fully support the idea that BES1-mediated ABA repression occurs through the suppression of *ABI3/ABI5*-mediated ABA signalling modules.

BR signalling-defective mutants displayed enhanced responses to ABA in seed germination and root growth inhibition of young seedlings (Fig. 1). To determine if BRI1-triggered BR signalling pathways genetically interact with *ABI3/ABI5*-mediated ABA signalling modules, we crossed the *bri1* knockout mutant to the loss-of-function *abi3-8* and *abi5-7*. Interestingly, the enhanced ABA sensitivity of *bri1* in seed germination was completely suppressed by *abi3-8* and *abi5-7* mutations (Fig. 5e and Supplementary Fig. 12c). Furthermore, the *abi3-8* or *abi5-7* mutation significantly reduced the enhanced ABA responses of *bri1* in root growth inhibition (Fig. 5f and Supplementary Fig. 12d). Finally, we found that *DWF4*-overexpressing seeds (*dwf4-D*) were also resistant to a GA biosynthetic inhibitor, paclobutrazol, during germination (Supplementary Fig. 13). These results support that the ABA signalling module via *ABI3* and *ABI5* is directly regulated by BR signalling pathways. Taken together, we propose that BR-activated BES1 signalling pathway reduces the expression of *ABI3* and consequently its downstream target, *ABI5*, a key ABA signalling component that inhibits seed germination and young seedling growth (Supplementary Fig. 14).

Discussion

The regulation of plant seed germination and young seedling establishment has both ecological and economic importance⁴. Diverse environmental factors, including light quality, water availability and temperature, are co-ordinately integrated into hormonal regulatory pathways that control early seedling development. Light signals induce the transition from heterotrophic to autotrophic growth of young seedlings by inhibiting skotomorphogenesis. In particular, BR is an essential factor for promoting the skotomorphogenic developmental programmes of germinated seedlings²⁰. Furthermore, BR has recently been shown to have important roles in light-, GA- and temperature-mediated regulation of plant development^{38,39}, suggesting the crucial biological relevance of BR in coordinating diverse signalling cues during early seedling development. In this study, we demonstrate the key roles of BES1-mediated BR signalling pathways in modulating the ABA responses during early seedling development. As germinated embryos and young seedlings are so vulnerable to variable environmental conditions, germination and stress tolerance of seedlings are expected to be regulated by elaborate mechanisms. The stress hormone ABA has long been considered as a major factor in the regulation of seed dormancy and stress tolerance. Indeed, under unfavourable conditions, such as desiccation and high salinity, ABA activity is greatly elevated in young seedlings, which maximizes stress tolerance, but arrests the seed-to-young seedling transition⁵. This growth checkpoint is known to be affected by the transcription factor *ABI5*, which is accumulated in response to *ABI3*, *ABI4* and *DWA1/2* (refs 40,41), which confer not only osmo-tolerance to

Figure 5 | BES1-mediated BR signalling is integrated into the ABI3-ABI5 module of ABA signalling. (a) ABI3 levels in ABI3-HA-overexpressing *bes1-D* lines (3–6). The protein expression levels of ABI3-HA in *bes1-D* 35S-ABI3-HA lines were detected with an anti-HA antibody. Actin was used as a loading control. (b) Ectopic expression of ABI3 eliminates the reduced sensitivity of *bes1-D* to ABA. En-2, *bes1-D* and three independent *bes1-D* 35S-ABI3-HA transgenic seeds (3, 4 and 6) were germinated on the 1/2 B5 agar plates containing 0, 0.5 or 1 μ M ABA for 4 days and the germinated seeds (radicle emergence) were counted (error bars indicate s.e.; $n = 3$; over 50 seeds were tested in each independent experiment). (c,d) Ectopic expression of ABI3 suppresses the ABA-insensitive root phenotype of *bes1-D*. Four-day-old 35S-ABI3-HA (3–6), *bes1-D* and En-2 seedlings were transferred to 1/2 B5 agar plate containing 0, 1, 2.5 or 5 μ M ABA. Representative 35S-ABI3-HA transgenic (5 and 6), *bes1-D* and En-2 seedlings grown on the ABA containing media for 5 days. (c) Five days later, root length was measured and relative growth was compared with that of plants grown on ABA-free medium (d), error bars indicate s.e.; $n > 30$). (e) The ABA hypersensitivity of *bri1* in seed germination was completely suppressed by the *abi3-8* or *abi5-7* mutation. Five-day-old *bri1 abi3-8* and *bri1 abi5-7* double mutant seedlings grown on 1/2 B5 medium containing 1 μ M ABA. (f) ABA-mediated root growth inhibition assay using *bri1 abi3-8* and *bri1 abi5-7* seedlings. Four-day-old Col-0, *bri1*, *bri1 abi3-8* and *bri1 abi5-7* seedlings were transferred onto 1/2 B5 agar plates containing 0, 1 or 2.5 μ M ABA. Root length was measured after a further 5 days and relative growth was compared with that of plants grown on ABA-free medium (error bars indicate s.e.; $n > 30$). Asterisks indicate statistically significant differences analysed by Student's *t*-test (** $P < 0.01$).

the germinated young seedlings, but also upregulate a number of critical genes that arrest the growth of the germinated seedlings¹². The expression of *ABI5* is upregulated by *ABI3*, and *ABI3* itself can also directly regulate the expression of key negative regulators involved in the seed-to-seedling transition⁴². These results indicate that the *ABI3-ABI5* signalling module acts as a central negative regulator of early seedling development, and that factors that promote the seed-to-seedling transition should attenuate *ABI3/ABI5*-mediated ABA signalling module. Consistently, the drastic downregulation of *ABI3* and *ABI5* was observed on seed imbibition and the loss-of-function mutation of *ABI3* and *ABI5* disrupted ABA-mediated young seedling growth arrest^{5,12}. Our work provides compelling evidence for the crucial roles of BES1 in the BR-mediated suppression of ABA responses via direct repression of the *ABI3-ABI5* signalling module in early seedling development. BR-activated BES1 directly binds to the *ABI3* promoter, but not to the *ABI5* promoter, and represses its expression by reducing histone acetylation at *ABI3* chromatin by recruiting TPL-HDA19 co-repressor complexes (Figs 2–4).

BRs promote cell elongation and inhibit photomorphogenesis, and these physiological features are also commonly regulated by GA. There are multiple overlaps in the gene sets regulated by BR and GA, and in the biological effects of these two hormones, and the BR-regulated transcriptional activity of BZR1 and BES1 is known to be required for proper GA responses in skotomorphogenic seedlings^{1,3}. In addition, similar to GAs, BRs play negative roles in ABA-inhibited early seedling development^{14–16}, and exogenous BR application or overexpression of BR biosynthetic *DWF4* can overcome the low seed germination phenotype of GA-defective mutants⁴³. Furthermore, central negative regulators of GA signalling, DELLA proteins, could directly inhibit the transcriptional activity of BES1 and BZR1, and BR activity was required for GA-mediated cell elongation^{1,3}. Collectively, these results suggest that GA signalling is integrated with the BES1-mediated repression of *ABI3*, to stimulate early seedling development. In addition, exogenous BR application dramatically increased tolerance to diverse abiotic stresses^{44,45}, indicating that the increased BR activity during early seedling development could promote seedling hardiness without arresting growth.

BES1 and BZR1 repress several target genes, including BR biosynthetic genes, light signalling components and chloroplast development-related genes^{46,47}. However, it has been an open question as to how these two transcription factors repress their target genes. Epigenetic reprogramming of gene expression through DNA methylation, histone modification and chromatin remodelling without the alteration of nucleotides provides a flexible means of regulating gene expression and coordinating developmental programmes in eukaryotic organisms⁴⁸. Plant-specific EAR motif-mediated epigenetic transcriptional repression has recently emerged as a key mechanism for modulating numerous hormonal, stress and developmental signalling pathways²⁹. In this study, we show that the repressor activity of BES1, which is mediated by TPL-HDA19 co-repressor complexes, is essential for BR-mediated plant growth and development (Figs 2 and 3 and Supplementary Figs 2–5). Interestingly, the EAR motif is evolutionarily conserved in all BR-related transcription factors, including BZR1 and BEH1-4 of *Arabidopsis* and other plant species. This suggests that TPL-mediated epigenetic histone remodelling mechanisms have critical roles in BR-mediated gene regulation and plant development. Histone methylation is frequently followed by deacetylation, which condenses chromatin into a structure that is highly repressive to gene transcription⁴⁹. Interestingly, TPL could also interact with histone methylation-related chromatin remodellers, including SUVH3 (SUV39H-like protein) and PKR1 (PICKLE homologue)²⁹. Thus, the BR-related

transcription factors can also use diverse chromatin remodelling mechanisms to repress their target genes. However, BES1, together with its binding partner, can also act as a transcriptional activator^{28,50}. BES1 can directly increase the demethylation of several target chromatin, including *TCH4*, by recruiting two Jumonji family histone demethylases, ELF6 (Early flowering 6) and REF6 (Relative of early flowering 6). The *elf6* and *ref6* loss-of-function mutations resulted in a semi-dwarf phenotype and enhanced the growth defects of *bri1-5* (ref. 50). Moreover, recent studies showed that BR and *ELF6* affected flowering time^{51,52}, indicating that by recruiting ELF6 and REF6, and functioning as a transcriptional activator, BES1 coordinates flowering and BR signalling pathways. BR signalling pathways have important roles in plant morphogenesis and many signalling stimuli are integrated into BR-mediated plant development²⁰. Therefore, the context-dependent switching of BES1 and its homologues between transcriptional activators and repressors plays fundamental roles in the BR-dependent coordination of multiple signalling networks. The ubiquitous expression of BES1 (ref. 25) suggests that the switching of BES1 properties is achieved by the tissue-specific expression or post-transcriptional/translational modification of its binding factors, such as TPL, ELF6 and REF6. The context-dependent regulation of gene expression in multicellular organisms imparts biological diversity and specificity to transcription factors, which may aid in accomplishing developmental programmes. It will be very interesting to determine how these multiple chromatin remodelling mechanisms facilitate BR-mediated plant growth and development.

Methods

Plant materials and growth conditions. *Arabidopsis thaliana* ecotype Col-0, En-2, Ler and WS-2 were used as wild-type controls and as the genetic backgrounds of transgenic lines. *Arabidopsis* seeds were germinated in media (pH 5.7) containing 1/2 × Gamborg B5 salts (Duchefa, Haarlem, The Netherlands), 1% sucrose and 0.8% phytoagar under long-day conditions (16-h light/8-h dark cycles) at 22–24 °C. The mutant seeds were kindly provided by Dr Sunghwa Choe at the Seoul National University (*bes1-D*, *bzr1-ID*, *dwf4-D* and *bri1*), Dr Jianming Li at the University of Michigan (*gsk* triple), Dr Zhi-Yong Wang at the Carnegie Institution for Science (*bes1* knockout) and Dr Eiji Nambara at University of Toronto (*abi3-8* and *abi5-7*). *tpl-1* seeds (CS65909) were provided by The Arabidopsis Biological Research Center. To generate *bri1 abi3-8* (L298F) or *bri1 abi5-7* (W75 Stop) mutants, genetic crosses were performed and double mutants were confirmed by DNA sequencing with gene-specific primer sets (Supplementary Table 2).

Protein-protein interaction assay. To identify interactions between BES1 and co-repressors (TPL, TPR2, TPR3, SIN3 or SAP18), AH109 yeast strains were co-transformed with *pGBKT7*-co-repressors (*TPL*, *tpl-1*, *TPR2*, *TPR3*, *SIN3* or *SAP18*) and *pGADT7-BES1* (*bes1-D*). For interaction test of BES1 with N-terminal domain of TPL, TPR1, TPR2 or TPR4, 600 bp of the coding region of each gene was cloned into the *pGBKT7* vector. Clones showing positive interactions were selected on synthetic medium containing 1 mM 3-aminotriazole without Leu, Trp and His. For the bimolecular fluorescence complementation and co-immunoprecipitation assays, *TPL*, *BES1* or *bes1-D-mEAR* was cloned into a plant expression vector containing nV (nVenus, 1–157 amino acids), cV (cVenus, 158–238 amino acids), HA, myc or FLAG tags in the C terminus driven by the 35S C4PPDK promoter as previously described^{53,54}. The *Venus* gene (NCBI accession number BAJ39289) for bimolecular fluorescence complementation assay was provided by Professor Inwhan Hwang at POSTECH. For protoplasts transient expression assays in protoplasts, 4 × 10⁴ protoplasts were transfected with 20–40 µg of plasmid DNA purified by CsCl-gradient ultracentrifugation and then incubated under constant light at 23 °C for 6 h. For BR treatment, the transfected protoplasts with *BES1-cV* and *TPL-nV* were further incubated for 3 h in the presence of 1 µM of epi-BL. The complemented Venus fluorescence was observed using a fluorescence microscope (Axioplan, Carl Zeiss MicroImaging, Inc.). For co-immunoprecipitation, total protein was extracted from the transfected protoplasts in IP buffer (50 mM Tris, pH 7.5, 75 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitor cocktail (Roche), 1 mM dithiothreitol) and incubated with 1/100 dilutions of a monoclonal anti-myc (Cell Signaling, catalogue number 2276) or FLAG (Sigma, catalogue number F1804) antibody. The protein-antibody complex was precipitated with Protein A/G PLUS-Agarose beads (Calbiochem). Immunoprecipitated proteins were subjected to SDS-PAGE (10% polyacrylamide) and blotted onto

polyvinylidene difluoride membranes (Millipore). The blots were probed with 1/2,000 dilution of a peroxidase-conjugated anti-HA antibody (Roche, catalogue number 12013819001), anti-FLAG antibody (Sigma, catalogue number A8592) or a monoclonal anti-myc antibody. All experiments were repeated three times with similar results.

Transgenic plants and immunoblotting assay. To generate transgenic plants overexpressing HA-tagged *ABI3* and *ABI5* in the *bes1-D* background, genomic DNAs were cloned into *pCB302ES* containing the 35S promoter and HA tag sequences as described previously^{53,54}. For *bes1-DmeAR*-overexpressing plants, *bes1-DmeAR* was subcloned into *pCB302ES*. For *pABI3-GUS* and *pABI3-gABI3-FLAG* lines, 3-kb of the *ABI3* promoter was cloned into either *pCambia1303* containing β -glucuronidase (*GUS*) or *pBI121* containing two copies of FLAG epitope-tagged genomic *ABI3*. Four putative BES1-binding E-box motifs (CAnnTG) located at -278 ~ 284, -321 ~ 327, -336 ~ 342 and -2,496 ~ 2,502 in the *ABI3* promoter were mutated to AAnnTG generating *pABI3m-GUS* and *pABI3m-gABI3-FLAG* lines. All transgenes were integrated into the *Arabidopsis* genome by *Agrobacterium*-mediated floral dipping. The transgene expression was verified by immunoblotting with 1/2,000 dilution of a monoclonal anti-HA antibody (Roche, catalogue number 12013819001). Total proteins from protoplasts or seedlings were extracted with protein extraction buffer (50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1 $\times$ protease inhibitor cocktail (Roche) and 1% Triton X-100). Next, 3–20 μ g of proteins were separated by SDS-PAGE (10% polyacrylamide), transferred to a polyvinylidene difluoride membrane and immunodetected using 1/2,000 dilution of peroxidase-conjugated high-affinity anti-HA (Roche), peroxidase-conjugated anti-FLAG (Sigma, catalogue number A8592) and anti-actin (MP Biomedicals, catalogue number 69100) antibodies. Full scans of all western blottings are provided in Supplementary Fig. 15.

Real-time qRT-PCR analysis. To determine the expression levels of transcripts, total RNA was isolated from 3- to 4-day-old seedlings using Trizol reagent (Invitrogen). Double-strand complementary DNA was synthesized from 1 μ g RNA with oligo dT primers and ImProm-II reverse transcriptase (Promega). Quantitative real-time PCR was performed with 1 μ l of cDNA and gene-specific primer sets according to the instructions provided for the Light Cycler 2.0 (Roche) and the SYBR Taq Premix system (Takara/Enzynomics) with the following condition: total 45 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 20 s and extension at 72 °C for 13 ~ 25 s. *ACT2* was used as an internal control. The data analysis and fold-change calculation was done with the delta Ct method^{55,56} provided for the Light Cycler 2.0 software. The sequences of all gene-specific primers used in this study are listed in Supplementary Table 2.

Physiological analysis and histochemical GUS staining. For seed germination assays, surface-sterilized homozygous seeds were kept at 4 °C for 4 days and plated on 1/2 Gamborg B5 medium containing 1% sucrose supplemented with or without the indicated concentrations of ABA or epi-BL. After 3 days, the seed germination (radicle emergence) rates were scored and normalized with those of the wild-type controls. For root elongation assays, 4-day-old seedlings grown vertically on Gamborg B5 medium containing 1% sucrose and 0.8% agar (pH 5.7) were transferred onto fresh medium (1.2% agar) containing various concentrations of ABA. The seedlings were further incubated for 5 days and the primary root lengths were measured. To investigate *GUS* expression patterns by BR treatments, 3-day-old seedlings of *pABI3-GUS* and *pABI3m-GUS* were incubated with 1 μ M epi-BL (Sigma) for 4 h. The seedlings were stained with GUS-staining buffer (100 mM Tris-HCl (pH 7.0), 2 mM ferricyanide and 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronidase)) for 12 h.

Chromatin immunoprecipitation. ChIP assay was performed as previously described⁵⁷. Five-day-old seedlings (~0.2 g) were harvested and immersed in cross-linking buffer (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM phenylmethanesulphonyl fluoride, 1% formaldehyde) under a vacuum for 10 min. Glycine was added to a final concentration of 0.1 M to stop the cross-linking and incubation was continued for 5 min under vacuum. The seedlings were ground in liquid nitrogen and resuspended in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 1 $\times$ protease inhibitor cocktail (Roche)). DNA was sheared by sonication to ~500–1,000-bp fragments. After centrifugation, the supernatant was precleared with 40 μ l salmon sperm DNA/Protein A/G agarose for 1 h at 4 °C. After centrifugation, the supernatant was diluted tenfold with dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 1 $\times$ protease inhibitor cocktail (Roche)) and transferred to 1.5 ml tubes. Next, 5 μ l of the appropriate antibody (anti-HA (Roche, catalogue number 11867423001) or anti-acetyl-Histone H3 (Millipore, catalogue number 06-599)) was added, and the samples were incubated overnight at 4 °C. Next, 40 μ l salmon sperm DNA/Protein A/G agarose was added to the samples and they were further incubated for 2 h at 4 °C. The beads were successively washed with 1 ml of 2 $\times$ lysis buffer, 2 $\times$ LNDET (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and 3 $\times$ TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunoprecipitated DNAs were

eluted by adding 100 μ l 10% Chelex 100 slurry, and analysed by quantitative real-time PCR. The sequences of primers used throughout this work are available in Supplementary Table 2.

References

- Bai, M. Y. *et al.* Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in *Arabidopsis*. *Nat. Cell Biol.* **14**, 810–817 (2012).
- Finkelstein, R., Reeves, W., Ariizumi, T. & Steber, C. Molecular aspects of seed dormancy. *Annu. Rev. Plant Biol.* **59**, 387–415 (2008).
- Oh, E., Zhu, J. Y. & Wang, Z. Y. Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. *Nat. Cell Biol.* **14**, 802–809 (2012).
- Rajjou, L. *et al.* Seed germination and vigor. *Annu. Rev. Plant Biol.* **63**, 507–533 (2012).
- Lopez-Molina, L., Mongrand, S. & Chua, N. H. A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* **98**, 4782–4787 (2001).
- Giraudat, J. *et al.* Isolation of the *Arabidopsis* ABI3 gene by positional cloning. *Plant Cell* **4**, 1251–1261 (1992).
- Nambara, E. *et al.* The role of ABI3 and FUS3 loci in *Arabidopsis thaliana* on phase transition from late embryo development to germination. *Dev. Biol.* **220**, 412–423 (2000).
- Parcy, F., Valon, C., Kohara, A., Misera, S. & Giraudat, J. The ABCISIC ACID-INSENSITIVE3, FUSCA3, and LEAFY COTYLEDON1 loci act in concert to control multiple aspects of *Arabidopsis* seed development. *Plant Cell* **9**, 1265–1277 (1997).
- Zhang, X., Garretton, V. & Chua, N. H. The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. *Genes Dev.* **19**, 1532–1548 (2005).
- Nambara, E., Keith, K., McCourt, P. & Naito, S. Isolation of an internal deletion mutant of the *Arabidopsis thaliana* ABI3 gene. *Plant Cell Physiol.* **35**, 509–513 (1994).
- Ng, D. W., Chandrasekharan, M. B. & Hall, T. C. The 5' UTR negatively regulates quantitative and spatial expression from the ABI3 promoter. *Plant Mol. Biol.* **54**, 25–38 (2004).
- Lopez-Molina, L., Mongrand, S., McLachlin, D. T., Chait, B. T. & Chua, N. H. ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *Plant J.* **32**, 317–328 (2002).
- Tanaka, M., Kikuchi, A. & Kamada, H. The *Arabidopsis* histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. *Plant Physiol.* **146**, 149–161 (2008).
- Steber, C. M. & McCourt, P. A role for brassinosteroids in germination in *Arabidopsis*. *Plant Physiol.* **125**, 763–769 (2001).
- Xue, L. W. *et al.* Brassinosteroids counteract abscisic acid in germination and growth of *Arabidopsis*. *Z. Naturforsch. C* **64**, 225–230 (2009).
- Zhang, S., Cai, Z. & Wang, X. The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. *Proc. Natl Acad. Sci. USA* **106**, 4543–4548 (2009).
- Nomura, T. *et al.* Roles of brassinosteroids and related mRNAs in pea seed growth and germination. *Plant Physiol.* **143**, 1680–1688 (2007).
- Li, J. & Chory, J. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90**, 929–938 (1997).
- Li, J. *et al.* BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* **110**, 213–222 (2002).
- Wang, Z. Y., Bai, M. Y., Oh, E. & Zhu, J. Y. Brassinosteroid signaling network and regulation of photomorphogenesis. *Annu. Rev. Genet.* **46**, 701–724 (2012).
- Choudhary, S. P., Yu, J. Q., Yamaguchi-Shinozaki, K., Shinozaki, K. & Tran, L. S. Benefits of brassinosteroid crosstalk. *Trends Plant Sci.* **17**, 594–605 (2012).
- Li, J., Nam, K. H., Vafeados, D. & Chory, J. BIN2, a new brassinosteroid-insensitive locus in *Arabidopsis*. *Plant Physiol.* **127**, 14–22 (2001).
- Wang, Z. Y. *et al.* Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. *Dev. Cell* **2**, 505–513 (2002).
- Yan, Z., Zhao, J., Peng, P., Chihara, R. K. & Li, J. BIN2 functions redundantly with other *Arabidopsis* GSK3-like kinases to regulate brassinosteroid signaling. *Plant Physiol.* **150**, 710–721 (2009).
- Yin, Y. *et al.* BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell* **109**, 181–191 (2002).
- He, J. X. *et al.* BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. *Science* **307**, 1634–1638 (2005).
- Sun, Y. *et al.* Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in *Arabidopsis*. *Dev. Cell* **19**, 765–777 (2010).

28. Yin, Y. *et al.* A new class of transcription factors mediates brassinosteroid-regulated gene expression in *Arabidopsis*. *Cell* **120**, 249–259 (2005).
29. Causier, B., Ashworth, M., Guo, W. & Davies, B. The TOPLESS interactome: a framework for gene repression in *Arabidopsis*. *Plant Physiol.* **158**, 423–438 (2012).
30. Szemenyei, H., Hannon, M. & Long, J. A. TOPLESS mediates auxin-dependent transcriptional repression during *Arabidopsis* embryogenesis. *Science* **319**, 1384–1386 (2008).
31. Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H. & Ohme-Takagi, M. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* **13**, 1959–1968 (2001).
32. Song, C. P. *et al.* Role of an *Arabidopsis* AP2/EREBP-type transcriptional repressor in abscisic acid and drought stress responses. *Plant Cell* **17**, 2384–2396 (2005).
33. Ryu, H., Kim, K., Cho, H. & Hwang, I. Predominant actions of cytosolic BSU1 and nuclear BIN2 regulate subcellular localization of BES1 in brassinosteroid signaling. *Mol. Cells* **29**, 291–296 (2010).
34. Nambara, E. *et al.* A screen for genes that function in abscisic acid signaling in *Arabidopsis thaliana*. *Genetics* **161**, 1247–1255 (2002).
35. Krogan, N. T., Hogan, K. & Long, J. A. APETALA2 negatively regulates multiple floral organ identity genes in *Arabidopsis* by recruiting the co-repressor TOPLESS and the histone deacetylase HDA19. *Development* **139**, 4180–4190 (2012).
36. Long, J. A., Ohno, C., Smith, Z. R. & Meyerowitz, E. M. TOPLESS regulates apical embryonic fate in *Arabidopsis*. *Science* **312**, 1520–1523 (2006).
37. Zhou, C., Zhang, L., Duan, J., Miki, B. & Wu, K. HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis*. *Plant Cell* **17**, 1196–1204 (2005).
38. Li, J., Nagpal, P., Vitart, V., McMorris, T. C. & Chory, J. A role for brassinosteroids in light-dependent development of *Arabidopsis*. *Science* **272**, 398–401 (1996).
39. Stavang, J. A. *et al.* Hormonal regulation of temperature-induced growth in *Arabidopsis*. *Plant J.* **60**, 589–601 (2009).
40. Bossi, F. *et al.* The *Arabidopsis* ABA-INSENSITIVE (ABI) 4 factor acts as a central transcription activator of the expression of its own gene, and for the induction of ABI5 and SBE2.2 genes during sugar signaling. *Plant J.* **59**, 359–374 (2009).
41. Lee, J. H. *et al.* DWA1 and DWA2, two *Arabidopsis* DWD protein components of CUL4-based E3 ligases, act together as negative regulators in ABA signal transduction. *Plant Cell* **22**, 1716–1732 (2010).
42. Park, J., Lee, N., Kim, W., Lim, S. & Choi, G. ABI3 and PIL5 collaboratively activate the expression of SOMNUS by directly binding to its promoter in imbibed *Arabidopsis* seeds. *Plant Cell* **23**, 1404–1415 (2011).
43. Divi, U. K., Rahman, T. & Krishna, P. Brassinosteroid-mediated stress tolerance in *Arabidopsis* shows interactions with abscisic acid, ethylene and salicylic acid pathways. *BMC Plant Biol.* **10**, 151 (2010).
44. Divi, U. K. & Krishna, P. Overexpression of the brassinosteroid biosynthetic gene AtDWF4 in *Arabidopsis* seeds overcomes abscisic acid-induced inhibition of germination and increases cold tolerance in transgenic seedlings. *J. Plant Growth Regul.* **29**, 385–393 (2010).
45. Kagale, S., Divi, U. K., Krochko, J. E., Keller, W. A. & Krishna, P. Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. *Planta* **225**, 353–364 (2007).
46. Song, L. *et al.* Genome-wide analysis revealed the complex regulatory network of brassinosteroid effects in photomorphogenesis. *Mol. Plant* **2**, 755–772 (2009).
47. Yu, X. *et al.* A brassinosteroid transcriptional network revealed by genome-wide identification of BES1 target genes in *Arabidopsis thaliana*. *Plant J.* **65**, 634–646 (2011).
48. Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33** Suppl 245–254 (2003).
49. Becker, P. B. & Horz, W. ATP-dependent nucleosome remodeling. *Annu. Rev. Biochem.* **71**, 247–273 (2002).
50. Yu, X. *et al.* Modulation of brassinosteroid-regulated gene expression by Jumonji domain-containing proteins ELF6 and REF6 in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* **105**, 7618–7623 (2008).
51. Domagalska, M. A. *et al.* Attenuation of brassinosteroid signaling enhances FLC expression and delays flowering. *Development* **134**, 2841–2850 (2007).
52. Noh, B. *et al.* Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of *Arabidopsis* flowering time. *Plant Cell* **16**, 2601–2613 (2004).
53. Ryu, H. *et al.* Nucleocytoplasmic shuttling of BZR1 mediated by phosphorylation is essential in *Arabidopsis* brassinosteroid signaling. *Plant Cell* **19**, 2749–2762 (2007).
54. Ryu, H., Cho, H., Kim, K. & Hwang, I. Phosphorylation dependent nucleocytoplasmic shuttling of BES1 is a key regulatory event in brassinosteroid signaling. *Mol. Cells* **29**, 283–290 (2010).
55. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K. & Scheible, W. R. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* **139**, 5–17 (2005).
56. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* **25**, 402–408 (2001).
57. Kim, D. *et al.* BLH1 and KNAT3 modulate ABA responses during germination and early seedling development in *Arabidopsis*. *Plant J.* **75**, 755–766 (2013).

Acknowledgements

We thank Professor Ilha Lee and Professor Tae-Houn Kim for critical reading of the manuscript and useful suggestions, and Daeseok Choi for illustration. This work was supported by grants from the Advanced Biomass R&D Center (2010-0029720), the National Research Foundation (2012R1A2A2A02014387) funded by the Korean Ministry of Science, ICT and Future Planning. H.R. was supported by a grant from the Research Program for Agricultural Science & Technology Development (PJ009991) by RDA and the National Research Foundation (2013R1A1A1076010) funded by the Korean Ministry of Science, ICT and Future Planning.

Author contributions

H.R., H.C. and W.B. performed experiments. H.R. and I.H. designed experiments and analysed the data. H.R. and I.H. wrote the manuscript.

Additional information

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

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>

How to cite this article: Ryu, H. *et al.* Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of *ABI3*. *Nat. Commun.* 5:4138 doi: 10.1038/ncomms5138 (2014).