TOPLESS mediates brassinosteroid control of shoot boundaries and root meristem development in Arabidopsis thaliana

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Summary Statement:
BES1 recruitment of the co-repressor TOPLESS to the CUC3 and BRAVO promoters plays a critical role in BR-mediated control of organ boundary formation and root QC quiescence.
ABSTRACT

The transcription factor bri1-EMS-SUPPRESSOR1 (BES1) is a master regulator of brassinosteroid (BR)-regulated gene expression. BES1 together with BRASSINAZOLE-RESISTANT1 (BZR1) drive activated or repressed expression of several genes, and have a prominent role in negative regulation of BR synthesis. Here, we report that BES1 interaction with TOPLESS (TPL), via its ERF-associated amphiphilic repression EAR motif, is essential for BES1-mediated control of organ boundary formation in the SAM and the regulation of the quiescent center (QC) cell division in roots. We show that TPL binds via BES1 to the promoters of the CUC3 and BRAVO targets and suppresses their expression. Ectopic expression of TPL leads to similar organ boundary defects and alterations in the QC cell division rate as the bes1-d mutation, while bes1-d defects are suppressed by the dominant interfering tpl-1 protein, with these effects respectively correlating with changes in CUC3 and BRAVO expression. Together, our data unveil a pivotal role of the co-repressor TOPLESS in the shoot and root meristems, which relies on its interaction with BES1 and regulation of BES1 target gene expression.
INTRODUCTION

Brassinosteroids (BR) are steroid plant hormones with an essential role in plant growth and development (Clouse 2011; Guo et al. 2013). In tight connection with environmental cues and other plant hormones, BRs control shoot and root growth and distinct developmental programs such as photomorphogenesis, organ boundary formation and vascular differentiation (Ibanes et al. 2009; Bell et al. 2012; Gendron et al. 2012; Wang et al. 2012). BR perception triggers a signalling cascade that ultimately leads to activation and accumulation of two homologous transcription factors, *bri1-EMS-SUPPRESSOR1* (*BES1*) and *BRASSINAZOLE RESISTANT1* (*BZR1*). In the nucleus, BES1 and BZR1 modulate the expression of thousands of genes with a role in cell elongation, BR synthesis, and in the control of multiple cellular processes (He et al. 2005; Yin et al. 2005). Such wide range of transcriptional effects relies on BES1 and BZR1 ability to interact with different families of transcriptional factors, which partly modify their DNA recognition motif and switch their transcriptional activity from a repressor to activation function (Yin et al. 2005; Oh et al. 2012). Although early studies showed that BZR1 binds a conserved BRRE (CGTGC/TG) element in the promoters of BR-biosynthetic genes (He et al. 2005), whereas BES1 activates gene expression by recognizing as a complex with the BIM1 (BES1 INTERACTING MYC1) bHLH factor an E-box (CANNTG) element in its target promoters (Yin et al. 2005), more recent studies have established that both factors have similar DNA binding and transcriptional activities (Sun et al. 2010; Yu et al. 2011). BES1 and BZR1 interact with the PHYTOCHROME-INTERACTING FACTOR (PIF) family of bHLH factors to co-regulate a large number of light and BR-responsive genes (Oh et al. 2012; Bernardo et al. 2014), and are blocked by the DELLA repressors via a similar sequestration mechanism as PIFs (Bai et al. 2012; Gallego-Bartolomé et al. 2012; Li et al. 2012). However, BES1 and BZR1 also play independent roles in other processes, like BES1-mediated attenuation of ABA signalling (Ryu et al. 2014) or BZR1 negative regulation of immune signalling (Lozano-Duran et al. 2013).

BES1 and BZR1 share a conserved ERF-associated amphiphilic repression (EAR) motif in the C-terminal end, recent studies showing that the repressive function of these factors involves direct interaction with the co-repressor
TOPLESS (Oh et al. 2014; Ryu et al. 2014). TOPLESS (TPL) and its TOPLESS-RELATED (TPR) homologues belong to the family of Groucho/TUP1 transcriptional co-repressors (Long et al. 2006), found to bind a wide range of transcription factors via the EAR motifs to repress their downstream targets (Kagale and Rozwadowski 2011; Causier et al. 2012). Repression by TPL/TPR has been associated to the recruitment of HISTONE DEACETYLASE 19 and 6 (HDA19 and HDA6), two closely related deacetylases that promote chromatin compaction and transcriptional inactivation (Long et al. 2006; Krogan et al. 2012; Wang et al. 2013). TPL/TPRs regulate gene expression in multiple hormone response pathways, including auxin, jasmonate and strigolactone, through their interaction with the Aux/IAA, JAZ and SMLX transcriptional repressors (Szemenyei et al. 2008; Pauwels et al. 2010; Wang et al. 2015); in addition to play a role in the central oscillator, through interaction with PRR5, PRR7 and PRR9 (Wang et al. 2013). Likewise, TPL modulates BZR1-regulated cell elongation (Oh et al. 2014), and mediates antagonistic effects of BRs on ABA signaling, a response that is specifically controlled by BES1 (Ryu et al. 2014).

BR-signaling is also critical to the control of cell proliferation in the shoot and root meristems. In the SAM, BRs specifically modulate limited growth of organ boundaries, a group of small rarely dividing cells that separate new forming organs from the meristem (Fletcher 2002; Reddy et al. 2004; Barton 2010). BZR1 fusions to the YFP fluorescent protein revealed that this factor is depleted in the boundaries, in opposite to bzr1-1D-CFP that shows uniform distribution in the SAM and boundary cells (Gendron et al. 2012). BZR1 directly represses expression of the organ boundary identity CUP-SHAPED COTYLEDON1, 2 and 3 (CUC1-3) genes, with constitutive bzr1-1D mutants found to display organ fusion defects indicative of impaired organ boundaries separation (Bell et al. 2012; Gendron et al. 2012).

Reduced BR signaling is likewise required to maintain quiescence at the root stem cell niche (Gonzalez-Garcia et al. 2011; Heyman et al. 2013). BRs promote QC cell division through a cell autonomous pathway that is independent of auxin and ethylene signalling (Gonzalez-Garcia et al. 2011; Lee et al. 2015) and that is mediated by the R2R3 MYB transcription factor BRAVO.
BRAV0 is specifically expressed in the QC and stele initials and maintains QC quiescence downstream from BRI1 (Vilarrasa-Blasi et al. 2014).

While recent studies evidenced a function of TPL/TPR in BES1/BZR1-mediated control of cell elongation, it is at present unknown whether this family of co-repressors is also involved in the promotion of cell proliferation in response to BR-signalling. Here, we show that mutation of the EAR domain in the bes1-D protein reverses both the organ boundary and the QC defects of bes1-D overexpressors. Increased TPL gene dosage aggravates the organ fusion and QC cell division phenotype of bes1-D mutants, while overexpression of the mutant tpl-1 protein largely overrides bes1-D effects. We show that TPL binds to conserved BRRE and G-box elements in the CUC3 and BRAVO promoters through complex formation with BES1, and that pTPL::TPL seedlings display similar organ fusion defects and increased QC division rates as bes1-D mutants. Together, these results unveil a pivotal role of the co-repressor TPL in BR-regulated expression in the root and shoot meristems, and demonstrate that this function is essential to organ boundary initiation and maintenance, and to the preservation of low QC cell division rates.

RESULTS

BES1-TPL interaction is required for BES1 transcriptional activity

BES1, BZR1 and BEH1 to BEH4, all contain a conserved EAR domain (LXLXL) at their C-terminal region. Since EAR domain proteins were identified in complexes with the co-repressor TOPLESS (Kagale et al. 2010), we investigated whether BES1 directly interacts with TPL. We analyzed interaction of these proteins, in in vitro yeast two-hybrid assay, and in vivo, by using bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (co-IP) studies in Nicotiana benthamiana leaves. As shown in Fig. S1A, BES1 and TPL were observed to interact in yeast cells, and this interaction is fully dependent on the presence of an intact EAR domain. Fluorescence of the reconstituted split YFP protein was observed in the nucleus of leaf cells co-
transfected with the BES1-eYFP\(^\text{N}\) and TPL-eYFP\(^\text{C}\) constructs, but not in cells expressing PIF4-eYFP\(^\text{N}\) and TPL-eYFP\(^\text{C}\), used as negative control (Fig. S1B). TPL-HA was also pulled-down out of leaf extracts co-expressing the BES1-GFP and TPL-HA proteins, after BES1-GFP immunoprecipitation. By contrast, a mutated version of BES1, where the three Leu residues in the EAR domain were replaced by Ala (BES1-EARm-GFP), was unable to pull-down TPL-HA (Fig. S1C), demonstrating that TPL and BES1 interact via the BES1 EAR domain.

To test whether this domain is required for BES1 function, we analyzed repressive activity of the wild-type and BES1-EARm proteins in transient assays, using the \(pDWF4::LUC\) construct as a reporter. \(N. \ benthamiana\) leaves were agro-infiltrated with the \(pDWF4::LUC\) construct alone, or in combination with \(35S\) constructs for the BES1, bes1-D, BES1-EARm or bes1-D-EARm proteins, and leaf discs were used to measure LUC activity. As shown in Fig. S2A, expression of the BES1 and bes1-D proteins efficiently repressed the \(DWF4\) promoter, but this repressive effect was not observed for the BES1-EARm or bes1-D-EARm mutated proteins. Also, expression of the TPL corepressor reduced LUC activity driven by the \(DWF4\) promoter, and enhanced the repressive effects of BES1 (Figure S2B), in opposite to a partial reversion of BES1 inhibitory effects observed on expression of the mutant tpl-1 protein. These effects were not observed when TPL or tpl-1 were co-expressed with BES1-EARm, in support of a function of TPL in repressing \(DWF4\) expression via interaction with the BES1 EAR motif (Figure S2B).

To confirm these results \textit{in vivo}, we generated \(35S::bes1-D\)-GFP and \(35S::bes1-D\)-EARm-GFP transgenic lines, and two \(bes1-D\)-EARm lines (L13 and L33) were further characterized (Fig. S3A). As expected, over-expression of the \(bes1-D\)-GFP protein phenocopied the \(bes1-D\) mutant, with a decreased response to the biosynthetic inhibitor brassinazole (BRZ), and the characteristic bent petioles and curled leaves of adult \(bes1-D\) plants (Fig. S3B-D). However, none of these phenotypes were recapitulated in \(bes1-D\)-EARm-GFP lines, neither in the stronger over-expressor (Fig. S3B-D), indicating that the EAR domain is essential for BES1 function.
BR biosynthetic gene expression confirmed that mutation of the EAR domain abolishes \textit{bes1-D} ability to repress \textit{CPD}, \textit{DWF4} and \textit{ROT3} genes (Fig. S3E). \textit{IAA19} and \textit{PRE5} gene activation was also impaired in \textit{bes1D-EARm} lines, suggesting that the EAR domain is not only essential for \textit{bes1-D} repressive activity but for the transcriptional activation of its target genes. Together, these results establish that the EAR domain is essential for BES1 transcriptional activity, mutation of this domain inactivating \textit{bes1-D} function.

**Loss of TOPELESS function abolishes the constitutive BR response phenotype of \textit{bes1-D} mutants**

TPL and the four TPL-related (TPR) Groucho/Tup1 co-repressors were identified by isolation of the temperature-sensitive \textit{tpl-1} mutant, which shows severe apical/basal axis defects and fused cotyledons, and at restrictive temperatures, the replacement of the shoot by an apical root (Long et al. 2006). The \textit{tpl-1} mutation has a semi-dominant character due to the dominant negative effect of the N176H substitution over the rest of TPL/TPRs proteins, (Long et al. 2006). Inactivation of all five \textit{TPL/TPR} genes is indeed required to recapitulate the \textit{tpl-1} phenotype, identical phenotypic alterations being also observed in lines ectopically expressing \textit{tpl-1} (Wang et al. 2013).

To obtain additional genetic evidence for BES1 and TPL interaction, we generated double \textit{tpl-1 OX;bes1-D} and TPL;\textit{bes1-D} lines, by crossing plants over-expressing the mutant \textit{tpl-1} protein (\textit{tpl-1 OX}) or that expressed an extra copy of the \textit{TPL} gene (\textit{pTPL::TPL}) into the \textit{bes1-D} mutant background. As shown in Fig. 1A,B, over-expression of \textit{tpl-1} abolished the BRZ-insensitive phenotype of the constitutive \textit{bes1-D} mutants, \textit{tpl-1 OX;bes1-D} seedlings showing shorter hypocotyls than \textit{bes1-D} or the WT, and a similar growth inhibition response to BRZ as WT plants. Expression of this mutant protein caused by itself the inhibition of hypocotyl elongation, and a hypersensitive response to BRZ, indicative of a function of TPL in BR-dependent promotion of hypocotyl growth.

BES1 and BZR1 promote plant growth via direct activation of multiple cell wall remodeling and auxin signalling genes, like \textit{IAA19}, \textit{SAUR15} and \textit{PRE5} (Sun et al. 2010; Oh et al. 2012). Expression of these gene targets was significantly
reduced in tpl-1 OX plants, the tpl-1 protein also suppressing activation of these genes in the bes1-D background (Fig. 1C). Moreover, as reported for the tpl;tpr1;tpr4 triple mutant (Oh et al., 2014), bes1-D caused a milder repression of the BR-biosynthetic DWF4, ROT3 and CPD genes in tpl-1 OX seedlings than in the wild-type background (Fig. 1E).

Notably, tpl-1 over-expression rescues the bent petioles and curly leaf phenotype of adult bes1-D plants, tpl-1 OX lines showing smaller and more compact rosettes, because of their shorter petioles (Fig. 1D). Upon flowering transition, tpl-1 OX inflorescences were also smaller and more compact than WT, and more detailed phenotypic studies showed that their compact aspect is associated to defects in pedicel elongation. By contrast, bes1-D inflorescences were larger than WT (Fig. 1E), and had bigger flowers as a result of increased expansion of sepals and petals (Fig. S4). All these phenotypes were rescued by tpl-1, inflorescences of tpl-1 OX;bes1-D plants being identical to those of tpl-1 OX plants (Fig. 1E and Fig. S4). Together, these results indicate that impaired TPL function interferes with BES1 transcriptional activity, and abolishes not only BES1 repressive function, but its ability to activate gene expression.

Increased TOPOLESS dosage results in organ fusion defects

Lines with an increased TPL dosage, due to expression of an extra TPL gene copy (pTPL::TPL), displayed similar organ fusion defects as bes1-D mutants (Fig. 2A,C). Fusion of the cauline leaves and pedicels to the main stem, and fused sepals and stamens (Fig. 2A,C) were observed in both bes-1D and pTPL::TPL lines, suggesting that an excess of TPL or BES1 function interferes with proper organ boundary formation. Similar defects were previously reported in bzr1-1D mutants (Gendron et al. 2012), indicating that BES1 and BZR1 redundantly control organ boundary formation.

Boundary cells are characterized by expressing a specific set of genes (Tian et al. 2014), including the CUP-SHAPED COTYLEDON 1-3 (CUC1-3) boundary identity genes. This NAC-type family of transcription factors restricts cellular proliferation and differentiation, and plays a pivotal role in organ separation during both the vegetative and reproductive stages (Takada et al. 2001; Vroemen et al. 2003). CUC1-3 have overlapping functions in boundary
maintenance, as indicated by the lack of phenotype of single loss-of-function mutants (Vroemen et al. 2003; Laufs et al. 2004; Hibara et al. 2006; Burian et al. 2015). Likewise, incomplete penetrance of their organ fusion defects suggest that other pathways converge to the control of boundaries (Johnston et al. 2014; Colling et al. 2015; Hepworth and Pautot 2015).

As for cuc mutants, sporadic organ fusion defects such as pedicel-stem fusions (Fig. 2A), fused stamens (Fig. 2B), and partially fused sepals (Fig. 2C), were observed in both bes1-D and pTPL::TPL plants. Penetration of this phenotype was similar in pTPL::TPL and bes1-D plants (2-4%, see Table 1), but was sensibly increased in the double TPL;bes1-D background (11% and 18%, see Table 1), suggesting a cooperative function of the BES1 and TPL proteins in mediating these alterations. Expression of the tpl-1 mutant protein, on the other hand, rescued the organ fusion phenotype of bes1-D plants, none of these defects being observed in tpl-1 OX or tpl-1 OX;bes1-D plants (Table 1).

A few percent of bes1-D (6%) and pTPL::TPL (11%) plants displayed floral patterning defects, such as extra petals, or a reduced number of petals of dissimilar size (Fig. 2D and Table S1). A related phenotype has been described in the EARLY EXTRA PETALS 1 (EEP1) mutant, encoding MIR164c that post-transcriptionally regulates CUC1 and CUC2, with eep1 mutants failing to repress CUC1 and CUC2 expression in the second whorl (Laufs et al. 2004; Baker et al. 2005). Although tpl-1 OX rescued the patterning defects of bes1-D plants, penetrance of these alterations was not increased in the TPL;bes1-D background (Table S1), suggesting that TPL controls petal initiation also via BES1-independent pathways, likely via regulation of auxin-signaling (Szemenyei et al. 2008).

TOPOLESS regulates organ separation via BES1 mediated CUC3 gene repression

To assess that fusion defects in bes-1D and pTPL::TPL lines were associated to down-regulation of the CUC genes, we examined the spatial pattern of CUC3 expression in these plants. To this aim, a pCUC3::GUS reporter line (Kwon et al. 2006) was crossed into the tpl-1 OX, pTPL::TPL, bes1-D and TPL;bes1-D backgrounds and GUS expression was analyzed by staining of the
inflorescences (Fig. 2E,F). During floral transition, the SAM is converted to an inflorescence meristem. This process involves the formation of meristem-organ boundaries between the central inflorescence meristem and the floral primordia, and organ-organ boundaries that separate the four concentric whorls and adjacent organs within a whorl. CUC3 is reported to be expressed in each of these boundaries (Vroemen et al. 2003) and, in agreement with previous reports, GUS expression in WT inflorescences was restricted to the adaxial side of the pedicel axils and to the boundaries between floral primordia in the SAM. In floral buds, it formed a ring at the bases of sepals and petals, and marked the boundaries between ovule primordia in the gynoecium (Fig. 2F). Notably, tpl-1 OX increased CUC3 expression in all these boundary regions, while GUS expression was reduced in both pTPL::TPL and bes1-D plants. Moreover, TPL;bes1-D plants showed an additive inhibition of GUS expression, indicating that TPL and bes1-D synergistically suppress the CUC3 gene (Fig. 2E,F).

In paraclade junctions between primary and secondary stems, CUC3 expression was restricted to the bases of the cauline leaf and the emerging axillary shoot (Fig. 3B). GUS activity was strongly reduced in bes1-D mutants, correlating with defective axillary branch separation (Fig. 3A,B). Reduced GUS expression was likewise detected in pTPL::TPL lines, in opposite to tpl-1 OX that showed an expanded area of CUC3 expression (Fig. 3B). Also, increased TPL dosage resulted in stronger CUC3 inhibition and more severe cauline leaf-branch fusions in TPL;bes1-D plants, whereas tpl-1 OX alleviated the fusion defects of bes1-D mutants (Fig. 3C). Similar trends in CUC3 expression were observed by RT-qPCR analyses of young seedlings, with reduced CUC3 transcript levels detected in bes1-D, pTPL::TPL and TPL;bes1-D lines, while in the tpl-1 OX;bes1-D and bes1-D-EARm backgrounds expression levels were similar to the WT (Fig. 3E). In these analyses, CUC3 levels in tpl-1 OX seedlings were found to be slightly lower than the WT, likely due to the delayed leaf differentiation in this genotype. Altogether, these results demonstrate that TPL and BES1 act in concert to repress CUC3 expression, impaired TPL function in tpl-1 over-expressors abolishing bes1-D mediated suppression of CUC3.
TPL is recruited to specific DNA promoter regions through interaction with different families of DNA-binding transcription factors. To test if TPL binds the same CUC3 promoter elements as BES1, we performed chromatin immunoprecipitation (ChIP) assays using both 35S::BES1-GFP plants and transgenic lines expressing the pTPL::TPL construct in the bes1-D mutant background. BES1-GFP ChIP-PCR studies confirmed that BES1 binds the CUC3 and DWF4 promoters with similar affinities, and associates to the same CUC3 promoter region as BZR1 (Fig. 3D) (Gendron et al. 2012). These two promoter fragments were also enriched by TPL-HA, although binding to the BES1-recognition sites was less efficient than for BES1-GFP (Fig. 3D), consistent with an indirect association of TPL to DNA. Together, these results demonstrate that BES1 recruits the TPL protein to the DWF4 and CUC3 promoters, pointing to a pivotal function of the TPL-BES1 module in the control of organ boundary maintenance, through direct repression of the CUC1-3 genes.

**TOPLESS modulates root meristem organization through BES1-mediated suppression of BRAVO**

Reduced BR-signaling is critical to the control of cell cycle progression in the root stem cell niche and to the correct organization of the meristem; in opposite to the role of increased BR-signaling in promoting cell elongation and differentiation in the root transition-elongation zone (Gonzalez-Garcia et al. 2011; Chaiwanon and Wang 2015). The BAS1 and SOB7 BR catabolic enzymes are expressed in the root cap, and reduce availability of bioactive BRs in the adjacent stem cell niche (Chaiwanon and Wang 2015). In the QC, BR-signaling targets the BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTRE (BRAVO) and ETHYLENE RESPONSE FACTOR 115 (ERF115) factors, which regulate QC quiescence in opposite ways (Heyman et al. 2013; Vilarrasa-Blasi et al. 2014). BRAVO is expressed in the QC and stele initials, and acts as a cell-specific repressor of QC division (Vilarrasa-Blasi et al. 2014). BRAVO is a repression target of BES1 and BZR1, reduced expression of this gene in bes1-D and bzr1-1D mutants leading to ectopic activation of QC division (Vilarrasa-Blasi et al. 2014; Chaiwanon and Wang 2015). BRAVO also
physically interacts and inactivates BES1, this negative feedback loop enabling high levels of QC expression, at the same time that prevents its suppression as a result of fortuitous activation of BR signaling (Vilarrasa-Blasi et al. 2014).

To assess whether TPL function was required to BR-mediated control of cell progression in the root meristem, we examined QC cell division in pTPL::TPL and tpl-1 OX roots. As shown in Fig. 4, expression of an extra TPL copy sensibly increased the number of plants with a divided QC, two QC cell layers being observed in 25% pTPL::TPL roots as compared to 5% in WT roots. In contrast, no QC cell divisions were observed in any of the tpl-1 OX roots analysed. Moreover, pTPL::TPL expression greatly increased the frequency of divided QC cells in bes1-D plants, a double QC layer or partially duplicated cells seen in 90% TPL;bes1-D roots (Fig. 4). Lines expressing the bes1-D-EARm protein, on the other hand, displayed a WT behavior, indicating that the EAR domain is required for BES1 promotion of QC cell division (Fig. 4A, C). To further prove that TPL and tpl-1 OX effects on QC division depend on BR-signaling, we tested whether altered QC division in these genotypes was restituted by BL or BRZ application. As shown in Figure 4B, C, increased QC division rates were observed in tpl-1 OX roots upon BL treatment, although divided cells were still less than in the WT, while the increased QC division phenotype of TPL roots was partially rescued by the inhibitor BRZ. Hence, altogether these results are consistent with a cooperative action of BES1 and TPL in promoting QC cell division.

We next analyzed if TPL effects on QC cell division correlated with suppressed BRAVO expression by crossing pBRAVO::GFP reporter lines into the pTPL::TPL and tpl-1 OX backgrounds. Unfortunately, pBRAVO::GFP was silenced in tpl-1 OX lines and we were unable to examine tpl-1 effects on expression of this gene. However, a notable decrease in GFP activity was observed in pTPL::TPL lines, evidencing that an increased TPL dosage leads to BRAVO suppression (Fig. 5A). Due to increased QC division, these plants displayed disorganized root meristems (see Fig. 5A), and such a phenotype was reverted by BRZ application (Fig. 5A, B). Western blot studies of pBRAVO::GFP and pBRAVO::GFP;TPL roots confirmed that TPL causes a similar reduction in BRAVO expression as seen in the WT in response to BL.
addition, BL further suppressed *BRAVO* expression in *pTPL::TPL* roots (Fig. 5C), suggesting an additive effect of TPL and BL in *BRAVO* suppression.

Finally, we tested whether TPL is recruited to the *BRAVO* promoter by performing ChIP-PCR studies on TPL;*bes1-D* lines. *BRAVO* contains a G-box and several BRRE elements in its 2.1 kb upstream region (Fig. 5D) and significant enrichment was observed for a promoter fragment including the G-box and one of the BRRE elements, previously shown to be recognized by BES1 (Vilarrasa-Blasi et al. 2014), indicating that TPL is recruited to this promoter region by BES1 (Fig. 5E). Additionally, ChIP-PCR experiments on *pTPL::TPL* and TPL;*bes1-D* seedlings grown on BRZ showed that BRZ impaired TPL binding to the *BRAVO* and *CUC3* promoters in *pTPL::TPL* plants, but not in the BRZ-insensitive TPL;*bes1-D* background (Figure 5F), hence establishing that BES1 is required for TPL recruitment to these promoters.

Altogether, our results demonstrate that interaction with TPL via its conserved EAR domain is essential for BES1 function in promoting QC cell division, and show that BL effects on QC division depend to a large extent on BES1 direct repression of the transcription factor *BRAVO*. Thus, these data unveil a novel cell-specific function of TPL in the root stem cell niche.

**DISCUSSION**

BES1 is a pivotal factor in BR signaling with dual roles as both transcriptional activator and repressor. Here, we show that the BES1 EAR domain is essential for its transcriptional activity, and that this conserved domain mediates interaction with the co-repressor TOPELESS (TPL), consistent with recent reports (Oh et al., 2014; Ryu et al., 2014).

Notably, over-expression of the mutant tpl-1 protein, caused de-repression of BES1/BZR1-repressed targets, such as *DWF4*, *ROT3* and *CPD*, and impaired activation of the induced *PRE5*, *IAA19* and *SAUR15* targets (Fig. 1C,E), suggesting that TPL is as well required for BES1/BZR1 transcriptional activation function. This effect was more evident in *tpl-1 OX;bes1-D* plants, in which *tpl-1* partially suppressed constitutive activation of these targets, in particular of *PRE5*. *tpl-1 OX* plants in fact showed shorter hypocotyls and petioles than WT,
and displayed a hypersensitive response to BRZ, while tpl-1 suppressed the BRZ-insensitive phenotype of bes1-D mutants, suggesting that the dominant negative function of tpl-1 impairs BR response.

Recent determination of the TPD crystal structure showed that the N176H substitution in tpl-1 does not play a relevant role in dimerization or EAR binding (Ke et al. 2015). Although the molecular basis for the dominant nature of this mutation is not well understood, our finding that tpl-1 interferes with BES1-target gene activation, suggest that TPL is implicated both in BR-repressed and activated gene expression. Related findings were also obtained by fusion of the bes1-D-mEAR protein to SDRX, TPL or HDA19 (Ryu et al., 2014), which restitutes constitutive BR-signaling activity of the protein and leads to elongated hypocotyl growth on BRZ, thus further supporting of a function of TPL in BES1/BZR1 target gene activation.

A role for TPL in shoot meristem maintenance has been previously reported through its interaction with the WUSCHEL (WUS) homeodomain and RAMOSA1 zinc-finger transcription factors (Kieffer et al., 2006; Sablowski 2007; Yadav et al. 2011; Gavallotti et al., 2010). Here, we provided biochemical and genetic evidence for a function of the BES1-TPL complex in direct suppression of the CUC3 and BRAVO genes, acting as cell-specific repressors of cell proliferation in the meristem boundaries and the root QC. We showed that increased TPL dosage causes similar organ fusion and QC division alterations as the constitutive BR-response bes-1D mutation. Moreover, TPL and bes1-D have synergistic effects in inhibiting boundary formation and QC quiescence, whereas tpl-1 expression abolishes bes-1D defects. Our findings show that BES1 recruits TPL to the CUC3 and BRAVO promoters, to repress boundary and QC cell specific expression of these genes.

Comparative analyses of BR-responsive gene expression and organ boundary specific transcriptomes (Tian et al. 2014) evidenced a significant overlap between boundary enriched transcripts and BR signaling repressed genes (Fig. S5). Most of the BR-repressed transcription factors were reported as BES1 and/or BZR1 direct targets, suggesting that BES1 and BZR1 modulate the expression of other boundary specific regulators in addition to CUCs. Interestingly, similar comparative studies of the QC cell transcriptome showed
that the only transcription factors targeted by BZR1 and repressed by BL were the *BRAVO*, *MONOPOLE* and *PLETHORA* genes (*PLT1*, *BABYBOOM/PLT4*) (Chaiwanon and Wang 2015), supporting a main function of BRAVO downstream of BES1/BZR1 in the root QC.

Reduced division of boundary cells is critical to the separation of young organs from the central meristem, and to the maintenance and organization of the meristem. Boundary cells express a specific set of genes that restrict cell division and auxin efflux carrier activity, while promote meristemmatic gene expression (Hepworth and Pautot 2015). These cells, similar to the root QC, function as a mode of organizing center regulating the patterning and development of adjacent organs (Zadnikova and Simon 2014; Yu and Huang 2016), thus highlighting a pivotal role of TPL in the organization of the shoot and root meristems. Consistent with this function, TPL is expressed to higher levels in the SAM and root meristem zone, and in young actively dividing tissues (Fig. S6). Moreover, our results provide evidence for a prevalent function of the BES1/BZR1-TPL module in coordinating the balance between cell proliferation and differentiation in both the root meristem and shoot boundary domains, therefore linking organogenesis to the maintenance of meristem activity.

A further intriguing question is why TPL activity is required for the activation function of BES1 and BZR1. Groucho/Tup1 co-repressors are believed to function as binding scaffolds for histone deacetylases and chromatin remodeling complexes (Long et al. 2006; Zhu et al. 2010; Krogan et al. 2012), but their exact mechanism of action is not yet understood. Although genetic evidence suggests that TPL acts through HDA19 (Long et al. 2006), high-throughput yeast two-hybrid approaches failed to identify HDA19 as a direct TPL interactor (Causier et al. 2012), while interaction of these proteins was observed in plant extract pull-down experiments (Zhu et al. 2010). This would indicate that additional factors bridge TPL and HDA19 and, in fact, yeast two-hybrid studies showed that TPL/TPR directly bind PKR1, an homolog of the PICKLE (PKL)/ ENHANCED PHOTOMORPHOGENIC1 (EPP1) chromatin-remodeling factor (Causier et al. 2012). Interestingly, PKL/EPP1 was recently shown to associate with PIF3 and BZR1, that recruit this chromatin-remodeling factor to the promoters of the *IAA19* and *PRE1* genes (Zhang et al. 2014).
Thus, it is possible that TPL forms chromatin modification complexes with opposite transcriptional outputs depending on its interaction with BES1 or the BES1-PIF heterodimer, an important task to the future being the identification of such complexes.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

tpl-1 OX, pTPL::TPL (Wang et al. 2013), pCUC3::GUS (Kwon et al. 2006) and pTPL::GUS (Tao et al. 2013) genotypes are in the Col-0 background. tpl-1 OX and pTPL::TPL plants were crossed to the bes1-D mutant (introgressed into Col-0, Ibanes et al. 2009) to obtain TPL;bes1-D and tpl-1 OX;bes1-D, respectively.

Seeds were surface-sterilized for 15 minutes in 70% (v/v) ethanol and 0.01% (v/v) Triton X-100, followed by two washes of 2 minutes in 96% (v/v) ethanol. Air dried seeds were then sown on half strength MS-agar plates with 1% sucrose and stratified for 3 days at 4°C in the dark. BL (24-epibrassinolide, Sigma-Aldrich) and brassinazole (Tokyo Chemical Industry, Japan) treatments were performed at 1.0 μM and 0.8 μM, respectively. Hypocotyls were measured using the ImageJ software.

**Plasmid constructs**

Full-length coding regions for the Arabidopsis BES1, TPL and PIF4 proteins were amplified with primers BES1-F/BES1-R, TPL-F/TPL-R and PIF4YFPf/PIF4YFPr, respectively. The bes1-D mutant ORF was amplified from an Arabidopsis bes1-D mutant cDNA, using primers BES1-F and BES1-R. To obtain the BES1-EARm and bes1-D-EARm constructs, primers BES1-F and BES1-EARm-R were used to introduce the EAR mutation into the corresponding ORFs, using as a template wild type and bes1-D cDNA, respectively. The PCR amplified fragments were cloned into pENTR/D-TOPO (Invitrogen) and used for subsequent LR reactions.
BES1, BES1-EARm, bes1-D and bes1-D-EARm full-length coding regions were cloned by LR clonase (Invitrogen) recombination into pGWB5, to obtain the 35S::BES1-GFP, 35S::BES1-EARm-GFP, 35S::bes1-D-GFP and 35S::bes1-D-EARm-GFP constructs.

The TPL coding region was inserted by LR clonase recombination into pGWB14, to create the 35S::TPL-HA binary vector.

The DWF4 promoter region was amplified using primers pDWF4-F and pDWF4-R, and cloned into LucTrap-3 to obtain the pDWF4::LUC reporter plasmid.

Transgenic plants

35S::bes1-D-GFP and 35S::bes1-D-EARm-GFP constructs were transformed into the Agrobacterium tumefaciens strain GV3101. Arabidopsis transformation was performed through the floral dip method. Homozygous Arabidopsis lines were identified by kanamycin resistance and lines with appropriate expression of the transgene selected by western blot immunodetection using an anti-GFP antibody (Roche).

Bimolecular Fluorescence Complementation assay (BiFC)

The TPL, PIF4 and BES1 coding sequences were inserted by LR-reaction (Invitrogen) into pBiFC binary vectors containing the N- and C- terminal YFP fragments (YFPN43 and YFPC43). Plasmids were transformed into the Agrobacterium tumefaciens GV3101 strain and infiltrated into Nicotiana benthamiana leaves. The p19 protein was used to suppress gene silencing. Two days after infiltration, leaves were observed under a Leica TCS SP5 laser scanning confocal microscope.

Co-immunoprecipitation

N. benthamiana leaves were co-infiltrated with Agrobacterium cultures bearing the 35S::BES1-GFP, 35S::BES1-EARm-GFP and 35S::TPL-HA plasmids in the appropriate combinations. After 48 hours leaves were homogenized in protein extraction buffer (20 mM Tris-HCl pH 7.5, 5 mM MgCl2, 75 mM NaCl, 2.5 mM EDTA, 25mM β-glycerophosphate, 0.1% Nonidet P-40, 10 mM NaF, 0.05% sodium deoxycholate, 5 mM β-mercaptoethanol, 10 μM MG-132, 1 mM PMSF and protease inhibitors (Roche)). Extracts were cleared by centrifugation at
13,000 g for 15 min at 4°C, and 1 ml of the supernatant was incubated at 4°C for 3 h with 50 μl of anti-GFP magnetic beads (μMACS Epitope Tag, Miltenyi Biotec). Beads were bound with the help of a magnet and washed five times with 500 μl extraction buffer. Immunocomplexes were eluted by boiling for 2 minutes in 50 μl of 2x SDS loading buffer. Antibodies anti-HA-Peroxidase (Roche) and anti-GFP-Peroxidase (Miltenyi Biotec) were used for immunodetection.

**Yeast Two-Hybrid Assay**

Yeast two-hybrid interaction assays were performed with the GAL4 Two-Hybrid System (Clontech). The complete ORFs for the TPL, BES1 and BES1-EARm proteins were introduced by LR clonase recombination into the pGADT7 and pGBK7 Gateway compatible vectors (Clontech). The NINJA-pGBT9 plasmid was a kind gift from Dr. Roberto Solano. Appropriate plasmid combinations were transformed into the yeast strain AH109 by the lithium acetate method and reporter gene activation was assayed by selection on SD-LWHA plates.

**Luciferase activity assays**

*Nicotiana benthamiana* leaves were co-infiltrated with *Agrobacterium tumefaciens* cultures bearing the pDWF4::LUC reporter construct, alone or in combination with 35S::BES1-GFP, 35S::BES1-EARm-GFP, 35S::bes1-D-GFP or the 35S::bes1-D-EARm-GFP effector constructs. Two days after inoculation, 0.5 cm diameter leaf discs were collected and transferred to 96 well microtiter plates filled with 165 μL 0.5X MS liquid media and 35 μL of 1x D-Luciferin substrate (20 μg/ml). At least 12 discs were measured per sample. Luciferase activity was measured with the LB 960 Microplate Luminometer Center (Berthold) using the MikroWin software.

**Quantitative RT-PCR analysis**

Total RNA was extracted from whole seedlings using the High Pure Isolation kit (Roche). One μg of RNA was used for first-strand cDNA synthesis using the SuperScript II Reverse Transcriptase (Invitrogen). One μl of the cDNA reaction was used for quantitative PCR using the FastStart Universal SYBR Green Master mix (Roche) and a 7500 Real Time PCR System (Applied Biosystems),
following manufacturer’s instructions. Expression levels were calculated relative to the PP2A gene, using the ΔΔ threshold cycle (Ct) method (Applied Biosystems). Primers used are listed in Supplementary Table S2. Results correspond to three biological replicates.

**GUS Staining**

Freshly harvested plant material was placed in cold 90% acetone for 20 minutes, washed once with water and transferred to staining solution (50 mM NaHPO4 buffer pH 7.2, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 2 mM X-glucuronide, and 0.2% Triton X-100). After 5 minutes vacuum infiltration, samples were placed at 37 °C overnight. Next day, they were incubated for 30 minutes in 20, 30 and 50% ethanol, fixed in FAA (50% ethanol, 5% formaldehyde, 10% acetic acid) and kept in 70% ethanol until visualization with a stereomicroscope.

**Chromatin Immunoprecipitation assays**

ChIP assays were performed as described previously (Lee et al. 2007). 3 grams of 6 days old Col-0, 35S::BES-GFP and pTPL-TPL-HA;bes1-D-GFP seedlings were used for chromatin preparation. The chromatin pellet was sonicated at 4ºC with a Diagenode Bioruptor to achieve an average DNA fragment size of ~0.3- to 0.8-kb. 1 µl of anti-GFP (MBL), 1 µl of anti-HA (2,2 µg) and 10 µl of protein G coupled to magnetic beads (Invitrogen) were used for chromatin immunoprecipitation. DNA was purified using the MiniElute Reaction CleanUp kit (Qiagen). An aliquot of untreated sonicated chromatin was reverse cross-linked and used as input DNA control for PCR amplification.

**Confocal microscopy**

Analysis of QC cell division rates and visualization of columella cells starch granules was carried out by imaging fixed stained primary roots obtained through a modified PseudoSchiff-PI staining method (Truernit and Haseloff 2008). For in vivo imaging experiments, roots were stained in 10 µg/ml propidium iodide for 1 minute, rinsed and mounted in dH2O. A Leica TCS SP5 laser scanning confocal microscope, with an Excitation Beam Splitter TD 488/561/633 and an Emission band width between 495 and 556nm was used to
visualize the samples. Dividing cells in the QC were manually counted from confocal stacks.

**Western blot analysis.**

Seedlings or roots were homogenized in extraction buffer (1XPBS, 0.1% SDS, 0.1% Triton X-100, 100 μM PMSF, 5 μM β-mercaptoethanol and protease inhibitors (Roche)). Extracts were cleared by centrifugation at 13,000 rpms for 15 min, and the protein concentration determined by Bradford assay (Bio-Rad). Protein samples were boiled in 2xSDS loading buffer, and loaded on SDS-PAGE gels. Blots were immunodetected with an anti-GFP antibody (Roche) and a secondary antibody peroxidase-conjugated. Anti-RPT5 was used as a loading control.
ACKNOWLEDGMENTS

We thank Dr. David Sommers (Ohio State University) for *tpl-1* OX and *pTPL::TPL* seeds, Dr. Patrick Laufs (INRA Versailles) for *pCUC3::GUS* seeds, and Dr. Genji Qin (Peking University) for the *pTPL::GUS* seeds.

COMPETING INTERESTS

No competing interests are declared.

AUTHOR CONTRIBUTIONS

S.P., A.E. and C.M. designed the experiments. M.L. performed initial studies. A.E. obtained the double mutants and performed their phenotypic and molecular characterization. A.E. and C.M. analyzed TPL and BES1 interaction and carried out the GUS expression studies. A.E. and S.P. performed the LUC transactivation studies and C.M. the ChiP experiments. *BRAVO* repression was analyzed by C.M.. N.F., N.B. and A.I.C.-D. studied QC cell division. A.E. and S.P. wrote the manuscript. All authors revised the manuscript.

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Fig. 1. Characterisation of *tpl-1 OX* and *tpl-1 OX;bes1-D* plants. (A) Phenotype of 7-day-old WT, *tpl-1 OX*, *bes1-D* and *tpl-1 OX;bes1-D* seedlings grown under short day conditions on MS growth media (Mock) or MS media supplemented with 0.5 µM brassinazole (BRZ). (B) Hypocotyl lengths of seedlings grown in the same conditions as (A). Hypocotyl growth reduction in BRZ compared to mock. Asterisks indicate significant difference in the BRZ response of *tpl-1 OX* compared to the corresponding background genotype (p<0.01, Student's t-test). Bars represent s. d. (n=20). (C) and (E) Quantitative real-time PCR analysis of BES1-activated and BR-biosynthesis repressed genes. Gene expression levels were normalized to *PP2A*. Error bars indicate s.d. (n=3 biological replicates). Asterisks indicate significant difference between each genotype and the WT (p<0.05, Student’s t-test). Numbers in (E) represent the relative expression levels. (D) Phenotypes of 4-week-old plants (F) Inflorescences of WT, *tpl-1 OX*, *bes1-D* and *tpl-1 OX;bes1-D* plants
Fig. 2. TPL and BES1 regulate postembryonic organ separation and cooperatively repress *CUC3* expression. Defects in organ boundary formation and maintenance observed in *pTPL::TPL* plants (TPL in the figure): pedicel fusion to the stem (A), fused stamens (B), partially fused sepals (C), flowers with 3 or 5 petals and petals of different size (D). (E) *pCUC3::GUS* expression in the inflorescence and branch junctions of *tpl-1 OX, WT, pTPL::TPL (TPL), bes1-D* and *pTPL::TPL;bes1-D* (TPL;bes1-D) plants. First primary inflorescences were collected for each genotype and used for staining. (F) Detail showing the boundary associated pattern of *pCUC3::GUS* expression in WT, TPL and TPL;bes1-D inflorescences.
Fig. 3. TPL and BES1 control leaf-branch separation and bind the CUC3 promoter. (A) Junction between the main stem, axillary branch and cauline leaf in the indicated phenotypes. (B) Detail showing pCUC3::GUS expression in the stem-branch junction of the indicated genotypes. (C) Length of fused region between the branch and the cauline leaf. Measurements were made on the lowest cauline leaf axil of plants of the same age (n=20). Significant differences by the Tukey’s multiple comparison test are indicated by letters above bars (p<0.01). (D) ChIP-qPCR analysis of BES1 and TPL binding to the CUC3 promoter region. DWF4 is included as a positive control, and the ORF of CUC3 and ACT as negative controls. The experiment was repeated three times with similar results. (E) Quantitative real-time PCR analysis of CUC3 expression. RNA was extracted from 6-days-old seedlings grown under long day conditions. Gene expression levels were normalized to those of PP2A. Error bars indicate s.d. (n=3 biological replicates). Asterisks indicate significant difference compared to the WT (p<0.05, Student’s t-test).
Fig. 4. TPL regulates QC cell division. (A) (B) Microscopy images of mPS-PI stained 6-day-old root tips of the indicated genotypes, grown in long day conditions on MS media (A) and MS media supplemented with 0.4 nM BL or 1 µM BRZ (B). Red arrows mark the QC position. The 35S::bes1-DEARm line corresponds to EARm L33. (C) Quantification of the QC cell divisions expressed as percentage, (n > 50 seedlings for each genotype).
Fig. 5. TPL and BES1 repress BRAVO expression. (A) Microscopy images of propidium iodide stained root tips of 6-days-old WT and pTPL::TPL (TPL) seedlings showing pBRAVO::GFP expression in QC cells and stele initials. Scale bar represents 30 µm. (B) Percentage of roots with disorganized meristems of the indicated genotypes. Seedlings were grown on MS medium for four days and 2 additional days on MS medium (Mock) or MS + 0.5 µM BRZ.
n>15, error bars represent ± s. d. (p<0.05, Student’s t-test). Asterisks indicate statistically significance difference between TPL and the WT in mock, and TPL seedlings grown in mock and BRZ (C) Western blot showing pBRAVO::GFP expression levels in WT and TPL roots. Hybridization with an anti-RPT5 antibody is included as a loading control. (D) Schematic representation of the BRAVO and DWF4 promoter regions showing the putative BES1/BZR1 binding elements. Bar indicates the region selected for qPCR. (E) ChIP-qPCR assay showing binding of TPL to the BRAVO promoter region indicated in (D). 6-days-old pTPL::TPL;bes1-D (TPL;bes1-D) seedlings were used to the assay. DWF4 amplification is included as positive control and the ORF of actin (ACT) as a negative control. (F) ChIP-qPCR assay showing differences in binding of TPL to the CUC3 and BRAVO promoters in pTPL::TPL (TPL) compared to TPL;bes1-D plants. 6-days-old seedlings grown in 0.5 µM BRZ were used. Experiments were repeated twice with similar results. Figures represent one of the two biological replicates.
Table 1. Percentage of flowers that contain stamen-stamen or pedicel-stem fusions.

<table>
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<th>Genotype</th>
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<th>pedicel-stem fusion</th>
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<th>%pedicel-stem fusion</th>
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<td>pTPL::TPL (TPL)</td>
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**Fig. S1.** BES1 interacts with TPL through the EAR domain. (A) Yeast two-hybrid assay showing TPL and BES1 interaction. TPL interacts with the intact BES1 protein but not with BES1-EARm (EARm), where core Leucine residues in the EAR motif (LXLXL) were substituted by Alanine. TPL interaction with NINJA is included as a positive control. Yeast cells were grown on the synthetic dropout minimal medium lacking Leu and Trp (-LW), or synthetic dropout without Leu, Trp, His and Ade (-LWAH). (B) BiFC showing BES1 and TPL interaction. Nuclear YFP fluorescence is observed in *N. benthamiana* leaves infiltrated with the BES1-eYFP\(^N\) and TPL-eYFP\(^C\) constructs. PIF4-eYFP\(^N\) and TPL-eYFP\(^C\) are included as a negative control. Red fluorescence corresponds to chlorophyll. Scale bars represent 14 µm in the BES1-TPL panel and 50 µm in the PIF4-TPL panel. (C) Co-IP assay. TPL-HA is pulled-down by immunoprecipitation of GFP-tagged BES1, but not by BES1-EARm (EARm-GFP). *N. benthamiana* leaves were agroinfiltrated with BES1-GFP, BES1-EARm-GFP and TPL-HA. Two days after agroinfiltration, total protein extracts were immunoprecipitated with an anti-GFP antibody. TPL-HA was detected in these fractions with an anti-HA antibody.
Fig. S2. The EAR domain is required for the repressor activity of the BES1 factor. (A) The *DWF4* promoter containing two BRRE elements was fused to the *LUC* reporter gene and co-transfected with the 35S::BES1, 35S::bes1-D, 35S::BES1-EARm or 35S::bes1-D-EARm effector constructs into *N. benthamiana* leaves. (B) TPL regulation of *DWF4* expression. Leaves were co-infiltrated with the *DWF4* reporter and combinations of *Agrobacteria* expressing the pTPL::TPL, 35S::BES1, 35S::BES1+pTPL::TPL, 35S::BES1+tpl-1 OX, 35S::BES1-EARm, 35S::BES1-EARm+ pTPL::TPL or 35S::BES1-LEARm+ tpl-1 OX, as indicated. Leaf discs were collected 48 hours after infiltration and luciferase activity was measured. Error bars represent SD (n=20). Schematic representation of the reporter construct shows the position of the BRRE elements. Asterisks indicate significant difference compared to the *DWF4* reporter (p<0.05, Student’s t-test).
Fig. S3. The EAR domain is essential for BES1 function. (A) Western blot of 2-weeks old WT, 35S::bes1-D-GFP (bes1-D) and 35S::bes1-D-EARm transgenic seedlings (EARm13 and EARm33). (B) Phenotype of adult WT, 35S::bes1-D-GFP and 35S::bes1-D-EARm-GFP plants. Plants were grown for 4-weeks under long day conditions (16h light/8h dark). (C) Phenotype of WT, 35S::bes1-D-GFP and 35S::bes1-D-EARm-GFP seedlings grown on BRZ. Seedlings were grown for 6 days under short day conditions (Light) or in continuous dark (Dark) in MS growth media (Mock) or MS media supplemented with 0.5 µM brassinazole (BRZ). (D) Hypocotyl length measurements of plants grown in (C). Bars represent s. d. (n=20). Similar results were obtained in three independent experiments. Asterisks indicate significant difference compared to the WT genotype (p<0.05, Student’s t-test). (E) Expression levels of the CPD, DWF4, ROT3, IAA19 and PRE5 genes in plants grown in short day conditions as in (D). Samples were taken at ZT0. Bars represent the standard deviation of three independent biological replicates. Asterisks indicate significant difference compared to the WT genotype (p<0.05, Student’s t-test).
**Fig. S4.** Phenotypic characterization of the inflorescences. Significant differences by the Tukey’s multiple comparison test are indicated by letters above bars (P < 0.05). (A) A representative flower of the indicated genotypes. (B) Measurement of the pedicel, bud, sepal and petal length of flowers in the primary inflorescences (n=10 plants and 20 flowers of each genotype). F: first open flower; 1, 2 and 3: three oldest buds.
Fig. S5

A

BR UP  2238  44  Boundary enriched  307  1865  BR DOWN

B

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<th>bzr1-1D (BRZ)</th>
<th>bes1-D</th>
<th>BES1 target</th>
<th>BZR1 target</th>
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C

Developmental process

lipid localization

seed maturation

embryo development

plant-type cell wall organization

reproductive system development

post-embryonic development

seed development
Fig. S5. Boundary-enriched BES1/BZR1-repressed targets include several boundary and patterning regulators. (A) Venn diagram showing the overlap of BR differentially expressed genes (Goda et al., 2004; Vert et al., 2005; Sun et al., 2010; Oh et al., 2012) and boundary enriched transcripts (Tian et al., 2014). BR-induced genes are not significantly enriched (44 found, 33 expected, hypergeometric test, p-value >0.01), while BR-repressed genes are strongly enriched in boundary-specific transcripts (94 found, 26 expected, hypergeometric test, p-value = 1.5 x 10^{-25}). (B) Heat-map representation of the expression profiles of the BR-repressed, boundary expressed transcription factors: *bzrl-1D* (Sun et al., 2010), *bzrl-1D BRZ* (Oh et al., 2012) and *besl-1D* (our own data). 8 out of 11 transcription factors were identified as direct BES1- or BZR1- targets (Sun et al., 2010; Yu et al., 2011). (C) Gene ontology analysis of the 94 boundary enriched, BR-repressed genes visualized with ReviGO.
Fig. S6. TPL is expressed in the shoot and root meristems. GUS staining of 6-day old *pTPL::GUS* seedlings. In the root, GUS staining was strong in the cell division and elongation zones. In aerial tissues, strong GUS staining was observed in the SAM and young, actively growing tissues.
**Supplemental Table 1.** Percentage of flowers that contain altered petal number.

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<td>tpl1-OX;bes1-D</td>
<td>105</td>
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### Supplemental Table 2. List of primers used in this study.

<table>
<thead>
<tr>
<th>Primers used for constructs</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>BES1-F</td>
<td>CACCATGACGTCTGACGGAGCAAC</td>
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<tr>
<td>BES1-R</td>
<td>ACCCGGGCAACTATGAGCTTTACCATTTC</td>
</tr>
<tr>
<td>TPL-F</td>
<td>CACCATGTCCTTCCTTAGTAGAGAGGTC</td>
</tr>
<tr>
<td>TPL-R</td>
<td>TCTCTGAGGCTGATCAGATGC</td>
</tr>
<tr>
<td>PIF4-YFP-F</td>
<td>CACCATGGAACACAAAGGTGG</td>
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<tr>
<td>PIF4-YFP-R</td>
<td>TCCGTGTTCAACAGGAACCGTCC</td>
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<tr>
<td>BES1-EARm-R</td>
<td>TCAACTATGAGCTTTACCATTTCAGCCTTTCATCAGGTC</td>
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<tr>
<td>pDWF4-F</td>
<td>CACCGAAATTACCGGTGTATGTAATATAG</td>
</tr>
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<td>pDWF4-R</td>
<td>TGGAGCTAGTTTCTCTCTCTCCTC</td>
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<thead>
<tr>
<th>Primers used for RT-qPCR</th>
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<tbody>
<tr>
<td>CPD-F</td>
<td>TGAAACAACCTCCACGATCA</td>
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<tr>
<td>CPD-R</td>
<td>TGCCCTAATCTTTTCTAGCCTCT</td>
</tr>
<tr>
<td>DWF4-F</td>
<td>CGACGTGGGAAACAACTAC</td>
</tr>
<tr>
<td>DWF4-R</td>
<td>CTGAAACCAGCAGCATACGCTGTG</td>
</tr>
<tr>
<td>ROT3-F</td>
<td>ATCCGTTGAGATGGGACA</td>
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<tr>
<td>ROT3-R</td>
<td>AACCAGGACATAGCCTTTTGC</td>
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<tr>
<td>IAA19-F</td>
<td>GAGCTGAGATGGGGGCT</td>
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<td>IAA19-R</td>
<td>CCGACGACGTCTATTCTCATCT</td>
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<tr>
<td>PRE5-F</td>
<td>TGCTTCTGAGATCTCGATGACCA</td>
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<tr>
<td>PRE5-R</td>
<td>GCCGTCTCCTAATCTCCGGGCA</td>
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<td>SAUR15-F</td>
<td>AAGGGAATCTCGTCGACAC</td>
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<tr>
<td>SAUR15-R</td>
<td>AAGTATGAAACCAGGCACCAC</td>
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<table>
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<tr>
<th>Primers used for ChIP-qPCR</th>
<th>Sequence</th>
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<tbody>
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<td>CUC3p-F</td>
<td>TGCATAAGTCGTGCAATCTAA</td>
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<tr>
<td>CUC3p-R</td>
<td>TACTGGAAGACACGGAGGCTT</td>
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<tr>
<td>CUC3 ORF-F</td>
<td>ACAGAGCAGCTTCCAGAAGGTA</td>
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<tr>
<td>CUC3 ORF-R</td>
<td>GCTGGAATCAAAGGGCATG</td>
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<tr>
<td>PP2A-F</td>
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<tr>
<td>PP2A-R</td>
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<td>DWF4p-2F</td>
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<td>DWF4p-2R</td>
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<tr>
<td>BRAVOp-F</td>
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<tr>
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