The TIE1 transcriptional repressor controls shoot branching by directly repressing BRANCHED1 in Arabidopsis

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Abstract

Shoot branching is a major determinant of plant architecture and is regulated by both endogenous and environmental factors. BRANCHED1 (BRC1) is a central local regulator that integrates signals controlling shoot branching. So far, the regulation of BRC1 activity at the protein level is still largely unknown. In this study, we demonstrated that TIE1 (TCP Interactor containing EAR motif protein 1), a repressor previously identified as an important factor in the control of leaf development, also regulates shoot branching by repressing BRC1 activity. TIE1 is predominantly expressed in young axillary buds. The gain-of-function mutant tie1-D produced more branches and the overexpression of TIE1 recapitulated the increased branching of tie1-D, while disruption of TIE1 resulted in lower bud activity and fewer branches. We also demonstrated that the TIE1 protein interacts with BRC1 in vitro and in vivo. Expression of BRC1 fused with the C-terminus of the TIE1 protein in wild type caused excessive branching similar to that observed in tie1-D and brc1 loss-of-function mutants. Transcriptome analyses revealed that TIE1 regulated about 30% of the BRC1-dependent genes, including the BRC1 direct targets HB21, HB40 and HB53. These results indicate that TIE1 acts as a positive regulator of shoot branching by directly repressing BRC1 activity. Thus, our results reveal that TIE1 is an important shoot branching regulator, and provide new insights in the post-transcriptional regulation of the TCP transcription factor BRC1.

Author summary

Shoot branching is a key factor that not only affects plant survival but also determines food productivity in crop species. BRANCHED1 (BRC1) integrates internal and external signals to determine shoot branching. However, the regulation of BRC1 at the protein level remains elusive. We found that TIE1 (TCP Interactor containing EAR motif protein...
plays an important role in the control of shoot branching in Arabidopsis. Higher TIE1 expression levels lead to bushier Arabidopsis plants. TIE1 directly interacts with BRC1 and represses BRC1 transcriptional activity. Furthermore, BRC1 downstream target genes are downregulated by TIE1. Our findings demonstrate that TIE1 acts as a key repressor of BRC1 activity and positively regulates shoot branching.

Introduction

Shoot branching greatly affects plant architecture, one of the most important agronomic traits. The manipulation of shoot branching patterns is an efficient way to promote and manage crop production [1]. Shoot branching is a developmental process with a high plasticity and tightly regulated by diverse endogenous and environmental stimuli. The development of shoot branches starts from the initiation of axillary meristems (AMs) in the leaf axils. The AMs then develop into small buds with a few leaves, which either remain dormant or grow to form branches in response to internal or external cues [2].

Genetic analyses have identified several important transcriptional regulators, which form a complex regulatory network during the initiation of AMs [3]. However, few transcriptional regulators have been found to control local bud activity. TEOSINTE BRANCHED1 (TB1) is an important domestication gene of maize that plays a central role in the control of shoot branching. TB1 is a founder member of the TCP (TB1/CYCLOIDEA/PCF) family of transcription factors conserved in the plant kingdom. In both monocots and dicots, orthologs of TB1 play a pivotal role in the control of bud activity. Examples of this are the rice FINE CULM 1/OsTB1, sorghum SbTB1, Arabidopsis BRANCHED1 (BRC1), and tomato, pea and potato BRC1-like genes [4–9]. The Arabidopsis BRC1 gene is predominantly expressed in developing axillary buds (axillary meristems, bud leaf primordia and subtending vascular tissue) and its expression levels decrease as buds grow out. BRC1 acts as a suppressor of bud activity: loss-of-function brc1 mutants display accelerated initiation of axillary meristem formation, faster bud development and more branches [6,10].

Increasing evidence indicates that BRC1 is an integrator of diverse internal and external signals that control bud activity. The branch-suppressing hormone strigolactone (SL) controls shoot branching in part by positively regulating BRC1 at the transcriptional level in Arabidopsis and pea [6,8,10–12]. The brc1 mutants are insensitive to SL treatments and epistatic to smx6 smxl7 smxl8 triple mutants [8,13, 14]. On the contrary, the hormone cytokinin (CK) negatively regulates BRC1 expression and promotes shoot branching in rice and pea [8,15], although the branching of Psbrc1 pea mutants still respond to CK treatments [8]. Likewise, sugar is an important nutritional and signaling element proposed to be necessary for axillary bud outgrowth. BRC1 transcript levels are reduced after sucrose application to buds [16–18], whereas low sucrose levels upregulate TB1 expression in wheat [19]. In addition to the endogenous signals, BRC1 is also regulated by numerous external inputs. For example, changes in light quality (i.e. a reduction in the red-to-far red light ratio) upregulate BRC1 and lead to suppression of bud growth [20,21].

A TB1 upstream regulator, IDEAL PLANT ARCHITECTURE1 (IPA1), has been identified in rice. IPA1 is a transcription factor that promotes the expression of OsTB1 by directly binding to its promoter region [22]. TB1 downstream targets [23,24] and also BRC1 targets begin to be characterized. Three HD-ZIP transcription factor-encoding genes, HOMEBOX PROTEIN (HB) 21, HB40 and HB53 have been shown to be directly regulated by BRC1 in Arabidopsis [18]. These HD-ZIPs and BRC1 together, upregulate 9-CIS-EPOXICAROTENOID DIOXIGENASE 3
(NCED3), which encodes a key enzyme in ABA biosynthesis to promote bud dormancy. However, although the transcriptional regulation upstream and downstream of BRC1 begins to be understood, the regulation of BRC1 activity at the protein level is still very poorly known.

TCP Interactor containing EAR motif protein 1 (TIE1) was identified as a nuclear transcriptional repressor that regulates leaf development [25]. Overexpression of TIE1 in the activation-tagging mutant tie1-D causes hypynastic leaves, while the disruption of TIE1 leads to epinastic leaves. The TIE1 protein interacts with CIN-like TCP transcription factors and it also recruits the transcriptional corepressors TOPOLESS (TPL)/TOPOLESS-RELATED (TPR). Formation of this protein complex leads to a repression of the activity of CIN-like TCP transcription factors. The association of TIE1 with these TCPs further leads to an altered expression of TCP target genes, such as LOX2, AS1 and IAA3. However, tie1-D and jaw-D, in both of which the TCP activity was downregulated, did not display completely identical phenotypes, which indicates that TIE1 may also bind other transcription factors and regulate additional biological processes. In addition, TIE1 is regulated by E3-ligase proteins termed TIE1-associated RING-type E3 ligases (TEARs) [26]. TEARs interact with TIE1 and are responsible for TIE1 degradation, which boosts CIN-like TCP activity during leaf development.

Here, we report that the transcriptional repressor TIE1 positively controls shoot branching by directly regulating BRC1 protein activity. We demonstrate that overexpression of TIE1 leads to higher bud activity and more branches, whereas disruption of TIE1 causes reduced bud activity and branch suppression. TIE1 is predominantly expressed in axillary buds and is negatively regulated as buds grow out. TIE1 represses the expression of BRC1 target genes, probably by directly interacting with BRC1 and antagonizing its activity. Our data reveals a novel molecular mechanism by which plants control BRC1 activity accurately and flexibly via TIE1 at the protein level to determine bud activity in response to endogenous and environmental cues.

Results

Overexpression of TIE1 causes excessive branching

We previously identified a transcriptional repressor, TIE1, essential for the control of leaf development [25]. The gain-of-function tie1-D mutant, obtained by T-DNA activation-tagging, displays strong leaf developmental defects. We noticed that tie1-D also produced an excessive number of branches (S1 Fig), suggesting a possible role of TIE1 in the control of shoot branching. To test this possibility, we first generated transgenic lines carrying the construct 35S-GFP-TIE1, in which the TIE1 coding sequence (CDS) fused to the GREEN FLUORESCENT PROTEIN (GFP) was driven by the Cauliflower Mosaic Virus 35S promoter (CaMV35S). The 35S-GFP-TIE1 plants displayed epinastic leaves as observed in tie1-D mutants (Fig 1A), which indicated that the GFP-TIE1 fusion protein was functional. We analyzed three 35S-GFP-TIE1 independent transgenic lines and found that all three lines produced more branches than the wild-type controls, and recapitulated the branching phenotype of tie1-D (Fig 1A–1C and S1 Fig). Because the homozygous tie1-D plants are sterile, we investigated the branching phenotype of plants of the fertile 35S-GFP-TIE1-19 line in detail. The results showed that although this TIE1 overexpression line generated fewer rosette leaves than the wild-type plants (Fig 1A–1C), almost all the buds grew out to form branches, whereas in the wild-type controls most buds remained small at this stage (Fig 1B and 1C). These results suggest that TIE1 is a positive regulator of axillary bud activity and shoot branching.

Disruption of TIE1 causes defects of shoot branching

TIE1 belongs to a gene family with a high functional redundancy [25]. To overcome the difficulties caused by such genetic redundancy, we used a dominant-negative strategy to interfere
Fig 1. Overexpression of TIE1 leads to excessive branching. (A) Branching phenotypes of 40-day-old wild-type plants and representative individuals of three independent 35S-GFP-TIE1 lines. The 35S-GFP-TIE1 mutants produce more branches than wild-type plants. Scale bar = 1 cm. On top, close-up details of the rosette base in wild type and TIE1 overexpression line. Schematic representations (left) and quantification (right) of rosette leaf axillary buds and branches of 40-day-old wild-type (B) and 35S-GFP-TIE1-19 plants (n = 8) (C). Left, arrows indicate
with the function of all the TIE family members. We generated a 35S-TIE1mEAR-VP16 construct in which the TIE1 repressor was changed into an activator by mutating the EAR motif of TIE1 and fusing it to the VP16 activation domain. This approach has been successfully used to disrupt TIE genes redundancy in our previous study [25]. We examined all the rosette leaf axils of wild-type and 35S-TIE1mEAR-VP16 transgenic plants (Fig 2). Under the same growth conditions in which wild-type controls produced several branches, TIE1mEAR-VP16 plants only had axillary buds but no branches (Fig 2A–2C). In addition, the degree of axillary bud development in the mutant and wild-type plants was classified into four classes, based on absence/presence of visible axillary leaf primordia and on axillary bud size (Fig 2D). The detailed analysis of the branching phenotypes showed that axillary bud development was obviously delayed in TIE1mEAR-VP16 plants when compared to wild-type controls (Fig 2D): TIE1mEAR-VP16 plants produced fewer branches than controls, and had more axils without a visible axillary bud. These results indicate that, like BRC1, TIE1 regulates shoot branching from the early stages of axillary bud development.

### TIE1 is expressed during axillary bud development

To characterize in more detail the spatial and temporal expression patterns of TIE1 during bud development, a 2790-bp genomic fragment upstream of the TIE1 translation start codon was fused to the β-GLUCURONIDASE (GUS) reporter gene to generate a TIE1pro-GUS construct [25]. GUS staining analyses of the TIE1pro-GUS transgenic lines revealed that TIE1 was predominantly expressed in developing axillary buds (Fig 3A–3G). In young axillary buds, signal was detectable throughout the leaf primordia (Fig 3A, 3C and 3E). As the buds developed, GUS signal became progressively more restricted to the base of the buds, and to the bud leaf vasculature (Fig 3B, 3D, 3F and 3G). When buds grew out into shoots, GUS activity was almost undetectable (Fig 3H). In addition, GUS accumulated in the stem vasculature, in particular in the phloem (Fig 3I), young leaf veins (Fig 3A and 3B) and sepal vasculature (Fig 3J). The expression patterns of TIE1 during axillary bud development resemble those of BRC1 [6]. These results are consistent with an important role of TIE1 in the control of axillary bud activity.

### TIE1 interacts with the transcription factor BRC1

To investigate the molecular mechanisms by which TIE1 regulates shoot branching, we performed a yeast two-hybrid screening of an Arabidopsis transcription factor library to identify TIE1 interactors, using as a bait of a protein containing the N-terminal (N-t) 108 amino acid residues of TIE1 [25]. The results showed that the N-t region of TIE1 interacted with BRC1 but not with BRC2. We therefore cloned the CDS of Arabidopsis BRC1 and BRC2 to verify this interaction. The yeast-two-hybrid assays confirmed that BRC1 interacted with TIE1, whereas BRC2 did not (Fig 4A). We then performed additional experiments to further investigate BRC1-TIE1 interaction in vitro and in vivo. First, we expressed BRC1 fused to the MALTOSE BINDING PROTEIN (MBP-BRC1) and His-tagged TIE1 in Escherichia coli and purified them for in vitro pull-down assays. Specific binding of His-tagged TIE1 was detected in the MBP-BRC1 after eluting six times, while no band of His-tagged TIE1 was observed in the control MBP, indicating that TIE1 interacted strongly with BRC1 in vitro (Fig 4B). Then, we confirmed the association of BRC1 with TIE1 in vivo by BiFC and firefly luciferase complementation.
Fig 2. Disruption of TIE1 leads to reduced shoot branching. (A) Expression of TIE1mEAr-VP16 chimeric protein causes defects in shoot branching. 45-day-old wild-type (left) and TIE1mEAr-VP16 transgenic plants (right). Rosettes of wild-type (B) and TIE1mEAr-VP16 plants (C). The primary inflorescence has been removed for better visualization of the axillary structures. Arrows point to branches (B) and buds (C). (D) Quantitative analysis of rosette axillary shoot development in 45-day-old wild-type and TIE1mEAr-VP16 plants. The bud developmental
stages were classified as follows: No bud: no axillary leaf primordia visible (grey); Small bud: axillary leaf primordia visible (green); Big bud: axillary leaf bearing many trichomes (yellow); Branch: visible elongating lateral inflorescence (blue). Representative images of these stages are shown in the right panels (from bottom to top). The white box indicates that no leaf is formed at the position. Scale bar = 1 mm.

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Fig 3. **TIE1 is expressed in the axillary buds.** GUS histochemical activity of Arabidopsis TIE1p:GUS. Side view of young (A) and older (B) vegetative axillary buds (red arrows) and their subtending rosette leaves. Notice the GUS accumulation in young bud leaf primordia (red arrows), rosette leaf vasculature (black arrowheads) and the bud leaves (white arrowheads). Top view of the rosettes buds around the stem; young (C) and older (D) axillary buds (arrows) are shown. In older buds, GUS activity is restricted to the base of the bud. (E to H) close-up view of axillary buds of sequential developmental stages. GUS signal becomes progressively restricted to the base of the bud (arrowhead in G). At the time of bud outgrowth the signal is no longer detectable (H). 3-mm transverse plastic-embedded section of a main stem showing GUS staining in phloem (ph) cells (I), x. xylem; c. cambium. (J) Close-up of a developing flower. Signal in the vasculature is indicated (white arrowhead). Scale bars = 1mm in (A) to (D), and (H); 50 μm in (E) and (I), 100 μm in (F) and (G).

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Fig 4. TIE1 interacts with BRC1 in vitro and in vivo. (A) Yeast two-hybrid assays of TIE1 with BRC1 and BRC2. The N-t of TIE1 (1 to 108 residues) was fused to GAL4–DBD to generate the bait protein. The transcription factors BRC1 and BRC2 were fused with the GAL4–AD to generate the prey protein. AD, activation domain; DBD, DNA binding domain. Co-transformed yeast cells were grown on medium lacking Leu and Trp (SD-Leu-Trp) and selected on medium lacking Leu, Trp and His (SD-Leu-Trp-His) with 5 mM 3-amino-1,2,4-triazole (3-AT). The empty vectors PDEST32 or PDEST22 were used as negative controls. The three spots are triplicates of the experiment. (B) Pull-down assays to test TIE1 interaction with BRC1 in vitro. The MBP-BRC1 was used to pull down TIE1-His with the MBP as a negative control. The mixtures MBP-BRC1/TIE1-His and MBP/TIE1-His were incubated with Amylose resin (NEB) and the beads were washed six times to avoid the false positive. The “elution 1” and “elution 6” mean the supernatant in the first wash and sixth experiment. (B) Pull-down assays to test TIE1 interaction with BRC1 MBP as a negative control. The mixtures MBP-BRC1/TIE1-His and MBP/TIE1-His were incubated with Amylose resin (NEB) and the beads were washed six times to avoid the false positive. The “elution 1” and “elution 6” mean the supernatant in the first wash and sixth wash of the MBP-BRC1/TIE1-His and MBP/TIE1-His beads, respectively. In the sixth wash, the TIE1-His was not detected in the supernatant, indicating the amylose resin was cleaned up and avoiding the false positive. After the six washes, the bound proteins in the beads (precipitate) were eluted with 2× SDS buffer and detected with anti-His6. The TIE1-His could be pulled down by MBP-BRC1 (the lane designated MBP-BRC1 output) but not by MBP control (the lane designated MBP output). (C) BicFC assay of the interaction between TIE1 and BRC1. GFP signal was observed in Nicotiana benthamiana leaves co-transformed with nYFP/TIE1 and cCFP-BRC1 vectors. cCFP-BRC1/empty nYFP and nYFP-TIE1/empty cCFP were used as negative controls. Yeast two-hybrid analyses showed that the TCP domain in yeast two-hybrid assays. Left, schematic representation of the different BRC1 deletions and BRC1 mutant proteins (in the TCP domain: tcp<sup>WL</sup>, tcp<sup>DRV</sup>, tcp<sup>PD</sup> or in the R domain: BRC1mR) that were fused to the GAL4 prey (AD). Yeast two-hybrid assays were carried out between these BRC1-AD proteins and an N-t of TIE1 (1 to 108 residues) fused to GAL4–DBD bait. The empty vectors pGADT7 or pGBK7 were used as negative controls. The three spots are triplicates of the experiment.

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imaging assays (Fig 4C and 4D). Finally, BRC1 and TIE1 interaction was further confirmed by acceptor photobleaching fluorescence resonance energy transfer (APB-FRET) using transient assays in Nicotiana benthamiana leaves (Fig 4E).

BRC1 contains several important domains including a TCP domain for dimerization and DNA binding, and an R domain of unknown function [27]. To map the regions of the BRC1 protein necessary for interaction with TIE1, we generated a series of BRC1 deletions lacking different regions of the protein (Fig 4F). Yeast two-hybrid assays showed that the TCP domain was necessary and sufficient for the interaction between TIE1 and BRC1, whereas the R domain was not required for the interaction. We further assayed three BRC1 proteins with point mutations in the TCP domain, two in the basic region and one in helix II. All three mutations disrupted the TIE1-BRC1 interaction (Fig 4F), whereas a point mutation in the R domain did not affect the interaction. These data demonstrate that BRC1 interacts with the N-t region of TIE1 through its TCP domain. These results together with the observed overlapping expression patterns of BRC1 and TIE1 in axillary buds support the existence of this interaction in planta.

We further examined whether other members of the TIE family could interact with BRC1. Yeast two-hybrid analyses showed that TIE2 and TIE4 also interacted with BRC1, while TIE3 did not (S2 Fig). These results suggest that several members of the TIE protein family may control shoot branching by directly interacting with BRC1.

**TIE1 represses BRC1 activity**

We then examined whether BRC1 transcriptional activity could be regulated by interaction with the transcriptional repressor TIE1. For that we used the reporter construct HB53<sub>pro</sub>-LUC, in which the LUCIFERASE (LUC) gene is driven by a 2000 bp promoter region of HB53, which is a BRC1 direct target gene [18]. We co-infiltrated Nicotiana benthamiana leaves with HB53<sub>pro</sub>-LUC and 35S-BRC1 and 35S-MYC-GFP control. LUC activity analysis indicated that BRC1 was able to activate directly the HB53<sub>pro</sub>-LUC. In contrast when HB53<sub>pro</sub>-LUC and
35S-BRC1 were co-infiltrated with 35S-TIE1-MYC-GFP, the activation of HB53pro-LUC was very much reduced (Fig 5A), suggesting that TIE1 inhibits BRC1 transcriptional activity. We have shown that TIE1 serves as a bridge between TCPs (with its N-t region) and the corepressors TPL/TPRs (with its C-t region) [25]. To further examine the effects of the TIE1-BRC1 interaction, we generated a fusion protein BRC1-TIE1C in which BRC1 was physically linked with the C-t of TIE1 (from the 108th amino acid residue to the stop codon). The fusion protein

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**Fig 5.** TIE1 inhibits BRC1 transcriptional activity. (A) A 2kb-long genomic region upstream of HB53 gene fused to LUC was used as a reporter for the transactivation assay. Results are represented as mean ± SEM (n = 6). Co-infiltration of BRC1 with TIE1 (light green line) considerably reduced the activation of the promoter as compared to the co-infiltration with the MYC-GFP epitope (red line; *** p < 0.005; ** p < 0.01 in a two-tailed Student’s T test). (B) Phenotype of 45-day-old transgenic plants expressing a chimeric BRC1-TIE1C (right) or a 35S-TIE1C control (left). Scale bar = 1 cm. (C) Quantification of buds and branches in the rosette leaf axils of 40-day-old 35S-TIE1C and 35S-BRC1-TIE1C plants (n = 8). Each box represents a rosette leaf node. Purple box indicate bud; blue, branch; grey, empty axil; white, no leaf formed at the position.

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was expressed in wild-type plants using a CaMV 35S promoter. The detailed branching phenotypes of these transgenic plants were analyzed (Fig 5B and 5C). The 35S-BRC1-TIE1C transgenic lines produced about six branches, whereas the control 35S-TIE1C transgenic plants produced two to three branches under the same growth conditions (Fig 5B and 5C). The increased branching of 35S-BRC1-TIE1C transgenic lines indicated that BRC1 protein activity was affected by the fusion with TIE1, probably due to the recruitment of TPL/TPRs corepressor by TIE1 C-t.

**TIE1 regulates genes involved in shoot branching**

To further elucidate the mechanisms by which TIE1 regulates shoot branching, we performed RNA-seq transcriptome analysis of rosette leaf axil tissue (highly enriched in axillary buds) of 35S-GFP-TIE1 overexpression lines and wild-type controls. We found that 1503 genes were upregulated and 1151 genes downregulated in the TIE1 overexpression line (q value < 0.05; fold change ≥ 1.5 and ≤ -1.5) (S1 Table). We compared these genes with a list of 307 BRC1-dependent genes (False Discover Rate < 0.05) [20], and found a negative correlation between TIE1-responding genes and the BRC1-dependent genes: TIE1-upregulated genes appeared at a much higher frequency than expected for a random gene list among the BRC1-downregulated genes (30% of the BRC1-downregulated genes, p value = 2.1E-18 in a hypergeometric test) (Fig 6A and S2 Table). Likewise TIE1-downregulated genes were significantly enriched among the BRC1-upregulated genes (22% of the BRC1-upregulated genes, p value = 1.5E-27 in a hypergeometric test) (Fig 6A and S2 Table), supporting that TIE1 acts to antagonize BRC1 activity during bud development. These enrichments were remarkably higher than those found when the comparison was done between TIE1-dependent genes and the BRC1-independent genes obtained in the same experiment (i.e. genes that respond to a low Red:Far red light ratio, both in wild type and brc1 mutants) [20].

Then we further compared TIE1-dependent genes with a particular subset of BRC1-dependent genes that also responded significantly to decapitation [28]. Genes upregulated in response to BRC1 and downregulated 24 hours after decapitation were termed Bud dormancy genes. Genes downregulated in response to BRC1 and upregulated 24 hours after decapitation were termed Bud activation genes [20]. Again, TIE1-dependent upregulated genes appeared among Bud activation genes at a much higher frequency than that expected in a random gene list (33%, p value = 6.55E-15) and TIE1-dependent downregulated genes were significantly enriched among Bud dormancy genes (29%, p value = 5.29–29).

Interestingly, three BRC1 direct targets, HB21, HB40 and HB53 [18], were among the TIE1-downregulated genes (Fig 6C, S2 and S3 Tables). Quantitative RT-PCR analysis confirmed that the transcriptional levels of these genes were significantly lower in the TIE1 overexpression line and in brc1-2 mutants than in wild-type controls (Fig 6D and 6F). These data indicate that TIE1 modulates the expression of sets of BRC1-dependent, bud activation and dormancy genes.

Finally, we also found that in our RNA-seq data BRC1 was downregulated (S3A Fig), and qRT-PCR analyses confirmed that BRC1 mRNA levels were much lower in TIE1 overexpression lines than in the wild-type control (S3B Fig). Conversely, BRC1 expression levels were significantly higher in three 35S:TIE1mEAR-VP16 lines than in the wild-type control (S3C Fig). These results suggest that TIE1 may also directly or indirectly regulate BRC1 at the transcriptional level.

**Discussion**

In this study, we discovered that the transcriptional repressor TIE1 is a regulator of shoot branching. The gain-of-function mutant tie1-D and transgenic plants overexpressing TIE1 produce more branches, whereas TIE1 loss-of function leads to lower bud activity and fewer
In addition, we demonstrated that TIE1 interacts, in vitro and in vivo, with BRC1, a transcription factor that plays an important role in the control of bud activity. Furthermore, TIE1 is expressed in young axillary buds and is regulated during bud development in patterns that overlap with those of BRC1 [6], further supporting the possibility that TIE1 interacts with BRC1 in planta. By binding to BRC1, TIE1 inhibits BRC1 activity and consequently represses the transcription of many BRC1 target genes (Fig 7A and 7B). Our data not only demonstrate that TIE1 is an important regulator in the control of shoot branching, but also provides evidence of a novel layer of regulation of BRC1 at the protein level.

TCP proteins are plant-specific transcription factors that group into class I and class II subclasses on the basis of sequence similarity [29,30]. The class II TCPs are further categorized into CINCINNATA-like TCPs and CYCLOIDEA/TB1 (CYC/TB1)-like TCPs [30]. The modulation of TCP activity at the protein level is important for plant development. Some proteins, including the SWI/SNF chromatin remodeling ATPase BRAHMA (BRM) and the ARMA-DILLO BTB ARABIDOPSIS PROTEIN1 (ABAPI) have been reported to interact with CIN-like TCPs and regulate their activity [31,32]. Recently, we found that the EAR-motif containing repressor TIE1 suppresses CIN-like TCP activity by recruiting the transcriptional co-

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**Fig 6.** TIE1 regulates BRC1-dependent genes involved in bud development. (A) to (C) Venn diagrams showing significant overlap between TIE1-regulated genes and BRC1-dependent genes. Detailed gene information is shown in S1–S3 Tables. (D) to (F) mRNA levels of HB21, HB40 and HB53 were quantified by qRT-PCR in wild type, 35S-GFP-TIE1 plants and brc1-2 mutants. The expression was normalized to AtUBQ10 levels, and was relative to wild-type levels. Data represents mean ± SD from three biological replicates. Significant differences are indicated ***P < 0.001 (two-tailed Student’s t-test).

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repressors TOPLESS (TPL)/TOPLESS-RELATED (TPR) proteins during leaf development [25]. A yeast two-hybrid screening revealed that TIE1 also interacts with BRC1 (a TCP factor of the CYC/TB1 subclade), a prominent bud-specific regulator of shoot branching [6,10]. However, the role of TIE1 in the control of shoot branching had not been identified so far. In this paper, our genetic and biochemical data indicate that TIE1 is not only an important factor regulating CIN-like TCPs, but also regulates BRC1 at the protein level. TIE1 interacts with BRC1 and prevents the transcription of BRC1 target genes. Like in the case of CIN-like TCPs, TPL/TPRs could be recruited by TIE1 to repress BRC1 activity during bud development (Fig 7A and 7B). This is consistent with the observation that plants expressing a BRC1 fusion protein carrying the C-terminal of TIE1 produce more branches.

Interestingly, other TPL/TPR-interacting proteins have been previously implicated in the control of shoot branching through SL signaling. Indeed, the rice SL signaling repressor D53, and its orthologs in Arabidopsis, SMXL6, SMXL7 and SMXL8, interact with TPL/TPR proteins [33–36]. This interaction may promote TPL/TPR oligomerization and formation of a repressor-corepressor nucleosome complex [37]. This interaction has been proposed to be responsible for the transcriptional repression of OsTB1/BRC1 although this is yet unclear.

Fig 7. Model for TIE1 function in the BRC1-dependent regulation of shoot branching. (A) In the absence of TIE1, BRC1 promotes the expression of bud dormancy genes such as HB53 and inhibits shoot branching. (B) and (C) When TIE1 binds BRC1, TIE1 recruitment of TPL/TPR co-repressors [25] leads to transcriptional repression of BRC1 targets (B) or TIE1 sequesters and prevents BRC1 binding to the promoters of target genes (C). Bud dormancy genes such as HB53 are downregulated and shoot branching is promoted.

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Our findings show that TPL/TPRs are also recruited by TIE1 to directly repress BRC1 at the protein level, suggesting that the TPL/TPRs use different molecular mechanisms to control shoot branching. Furthermore, rice D53 interacts with Ideal Plant Architecture 1 (IPA1), another negative regulator of shoot branching [40–42] that binds the OsTB1/FINE CULM1 promoter [22], and may affect its expression. This interaction, conserved in wheat, leads to suppression of the transcriptional activity of IPA1-like factors [42]. These observations indicate that TPL/TPRs-interacting proteins, such as TIE1-like and D53-like proteins, play important roles in the control of shoot branching both in dicots and monocots. The rice genome has six TIE1 homologs [43,44]. It will be very interesting to determine whether OsTIEs interact with OsTB1/FINE CULM1 and TPL/TPR corepressors.

Remarkably, our deletion and mutation analysis suggested that TIE1 interacts with the TCP domain of BRC1, responsible for DNA binding [30]. The TCP domain of BRC1 is necessary and sufficient for the TIE1 and BRC1 interaction, because point mutations in the TCP domain completely abolish this interaction. This raises the alternative possibility that TIE1 represses BRC1 activity by preventing BRC1 binding to DNA as it has been described for DELLA proteins, which repress the activity of TCP14 and TCP15 by interacting with their TCP domains [45]. Therefore, TIE1 is likely to inhibit BRC1 activity either by recruiting the transcriptional repressor machinery and/or by hindering the TCP domain of BRC1 from binding the promoters of target genes (Fig 7B and 7C). These two mechanisms may work together to precisely regulate BRC1 activity and shoot branching in response to internal and external cues. In addition, we found that BRC1 itself was down-regulated by TIE1, which indicates that TIE1 may also control directly or indirectly BRC1 at the transcriptional level. Investigating how this transcriptional and post-transcriptional regulation of BRC1 by TIE1 affects plant architecture remains to be determined.

Recently, ABA has been reported to negatively regulate axillary bud growth in Arabidopsis [46]. BRC1 is an important regulator of ABA signaling in buds partly through the regulation of three genes, HB21, HB40 and HB53, encoding HD-ZIP transcription factors [18]. Our results showed that TIE1 regulates about one third of the BRC1-dependent genes induced in dormant buds including HB21, HB40 and HB53, which raises the possibility that TIE1 helps BRC1 finely tune the transcriptional level of these branching control genes.

It is worth noting that the phenotype of TIE1 gain-of-function plants is not identical to that of brc1 mutants: in addition to an excess of branching, the former display many other phenotypes including epinastic leaves, dwarfism and early flowering [25]. Indeed, TIE1 expression in tissues other than axillary buds (e.g. leaf, sepal and stem vasculature) as well as the reported interaction of TIE1 with other transcription factors and TPL/TPRs (see above) may account for these phenotypes unrelated to shoot branching of TIE1 gain-of-function lines.

We have recently found that TIE1 is ubiquitinated by several E3-ligase proteins TEARs (TIE1-associated RING-type E3 ligases) for degradation [26]. Interestingly, disruption of TEARs using the dominant-negative strategy and sextuple tear mutants also cause excessive branching [26], this is consistent with our observation in this study that overexpression of TIE1 promotes shoot branching. The characterization of potential additional components of the molecular machinery that controls shoot branching via modulation of the activity of the BRC1 protein will help us further understand the complex regulatory mechanisms that determine plant shoot architecture in response to environmental cues.

Materials and methods

Plant materials and growth conditions

The Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used in this study. The mutants tie1-D and brc1-2 were described previously [6, 25]. Half-strength Murashige and Skoog
medium with or without 20 μg/mL DL phosphinothricin or 50 μg/mL kanamycin were used for growing or screening the plant seeds. The plates with seeds were placed at 4°C for 2 d synchronization before being incubated at 22°C under long-day conditions (16-h light and 8-h dark, 70% relative humidity). The seven-day-old seedlings were transferred to soil and were grown under the same conditions as described above.

**Generation of binary constructs and transformation**

To generate the TIE1 overexpression line, the TIE1 coding sequence was amplified from Arabidopsis seedling cDNA using the primer pairs TIE1-F/R (S4 Table). The PCR product was cloned into pENTR/D TOPO (Invitrogen) to generate pENTR-TIE1. Then, the overexpression construct 35S-GFP-TIE1 was generated by an LR reaction between pENTR-TIE1 and pB7GWF2 (Ghent University). To examine the temporal and spatial expression pattern of TIE1, the 2790-bp genomic fragment upstream of TIE1 start codon was amplified using the primers TIE1P-F and TIE1P-R and was cloned into pENTR/D-TOPO to generate pENTR/D-TIE1P. TIE1P-GUS was generated by LR reaction between pENTR/D-pTIE1 and pKGWFS7 (Ghent University). To generate 35S-BRC1-TIE1C construct, the coding region of BRC1 without a stop codon was amplified from Arabidopsis seedling cDNA with primers BRC1-F1/R1 and further was cloned into pENTRY/D-TOPO to generate pENTRY-BRC1N. The CaMV 35S promoter was amplified from vector pWM101 with primers p35S-F/R. The fragment was cloned into pDONRP4P1r (Invitrogen) to generate pENTRY-L4-35S-R1. The C-t of TIE1 sequence was amplified from pENTR-TIE1 using primers TIE1C-F/R and was cloned into pDONRP2rP3 (Invitrogen) to generate pENTRY-R2-TIE1C-L3. The 35S-BRC1-TIE1C construct was generated by LR reaction from pENTRY-L4-35S-R1, pENTRY-BRC1N, and pENTRY-R2-TIE1C-L3 and pK7m3GW (Ghent University). To generate 35S-TIE1mEAR-VP16, TIE1mEAR was amplified with primers TIE1m-F/R and the PCR product was cloned into NTRY/D-TOPO to generate pENTRY-TIE1mEAR. The coding region of VP16 was amplified from pTA7002 [47] and was cloned into pQDR2L3 with primers VP16-F/R to generate pENTRY-R2-TIE1C-L3. The 35S-TIE1mEAR-VP16 construct was generated by LR reactions among plasmids pK7m3GW, pENTRY-L4-35S-R1, pENTRY-TIE1mEAR and pENTRY-R2-VP16-L3. These constructs were transformed into Agrobacterium tumefaciens GV3101/pMP90 by electroporation method and then into Arabidopsis as described previously by floral dip method [48].

**Histochemical GUS staining**

For GUS staining, tissues from TIE1pro-GUS lines were soaked in 90% acetone solution for 20 mins on the ice and washed by phosphate buffer twice. Then the samples were incubated in GUS staining buffer containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide and vacuumed for 30 min before incubation overnight at 37°C. The staining buffer was then replaced by 70% ethanol for decolorizing before microscopy analysis. Plastic embedding and sectioning of GUS-stained stem fragments of adult plants was carried as described in Chevalier et al. [49].

**Gene expression assays**

For quantitative RT-PCR, total RNAs of the tissues around leaf axils from 25-day-old wild-type, 35S-GFP-TIE1 and brc1-2 were extracted using TRIzol reagent (Invitrogen). Reverse transcription was carried out using Superscript II Reverse Transcriptase Kit (Invitrogen). Quantitative RT-PCR was performed with three biological repeats using SYBR Green Realtime PCR Master Mix (Toyobo) and using the diluted cDNA as the template. The 2^-ΔΔCT method was
used to calculate the relative expression level of each gene [50]. Primers used were listed in S4 Table. *AtUBQ10* gene was used as an internal control.

For RNA-seq, total RNAs of the tissue surrounding leaf axils enriched in axillary buds from 25-day-old wild-type and 35S-GFP-TIE1 plants were extracted using TRizol reagent. The RNA-seq was performed on the Illumina HiSeq 2000 platform (Illumina) at the Biodynamic Optical Imaging Center (BIOPIC) of Peking University. The bioinformatic and statistical analysis of the RNA-seq data was performed according to the procedures described previously [51]. Genes with changes of more than 1.5-fold (Q-value ≤ 0.05) were defined as differentially expressed genes. The hypergeometric test was performed in the software environment R (CRAN) using the phyper function.

**Yeast two-hybrid assays**

To test the interaction between TIE1 and BRC1/BRC2, the N-t of TIE1 (1–108) and the coding sequences of BRC1/BRC2 were amplified with primers TIE1N-F/R and BRC1-F/R or BRC2-F/R listed in S4 Table. The products were cloned into pENTR/D-TOPO to generate pENTRY-TIE1N and pENTRY-BRC1/BRC2. The bait construct DBD-TIE1N was generated by LR reaction between pDEST32 (Invitrogen) and pENTRY-TIE1N. The prey construct AD-BRC1/BRC2 were generated by LR reaction between pENTRY-BRC1/BRC2 and pDEST22 (Invitrogen). The bait construct and each prey one were co-transformed into the yeast strain AH109. Medium without Leu, Trp and His and with 5 mM 3-amino-1,2,4-triazole (3-AT) was used for selection.

To determine which region of BRC1 interacts with TIE1, fragments of BRC1 where amplified by PCR and cloned in pDONR207 by BP clonase (Thermofisher) and then inserted in pGADT7-GW by LR recombination using Gateway LR clonase II (Thermofisher). The TIE truncated in the C-t part (TIE1(1–108)) was also cloned in pDONR207 and inserted afterwards in pGBKT7-GW (YTH assays; Thermofisher). Vectors were transformed in yeast strain AH109 and medium without Leu, Trp and His and with 5 mM 3-amino-1,2,4-triazole (3-AT) was used for selection.

**Pull-Down assays**

The MBP-BRC1 construct was generated by LR reaction between pENTR/D-BRC1 and pMAL-GW modified from pK2GW7 (Ghent University). The pET28-TIE1-His construct was generated by enzyme digestion reaction with the EcoRI and SalI sites of pET-28a (+) (Novagen). The constructs were introduced into *E. coli* BL21 (DE3) competent cells for protein expression. The transformed cells were cultured in LB medium at 37˚C until the OD600 reached 0.5 and then moved to 18˚C condition for 12h in the presence of 0.5mM IPTG for the induction of protein expression. The proteins were extracted in buffer containing 20 mM Tris-HCl [pH7.4], 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1×C-complete protease inhibitor [Roche]. Bacterial lysates in extraction buffer contained 50 μg MBP-BRC1 or the control MBP proteins were mixed with lysates containing 50 μg TIE1-His fusion protein. The mixtures were incubated with Amylose resin (NEB) at 4˚C for 3 h. Beads were washed six times with the column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1mM EDTA, 1mM PMSF, and 1×C-complete protease inhibitor [Roche]). The supernatant in the first wash (elution 1) and sixth wash (elution 6) of the beads was boiled with 2×SDS buffer for further immunoblot analysis. After the sixth washes, the bound proteins (precipitate) were eluted with 2×SDS buffer and boiled for 5 min. Immunoblot analysis was then performed to detect the proteins with anti-His antibody (Sigma-Aldrich).
BiFC assays and LCI assay

The constructs nYFP-TIE1 and cCFP-BRC1 were generated by LR reactions between pENTR-TIE1 and pnYFPXGW or between pENTR-BRC1 and pcCFPXGW [52]. The above constructs were first transformed into A. tumefaciens GV3101 and then the nYFP-TIE1 and cCFP-BRC1 were co-infiltrated into the leaves of N. benthamiana. The plants were grown in the dark for 12 h followed by 48 h in a growth chamber under normal conditions. The fluorescence signal of GFP in N. benthamiana leaf cells was observed under a Leica SPE confocal microscope (Leica). A DAPI (Sigma) solution was used to stain the nuclei. The excitation laser was set at 488 nm for GFP and 405 nm for DAPI staining. For LCI assay, the constructs TIE1-nLUC and cLUC-BRC1 were generated by cloning the TIE1 gene into pCAMBIA-nLUC-GW and by cloning BRC1 gene into pCAMBIA-cLUC-GW [26]. The above constructs were first transformed into A. tumefaciens GV3101 and then the different combinations of the constructs, i.e. cLUC-BRC1 and TIE1-nLUC, cLUC and TIE1-nLUC, cLUC-BRC1 and nLUC, were co-infiltrated into the N. benthamiana leaves. The plants were placed in the dark for 12 h followed by 48 h in a growth chamber under normal condition. The infiltrated N. benthamiana leaves were sprayed with luciferin (100 mM) and kept in dark for 10 mins. The leaves were observed under a low-light cooled charge-coupled device (CCD) imaging apparatus Lumazone_1300B (Roper Bioscience).

Acceptor photo-bleaching fluorescence resonance energy transfer (APB-FRET)

The TIE1 and BRC1 full sequences without codon stop were cloned in pDONR207 and then inserted by Gateway cloning (Thermofisher) in pABindGFP, pABindmCherry and pABind-FRET allowing production of the proteins fused to GFP, mCherry or GFP-mCherry, respectively. Vectors were Agro-infiltrated in N. benthamiana leaves and protein production was inducted 24h after infiltration and APB-FRET assays were performed 16-20h after induction. APB-FRET conditions and FRET efficiency were as described in Nicolas et al. [10].

Transactivation assay

To perform the transactivation assay, we used a 2-kb promoter of the BRC1 direct target gene HB53 cloned in pGWB435 [53] for fusion with the LUCIFERASE reporter gene as described in González-Grandío et al. [18]. TIE1 and BRC1 were cloned in the destination vector pGWB2 for their constitutive expression under the CaMV 35S promoter. The different constructs were co-infiltrated in tobacco leaves and the LUC activity was measured 16–20 h after infiltration in a LB 960 Microplate Luminometer (Berthold) as described in Nicolas et al. [9].

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: TIE1, At4g28840; TIE2, AT2g20080; TIE3, At1g29010; TIE4, At2g34010; BRC1, AT3g18550; HB21, At2g18550; HB40, AT4g36740; HB53, AT5g66700.

Supporting information

S1 Fig. The shoot branching phenotype of tie1-D. (A) Branching phenotypes of 35-day-old wild-type plants and tie1-D mutants. Scale bar = 1 cm. (B) and (C) Close-up views of rosette leaf branches in the wild type and tie1-D mutant. Scale bars = 1 mm. (D) Number of primary rosette branches of 35-day-old wild-type plants and tie1-D mutants (n = 10). (TIF)
S2 Fig. **TIE2 and TIE4 also interact with BRC1.** Yeast two-hybrid assays of TIE2, TIE3 or TIE4 with BRC1. AD, activation domain; DBD, DNA binding domain. Co-transformed yeast cells were grown on medium lacking Leu and Trp (SD-Leu-Trp) or in selective medium lacking Leu, Trp and His (SD-Leu-Trp-His) with 2.5 mM 3-amino-1,2,4 triazole.

(TIF)

S3 Fig. **TIE1 may regulate BRC1 at the transcriptional level.** (A) The Fragments Per Kilobase Million (FPKM) Value of BRC1 in RNA-seq. (B) The BRC1 mRNA levels were quantified by qRT-PCR in wild type and 35S-GFP-TIE1. (C) The BRC1 mRNA levels were quantified by qRT-PCR in wild type and 35S-TIE1mEAR-VP16 lines. The expression was normalized to AtUBQ10 levels, and was relative to wild-type levels. Data represents mean ± SD from three biological replicates. Significant differences are indicated ***p< 0.001 (two-tailed Student’s t-test).

(TIF)

S1 Table. **List of genes regulated by TIE1.**

(XLS)

S2 Table. **List of BRC1-dependent and TIE1-dependent genes.**

(XLS)

S3 Table. **List of bud activation and bud dormancy genes co-regulated by TIE1 and BRC1.**

(XLS)

S4 Table. **List of primers used in this study.**

(XLS)

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