RESEARCH ARTICLE

The Arabidopsis RING-type E3 Ligase TEAR1 Controls Leaf Development by Targeting the TIE1 Transcriptional Repressor for Degradation

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Short title: Targeted degradation of TIE1 by TEAR1

One-sentence summary: TEAR1 is an active E3 ligase that controls leaf development by promoting CIN-like TCP activity by targeting the TCP repressor TIE1 for degradation via the ubiquitin-proteasome system.

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ABSTRACT

The developmental plasticity of leaf size and shape is important for leaf function and plant survival. However, the mechanisms by which plants form diverse leaves in response to environmental conditions are not well understood. Here, we identified TIE1-ASSOCIATED RING-TYPE E3 LIGASE 1 (TEAR1), and found that it regulates leaf development by promoting the degradation of TCP INTERACTOR-CONTAINING EAR MOTIF PROTEIN 1 (TIE1), an important repressor of CINCINNATA (CIN)-like TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) transcription factors, which are key for leaf development. TEAR1 contains a typical C3H2C3-type RING domain and has E3 ligase activity. We show that TEAR1 interacts with the TCP repressor TIE1, which is ubiquitinated in vivo and degraded by the 26S proteasome system. We demonstrate that TEAR1 is co-localized with TIE1 in nuclei and negatively regulates TIE1 protein levels. Overexpression of TEAR1 rescued leaf defects caused by TIE1 overexpression, whereas disruption of TEAR1 resulted in leaf phenotypes resembling those caused by TIE1 overexpression or TCP dysfunction. Deficiency in TEAR partially rescued the leaf defects of TCP4 overexpression line, and enhanced the wavy leaf phenotypes of jaw-5D. We propose that TEAR1 positively regulates CIN-like TCP activity to promote leaf development by mediating the degradation of the TCP repressor TIE1.

INTRODUCTION

Compared to mobile animals, sessile plants evolved developmental plasticity as an important strategy to adapt to different environmental conditions (Gaillochet and Lohmann, 2015). Leaves are the main photosynthetic organs in plants and have high developmental plasticity (Szymanski, 2014; Ichihashi and Tsukaya, 2015). Although alteration of leaf size and shape is important for plant survival and global food security, the molecular mechanisms by which plants control the formation of leaves are not well understood.

A plant leaf develops from a group of cells called the leaf primordium, which is initiated at the periphery of the shoot apical meristem (SAM) (Ichihashi and Tsukaya, 2015; Sluis and Hake, 2015). Initially, all cells in the leaf primordium actively divide to convert the cylindrical peg-like primordium into a flat nascent leaf with proximal-distal, adaxial-abaxial and medial-lateral polarities. Subsequently, cell division, cell differentiation, and cell expansion are temporally and spatially coordinated to convert the
nascent leaf into a mature leaf with a specific leaf size and shape (Szymanski, 2014; Sluis and Hake, 2015). Several important transcription factors, such as class I KNOX family proteins, ASYMMETRIC LEAVES 1 (AS1), AS2, the class III homeodomain-leucine zipper (HD-ZIP) proteins, AUXIN RESPONSE FACTOR 3 (ARF3), ARF4, KANADI, the YABBY family proteins, ANGUSTIFOLIA3 (AN3) and CINCINNATA (CIN)-like TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) proteins, have been shown to regulate different stages of leaf development (Poethig and Sussex, 1985; Nath et al., 2003; Scanlon, 2003; Ha et al., 2010; Hay and Tsiantis, 2010; Piazza et al., 2010; Moon and Hake, 2011; Bolduc et al., 2012; Vercruyssen et al., 2014; Hur et al., 2015; Husbands et al., 2015; Sluis and Hake, 2015). Among these factors, CIN-like TCP genes play pivotal roles in leaf formation by controlling leaf cell division and differentiation (Nath et al., 2003; Koyama et al., 2007; Ori et al., 2007; Efroni et al., 2008; Koyama et al., 2010; Martin-Trillo and Cubas, 2010).

TCP proteins are plant-specific transcription factors and are conserved in the plant kingdom (Navaud et al., 2007). CIN was the first TCP transcription factor shown to control leaf cell differentiation and thus leaf flatness in Antirrhinum majus (Nath et al., 2003). In Arabidopsis thaliana, the TCP family consists of 24 members classified into 13 class I group TCPs and 11 class II group TCPs based on the conserved non-canonical basic helix-loop-helix domain. The class II TCPs are further grouped into 8 CIN-like TCPs and 3 CYC/TB1-like TCPs (Martin-Trillo and Cubas, 2010). The CIN-like TCPs have high functional redundancy in Arabidopsis. Whereas the tcp single mutant shows no obvious leaf phenotypes, disruption of multiple CIN-like TCPs causes leaf curvature and wavy leaf margins in a dose-dependent manner (Schommer et al., 2008; Koyama et al., 2010), suggesting that TCP activity is central for the elaboration of leaf forms. The genetic alteration of TCP activity leads to different leaf sizes and shapes, further
confirming that the tight control of TCP activity at both the temporal and spatial levels is fundamental in determining leaf form (Efroni et al., 2008; Shleizer-Burko et al., 2011).

Plants have evolved several strategies to tightly control the activity of CIN-like TCPs during leaf development. First, the activity of CIN-like TCPs is regulated at the transcriptional level. For example, we identified an activation-tagging mutant, iamt1-D, and found that indole-3-acetic acid (IAA) carboxyl methyltransferase (IAMT1) negatively regulates the transcripts of several CIN-like TCP genes to control leaf development by converting active IAA to inactive methyl IAA (Qin et al., 2005). Second, TCP activity is tightly regulated at the post-transcriptional level. Overexpression of the microRNA miR319 in the activation-tagging mutant jaw-D causes leaf curvature by targeting five CIN-like TCP genes (Palatnik et al., 2003). The regulation of TCP activity by miR319 is important for plant leaf development; the expression of TCPs with synonymous mutations that resulted in resistance to miR319-mediated cleavage caused severe leaf phenotypes or even seedling death in Arabidopsis and changed compound leaves into simple leaves in tomato (Palatnik et al., 2003; Ori et al., 2007; Palatnik et al., 2007). Third, the activity of CIN-like TCPs is modulated at the protein level. For example, ARMADILLO BTB ARABIDOPSIS PROTEIN 1 (ABAP1) may modify TCP24 activity to regulate leaf cell proliferation (Masuda et al., 2008). The SWI/SNF chromatin remodeling ATPase BRAHMA (BRM) mediates CIN-like TCP activity to reduce leaf sensitivity to cytokinin by increasing the expression of the negative cytokinin regulator ARABIDOPSIS RESPONSE REGULATOR 16 (ARR16) (Efroni et al., 2013). We previously characterized a transcriptional repressor, TCP INTERACTOR-CONTAINING EAR MOTIF PROTEIN 1 (TIE1) (Tao et al., 2013). The overexpression of TIE1 in the activation-tagging mutant tie1-D resulted in hyponastic leaves, whereas disruption of TIE1 caused epinastic leaves. TIE1 regulates leaf development by physically interacting with CIN-like TCPs. We further demonstrated that TIE1 is a key transcriptional repressor
of TCP activity that acts as a bridge between TCPs and the corepressor TOPLESS (TPL) (Tao et al., 2013). However, the molecular mechanism by which TIE1 temporally and spatially regulates TCP activity is still unknown.

The ubiquitin (Ub)-proteasome system is a major protein degradation system that flexibly regulates the activity of transcription regulators and is important in hormone, stress and other signaling pathways (Potuschak et al., 2003; Mach, 2008; Chen and Hellmann, 2013; Duplan and Rivas, 2014; Shabek and Zheng, 2014; Noir et al., 2015). Plants utilize the Ub-proteasome system for morphological plasticity and altering physiological states in response to internal and external stimuli via the rapidly changing proteome (Chen and Hellmann, 2013). The Ub-proteasome system comprises the 26S proteasome and three types of enzymes for substrate protein ubiquitination. These enzymes are the Ub-activating enzymes (E1), the Ub-conjugating enzymes (E2) and the Ub ligases (E3). Among them, E3 ligases are key for defining the specificity of substrates by transferring Ub from the E2-Ub intermediate to the target proteins. The four major types of E3 include RING (REALLY INTERESTING NEW GENE), CULLIN-RING, U-box and HECT (HOMOLOGOUS TO E6-ASSOCIATED PROTEIN C-TERMINUS) (Chen and Hellmann, 2013; Shabek and Zheng, 2014). The Arabidopsis thaliana genome is predicted to contain more than 460 RING-containing proteins (Stone et al., 2005). Although the function of several RING proteins has been studied extensively (Dong et al., 2006; Stone et al., 2006; Lau and Deng, 2012; Lazaro et al., 2012; Liu and Stone, 2013; Lazaro et al., 2015; Lee and Seo, 2015; Pauwels et al., 2015; Wang et al., 2015a), the E3 ligase activity and the targets of most RING proteins in Arabidopsis have yet to be elucidated.

Here, using a yeast two-hybrid screen with an Arabidopsis cDNA library we identified a TIE1-interacting protein that we named TIE1-ASSOCIATED RING-TYPE E3 LIGASE 1 (TEAR1). TEAR1 contains a C3H2C3-type RING domain, and we
demonstrate that TEAR1 has E3 ligase activity and that TIE1 is an unstable protein. We further show that TIE1 is ubiquitinated in vivo and is degraded by the 26S proteasome. TEAR1 is co-localized with TIE1 in the nuclei and interacts with TIE1 in vivo and in vitro. Disruption of TEAR1 leads to defective leaves with wavy margins similar to those observed in tcp multiple knock-out mutants and TIE1 overexpression lines. Our data suggest that TEAR1 plays important roles in the control of leaf development by enhancing TCP activity via degradation of the TCP repressor TIE1.

RESULTS

Identification and Cloning of TEAR1 and TEAR2

We previously identified TIE1 transcriptional repressors by characterizing an activation-tagging mutant, tie1-D, in Arabidopsis. TIE1 and its close homologs TIE3 and TIE4 play important roles in the leaf formation by controlling the activity of CIN-like TCP transcription factors. To further elucidate how TIE1 regulates TCP activity temporally and spatially, we searched for TIE1 interactors using yeast two-hybrid screening. Because the full-length TIE1 activated the reporter gene in yeast cells, whereas the full-length TIE4 did not (Tao et al., 2013), we used the full-length coding region of TIE4 as the bait to screen an Arabidopsis cDNA library. Sequencing analysis showed that the insert sequences of 13 positive clones encoded 5 different RING-type proteins. Among them, At1g53190 and At3g15070 were shown to be closely related in the phylogenetic tree (Supplemental Figures 1A and 1B, Supplemental Data Set 1). Therefore, we first focused our analysis on At1g53190 and At3g15070 and named them TIE1-ASSOCIATED RING-TYPE E3 LIGASE 1 (TEAR1) and TEAR2 for the reasons described below.

We cloned the full-length coding regions of both TEAR1 and TEAR2 to verify their interactions with TIE4. The results confirmed that both interacted with TIE4 in yeast cells.
TEAR1 and TEAR2 are predicted to contain a canonical RING domain with eight metal-binding residues coordinating two zinc ions in a cross-brace arrangement (Supplemental Figure 1A; Stone et al., 2005). The RING domain in TEAR1 and TEAR2 is a C3H2C3-type or RING-H2 type containing two histidine (His) residues at metal ligand positions 4 and 5 (Supplemental Figure 1A; Stone et al., 2005). To determine whether the RING domains of TEAR1 and TEAR2 are required for their interactions with TIE4, we mutated the two conserved His residues into tyrosine (Tyr) residues in the RING domains to generate mTEAR1 and mTEAR2 (Supplemental Figure 1C). The yeast two-hybrid assays showed that both mTEAR1 and mTEAR2 were still able to interact with TIE4 (Figure 1A), suggesting that the RING domain was dispensable for the interactions of TIE4 with TEAR1 or TEAR2. The results are consistent with the previous reports that the RING domain forms a platform for interacting with ubiquitin conjugases (UBC), while the other domains bind to substrate (Metzger et al., 2014).

TEAR1 and TEAR2 Interact with TIE1 In Vitro and In Vivo

To determine whether TEAR1 and TEAR2 also interact with the TIE4 homolog TIE1, we first expressed a maltose-binding protein (MBP) fusion with TEAR1 or TEAR2 and His-tagged TIE1 proteins. In vitro pull-down assays showed that both MBP-TEAR1 and MBP-TEAR2 could bind to TIE1, whereas the control MBP could not (Figure 1B). We then expressed MBP-mTEAR1 and MBP-mTEAR2 and assessed their interactions with TIE1, and the results showed that MBP-mTEAR1 and MBP-mTEAR2 could also pull down the TIE1 protein, further indicating that the RING domain was not required for the interaction of TIE1 with TEAR1 or TEAR2 (Figure 1C). The protein level of MBP-TEAR1, MBP-TEAR2, MBP-mTEAR1 and MBP-mTEAR2 was comparable to or lower than that of the control MBP, based on immunoblots using anti-MBP antibody (Supplemental Figures 2A and 2B). Considering that the RING domain is dispensable for
TIE1 interactions with TEAR1 or TEAR2 (Figure 1C) and TEAR1 and TEAR2 may lead to TIE1 degradation in planta, we further investigated the interactions in vivo using mTEAR1 and mTEAR2 but not TEAR1 and TEAR2 in firefly luciferase complementation imaging assays. Clear fluorescence was observed in the leaf areas co-transformed with the C-terminus of LUC fused to mTEAR1 or mTEAR2 and the N-terminus of LUC fused to TIE1, whereas no obvious fluorescence was observed in the areas of the same leaf co-transformed with the control plasmids, further confirming that TIE1 interacts with TEAR1 and TEAR2 in vivo (Figure 1D and Supplemental Figure 2C).

Many RING-type proteins contain other domains in addition to RING domains which bind to E2s, while the other domains are possibly responsible for interaction with substrates (Stone et al., 2005; Metzger et al., 2014). TEAR1 and TEAR2 contain an unidentified domain associated with RING 1 (DAR1) which is also present in At5g42940 (MBRL2, see below) (Stone et al., 2005). To identify which domain or region in TEAR1 could interact with TIE1, we first partitioned TEAR1 into the N-terminus (TEAR1ΔC1) and the C-terminus containing the DAR1 domain (residues 374 to 711 in TEAR1) had a minor role in the interaction (Figures 1E and 1F). We then further partitioned TEAR1 into TEAR1ΔC2, TEAR1ΔNC1, TEAR1ΔNC2 and mTEAR1ΔN2 (Supplemental Figure 2D). The imaging assays showed that TEAR1ΔNC1, which included the amino acid residues from 141 to 260, was the major domain that interacted with TIE1 (Figure 1G), consistent with the above results indicating that the RING domain is not required for the interaction between TEAR1 and TIE1. To further confirm the results, we expressed MBP fusions with TEAR1ΔC1, mTEAR1ΔN1 or DAR1. The protein levels of MBP-TEAR1ΔC1, MBP-mTEAR1ΔN1
MBP-DAR1 were comparable to that of the control MBP (Supplemental Figure 2E). In vitro pull-down assays showed that MBP-TEAR1ΔC1 could pull down TIE1, whereas MBP-mTEAR1ΔN1 could pull down only trace amounts of TIE1, and both MBP-DAR1 and the control MBP pulled down none (Figure 1H). These results were consistent with those obtained above in firefly luciferase complementation imaging assays. These findings demonstrate that TEAR1 utilizes the N-terminus to recognize TIE1.

TEAR1 and TEAR2 can Localize to Nuclei

Both TEAR1 and TEAR2 are previously uncharacterized RING domain-containing proteins. To determine the subcellular localization of TEAR1 and TEAR2, we fused TEAR1 or TEAR2 to green fluorescent protein (GFP). Transient expression of GFP-TEAR1 or GFP-TEAR2 in Nicotiana benthamiana leaves showed that both TEAR1 and TEAR2 could localize to nuclei (Figures 2A and 2B). When we co-expressed GFP-mTEAR1 and TIE1-RFP in Arabidopsis leaf protoplasts, mTEAR1 co-localized with TIE1 in nuclei (Figure 2C), further supporting the likelihood of interaction between TIE1 and TEAR1.

The Expression of TEAR1 and TEAR2 is Developmentally Regulated in the Leaves

To determine the expression patterns of TEAR1 and TEAR2, we cloned the 2119 bp promoter of TEAR1 and the 1974 bp promoter of TEAR2. We generated the constructs, TEAR1pro-GUS and TEAR2pro-GUS, in which the TEAR1 promoter or TEAR2 promoter was used to drive the GUS reporter gene. Eighteen TEAR1pro-GUS transgenic lines and fifteen TEAR2pro-GUS lines displayed two types of staining patterns. GUS staining showed that both TEAR1 and TEAR2 were expressed predominantly in the SAM and in whole nascent and young leaves (Figures 3A to 3G and Figures 3L to 3P). Interestingly, as the leaves aged, GUS staining gradually faded from the distal to
proximal regions and the medial regions to margins in the leaves of TEAR1pro-GUS and TEAR2pro-GUS lines (Figures 3E to 3K and Figures 3O to 3T). We observed the specific expression driven by the promoters of TEAR1 and TEAR2 in the proximal half (Figures 3I and 3R) and in the proximal leaf margins (Figures 3J and 3S) of moderately old leaves. Nearly no GUS staining was found in the old leaves (Figures 3K and 3T). The expression patterns of TEAR1 and TEAR2 overlapped with each other and with that of TIE1 (Tao et al., 2013), further providing temporal and spatial evidence for the interactions between TIE1 and TEAR1 or TEAR2. These data suggest that TEAR1 and TEAR2 may redundantly regulate leaf development by interacting with TIE1.

TEAR1 and TEAR2 are Functional E3 Ligases for Ubiquitination

RING domain-containing proteins were previously reported to independently function as E3 ligases (Stone et al., 2005). As TEAR1 and TEAR2 contain typical C3H2C3-type RING domains (Supplemental Figure 1A), we investigated whether TEAR1 and TEAR2 had E3 ligase activity. We expressed MBP-TEAR1, MBP-mTEAR1, MBP-TEAR2 and MBP-mTEAR2 in Escherichia coli cells and purified the fusion proteins. We first tested the protein level of MBP, MBP-TEAR1, MBP-mTEAR1, MBP-TEAR2 and MBP-mTEAR2 by immunoblots using anti-MBP before performing in vitro E3 ligase activity assays. The protein level of the control was comparable to or higher than that of MBP-TEAR1 or MBP-TEAR2 (Figure 4). Then we used the same amounts of the proteins to perform the assays. Clear Ub ladders were found in the lanes with MBP-TEAR1 or MBP-TEAR2 combined with E1, E2 and Ub, whereas no Ub ladders appeared in the lanes with combinations lacking E1, E2 or Ub and in the combination of MBP plus E1, E2 and Ub, indicating that TEAR1 and TEAR2 had E3 ubiquitination activity (Figure 4). However, Ub ladders were not present in the lanes with MBP-mTEAR1 or MBP-mTEAR2 plus E1, E2 and Ub (Figure 4). These results suggest
that both TEAR1 and TEAR2 are active E3 ligases, and that the RING domains are
required for their E3 ligase activity.

**TIE1 is Ubiquitinated and Regulated by the Ub-Proteasome System In Planta**

The E3 ubiquitination activity of TEAR1 and the interaction between TEAR1 and TIE1
demonstrated above suggested that TIE1 was likely ubiquitinated in planta and degraded
by the 26S proteasome. To test this hypothesis, we first generated the construct
35Spro-TIE1-Flag, in which TIE1 was fused with 3×FLAG tags and driven by the CaMV
35S promoter. We transformed 35Spro-TIE1-Flag into wild-type plants. We obtained
several transgenic plants showing more serrations, as observed in the TIE1
overexpression lines, indicating that FLAG-tagged TIE1 was functional (Supplemental
Figure 3). We then used a tandem-repeated ubiquitin-binding entities (TUBEs) method,
which was previously developed to efficiently purify poly-ubiquitinated proteins from
cell extracts in native conditions (Hjerpe et al., 2009). The poly-ubiquitinated proteins
were isolated from the leaf cell extracts of 35Spro-TIE1-Flag transgenic lines or
wild-type plants using TUBEs agarose resin. Immunoblot analysis using an anti-Ub
antibody indicating that the poly-ubiquitinated proteins were successfully purified from
either 35Spro-TIE1-Flag or wild type but not from the agarose control (Figure 5A).
Moreover, the smeared ladders formed by the poly-ubiquitinated proteins were stronger
after treatment with the proteasome inhibitor MG132 in both the 35Spro-TIE1-Flag and
wild type (Figure 5A). We then tested FLAG-tagged TIE1 using an anti-Flag antibody.
Clear signals of poly-ubiquitinated TIE1 proteins were observed in the 35Spro-TIE1-Flag
extracts, while no signals were found in the wild-type extracts (Figure 5B). Furthermore,
the poly-ubiquitinated TIE1 proteins showed greater accumulation after MG132
treatment, indicating that TIE1 was regulated by the 26S proteasome (Figure 5B).
To further confirm that TIE1 is degraded via the Ub-proteasome system, we detected the FLAG-tagged TIE1 levels in 35Spro-TIE1-Flag transgenic lines over time. After treatment with the protein biosynthesis inhibitor cycloheximide (CHX), the TIE1 bands were gradually attenuated over time, whereas the control ACTIN band was not (Figure 5C). After 24 h of treatment, few TIE1 proteins could be detected (Figure 5C). However, after treatment with both CHX and MG132, the TIE1 protein abundance was not changed, even 24 h after treatment, indicating that TIE1 degradation required the 26S proteasome (Figure 5C).

These results demonstrate that TIE1 is an unstable protein degraded by ubiquitination and the 26S proteasome.

**TIE1 Levels are Negatively Regulated by TEAR1**

To investigate the biological roles of the interaction between TEAR1 and TIE1, we performed four experiments. First, we co-transformed TIE1-nLUC with cLUC-TEAR1/cLUC-TEAR2 or cLUC-mTEAR1/cLUC-mTEAR2 in two halves of the same *N. benthamiana* leaves. The fluorescence signals in the halves co-transformed with TIE1-nLUC and cLUC-mTEAR1 or cLUC-mTEAR2 were always stronger than those with TIE1-nLUC and cLUC-TEAR1 or cLUC-TEAR2 in six repeat experiments, suggesting that the cLUC-TEAR1 or cLUC-TEAR2 binding to TIE1-nLUC caused TIE1-nLUC degradation, resulting in the weak fluorescence signals, while cLUC-mTEAR1 or cLUC-mTEAR2 without E3 ligase activity did not (Figures 6A and 6B). Second, our previous study showed that TIE1 has transcriptional repression activity. Thus, we generated the reporter 35Spro-UAS-LUC, in which the luciferase gene *LUC* was driven by a CaMV 35S promoter fusion with six copies of the GAL4 binding sites (UAS), and the DBD-TIE1 construct, in which TIE1 fused to the GAL4 DNA binding domain (DBD) was driven by the CaMV 35S promoter. Compared to the control DBD,
DBD-TIE1 indeed repressed the expression of the *LUC* reporter gene, consistent with the previous report (Tao et al., 2013). However, when we co-transformed DBD-TIE1 and 35Spro-UAS-LUC together with 35Spro-TEAR1, in which *TEAR1* was driven by the CaMV 35S promoter, the repression of the reporter gene by TIE1 was significantly released, suggesting that TEAR1 could dampen the repression activity of TIE1 possibly by promoting TIE1 degradation (Figure 6C). Third, we transformed 35Spro-TEAR1 into wild-type Arabidopsis and obtained several *TEAR1* overexpression lines. We crossed one line, 35Spro-TEAR1-4, with the *TIE1* overexpression line 35Spro-TIE1-Flag-19, which produced small and serrated leaves. In the F2 generation, the 35Spro-TIE1-Flag-19 lines containing 35Spro-TEAR1-4 recovered a normal appearance, whereas the 35Spro-TIE1-Flag-19 siblings without 35Spro-TEAR1-4 still showed the small and serrated leaf phenotypes (Figures 6D and 6E). Although the level of *TIE1* transcripts was similar, the TIE1 protein level in the 35Spro-TIE1-Flag-19 lines with 35Spro-TEAR1-4 was much lower than that in the 35Spro-TIE1-Flag-19 siblings without 35Spro-TEAR1-4 (Figure 6E). Finally, to further confirm that TEAR1 promotes TIE1 degradation, we performed TIE1 *in vitro* degradation assays using leaf extracts from 35Spro-TIE1-Flag or 35Spro-TEAR1 35Spro-TIE1-Flag plants. The TIE1 protein level was much lower in 35Spro-TEAR1 35Spro-TIE1-Flag than 35Spro-TIE1-Flag (Figures 6F and 6G), consistent with the result shown in Figure 6E. As time elapsed, the TIE1 degraded much faster in 35Spro-TEAR1 35Spro-TIE1-Flag than 35Spro-TIE1-Flag, while the proteasome inhibitor MG132 inhibited the TIE1 degradation (Figures 6F and 6G), suggesting that TEAR1 promotes TIE1 degradation through 26S proteasomes.

These results demonstrate that TEAR1 regulates TIE1 activity by negatively affecting TIE1 protein levels.

**Disruption of TEAR1 Family Proteins Causes Leaf Defects**
To further elucidate the function of TEAR1 and TEAR2 during leaf development, we generated a *TEAR1* mutant, *tear1*, using the CRISPR/Cas9 system and identified a *TEAR2* T-DNA insertion mutant named *tear2* (Supplemental Figures 4A and 4B). The *tear1* mutant contained a 1-bp insertion in the first exon of *TEAR1* and caused premature translational termination (Supplemental Figure 4A), while the T-DNA insertion was located in the first exon of *TEAR2* and led to TEAR2 loss of function (Supplemental Figure 4B). Neither mutant displayed obvious phenotypes. We then generated a *tear1 tear2* double mutant by genetic crossing. No phenotypes were observed in *tear1 tear2*. Because TEAR1 and TEAR2 belong to a large RING domain-containing protein family (Supplemental Figures 1A and 1B), we hypothesized that other members including the previously identified MEDIATOR 25 (MED25)-BINDING RING-H2 PROTEIN 1 (MBR1), MBR2, MBRL1 and MBRL2 (Inigo et al., 2012) could be functionally redundant to TEAR1 and TEAR2. To test the hypothesis, we first cloned the promoters of *MBR1* and *MBRL1*. The constructs of *MBR1* pro-GUS and *MBRL1* pro-GUS were generated by putting the GUS reporter gene under the control of the two promoters, respectively. GUS staining showed that the two genes were expressed in leaves (Supplemental Figure 5), in agreement with previous RT-PCR results (Inigo et al., 2012). Furthermore, the GUS staining caused by the *MBR1* and *MBRL1* promoters was strong in young leaves and gradually diminished as the leaves aged, indicating that the expression of *MBR1* and *MBRL1* temporally and spatially overlapped with that of *TEAR1* and *TEAR2* (Supplemental Figure 5). We then investigated the interactions between six TEAR family proteins, i.e. TEAR1, TEAR2, MBR1, MBR2, MBRL1 and MBRL2, and three TIE family members i.e. TIE1, TIE3 and TIE4, which are reported to be functionally redundant in leaf development (Tao et al., 2013) to assess the specificity of the interactions between the two families. The firefly luciferase complementation imaging assays showed that TEAR1, TEAR2, MBR1 and MBR2 each interacted with all
of the three TIEs (Supplemental Figure 6). However, MBRL1 had no interaction with
TIE1, but interacted with TIE3 and TIE4 (Supplemental Figure 6). MBRL2 interacted
only with TIE4 (Supplemental Figure 6). These results suggested that MBR1, MBR2,
MBRL1 and MBRL2 could function redundantly with TEAR1 and TEAR2, and had
some specificities for association with TIE family members.

To overcome the high functional redundancy of TEARs, we implemented a
dominant-negative strategy by overexpression of mTEAR1 based on our above data
indicating that mTEAR1 had no E3 ligase activity but interacted with TIE1. This system
should allow mTEAR1 to compete for the binding sites with the endogenous TEAR
family proteins. We generated the construct 35Spro-mTEAR1, in which mTEAR1 was
driven by the CaMV 35S promoter. Interestingly, fifteen 35Spro-mTEAR1 transgenic
lines produced defective leaves with excessive serrations and wavy margins similar to
those observed in the TIE1 overexpression lines or tcp multiple mutants (Figures 7A to
7E) (Efroni et al., 2008; Koyama et al., 2010; Tao et al., 2013). These 35Spro-mTEAR1
lines also displayed other phenotypes including semi-dwarfism, more branches and low
fertility, similar to those exhibited by the TIE1 overexpression lines at the adult stage
(Supplemental Figure 7A). We then analyzed the leaf phenotypes of VC330 and AE284
transgenic lines, in which MBR1, MBR2, MBRL1 and MBRL2 were knocked down by two
different tandem artificial microRNAs (Inigo et al., 2012). Both VC330 and AE284
displayed severely wavy leaf margins like 35Spro-mTEAR1 lines (Supplemental Figure
7B), consistent with the previous observations (Inigo et al., 2012). VC330 and AE284 also
displayed semi-dwarfism and more branches, similar to 35Spro-mTEAR1 (Supplemental
Figure 7C). However, the low fertility phenotype in 35Spro-mTEAR1 was not observed
in VC330 or AE284 (Supplemental Figure 7C), suggesting that TEAR1 or TEAR2 may
also play important roles at reproductive stage.
To further confirm the functional significance of TEAR-related proteins during leaf development, we generated and identified \textit{mbr1}, \textit{mbr2}, \textit{mbrl1} and \textit{mbrl2} single mutants (Supplemental Figure 8A to 8D). No obvious leaf defects were observed in these single mutants. We then generated the multiple \textit{tear} mutants by genetic crossing. Both the quadruple \textit{tear1 tear2 mbr2 mbrl1} mutant and the quintuple \textit{tear1 tear2 mbr2 mbrl1 mbrl2} mutant displayed no obvious defective-leaf phenotypes (Supplemental Figure 8E). However, the sextuple \textit{tear1 tear2 mbr1 mbr2 mbrl1 mbrl2} mutant did produce wavy and serrated leaves like those observed in 35Spro-mTEAR1 lines and \textit{VC330} (Figure 7F, 7G and Supplemental Figure 8E), further indicating that TEAR family proteins play pivotal roles during leaf development by targeting TIE proteins for degradation.

### Genetic Interactions of TEAR and TCP Family Genes

To further explore the regulation of TCP activity by TEAR E3 ligases, we analyzed the genetic interactions of the mutants for \textit{TEAR} and \textit{TCP} family genes. We first crossed the \textit{TEAR} family-deficient line \textit{VC330} and a \textit{jaw-5D} mutant in which \textit{miR319b} is overexpressed to cause the decrease of \textit{TCP2}, \textit{TCP3}, \textit{TCP4}, \textit{TCP10} and \textit{TCP24} transcripts (Tao et al., 2013). Compared to the leaf curvature and wavy margins of \textit{VC330} or \textit{jaw-5D}, the \textit{VC330 jaw-5D} double mutants displayed much more severe phenotypes by producing highly unflattened leaves with severely wavy margins, suggesting that \textit{VC330} interacted with \textit{jaw-5D} synergistically (Figures 7H and 7I). We then crossed \textit{VC330} to 35Spro-mTCP4 in which a \textit{miR319-resistant mTCP4} was overexpressed using CaMV 35S promoter (Tao et al., 2013) and small leaves were produced with smooth margins and very few trichomes as previously reported (Figures 7J and 7K) (Efroni et al., 2013). The \textit{VC330} 35Spro-mTCP4 double mutant lines produced flattened leaves that had normal trichomes and were bigger than the leaves of 35Spro-mTCP4, indicating that overexpression of mTCP4 completely rescued the leaf curvature of \textit{VC330} and \textit{VC330}.
partially rescued the small size and few trichome defects in 35Spro-mTCP4 (Figures 7J and 7K). These results confirmed that TEAR family proteins control leaf development by positively regulating the activity of TCPs.

To further verify the regulation of TCP activity by TEAR family proteins, we investigated the expression of TCP target genes in TEAR-deficient line VC330 and 35Spro-mTCP4 using quantitative Reverse Transcription PCR (qRT-PCR). We selected LOX2, ARR16, IAA3 and SMALL AUXIN UP RNA gene At1g29460, all of which have been identified as TCP direct-target genes (Schommer et al., 2008; Koyama et al., 2010; Efroni et al., 2013). All four genes were down-regulated significantly in VC330, but were up-regulated in TCP4 overexpression line 35Spro-mTCP4 (Figure 7L). This is consistent with the previously reported results that the TCP target genes are repressed in TIE1 overexpression line tie1-D (Tao et al., 2013). The results again suggest that TEAR proteins regulate TCP activity by targeting the TCP-repressing TIE proteins for degradation.

**DISCUSSION**

In this study, we identified an E3 ligase, TEAR1, which contains a typical C3H2C3 RING-domain and controls leaf sizes and shapes by modulating CIN-like TCP activity. We showed that TEAR1 is a functional E3 ligase and interacts with the TCP repressor TIE1. We demonstrated that TIE1 can be modified by ubiquitination and degraded through the 26S proteasome. In addition, we propose a working model for TEAR1 function (Figure 8) in which TIE1 functions as a transcriptional repressor that recruits corepressor TPL/TPRs to CIN-like TCP transcription factors and thus suppresses TCP activity (Tao et al., 2013). TEAR1 functions as an active E3 ligase and targets the TIE1 protein for degradation via the Ub-proteasome system, releasing the TIE1 repression of
RING-type E3 ligases play central roles in alteration of physiological and/or morphological properties of plants in response to different intrinsic and environmental signals (Zhang et al., 2005; Disch et al., 2006; Dong et al., 2006; Stone et al., 2006; Zhang et al., 2007; Liu et al., 2008; Qin et al., 2008; Lau and Deng, 2012; Sakai et al., 2012; Chen and Hellmann, 2013; Marino et al., 2013; Xia et al., 2013; Duplan and Rivas, 2014; Shabek and Zheng, 2014; Ding et al., 2015; Lazaro et al., 2015; Sarid-Krebs et al., 2015; Zhang et al., 2015). However, the Arabidopsis genome contains more than 460 RING-type proteins (Stone et al., 2005), most of which have yet to be characterized in detail. Here, we demonstrated that TEAR1 is a RING domain-containing protein and has E3 ligase activity. The expression of TEAR1 in leaves is developmentally regulated, with high levels in young leaves and low levels in old leaves. Disruption of TEAR1 causes obvious leaf developmental defects, indicating that TEAR1 plays pivotal roles in leaf development. As the tear1 tear2 double mutant displayed no obvious phenotypes, we hypothesize that TEAR1 functions redundantly not only with TEAR2 but also possibly with the other members of TEAR family proteins, including the previously identified MEDIATOR 25 (MED25)-BINDING RING-H2 PROTEIN 1 (MBR1), MBR2, MBRL1 and MBRL2 (Inigo et al., 2012). MBR1 and MBR2 have been shown to be RING-type E3 ligases indirectly by associating with ubiquitin conjugases (UBC) and promote flowering by targeting PHYTOCHROME AND FLOWERING TIME 1 (PFT1)/MED25 for degradation (Inigo et al., 2012). Interestingly, disruption of MBR1, MBR2, MBRL1 and MBRL2 by amiRNAs in VC330 and AE284 (Inigo et al., 2012) resulted in leaves with wavy margins similar to those observed in the TEAR1 dominant-negative transgenic lines expressing mTEAR1. Our current results indicated that the sextuple tear mutant
produced serrated leaves with wavy margins as well. These data suggest that MBR1, MBR2, MBRL1 and MBRL2 function in a redundant manner with TEAR1 and TEAR2 to regulate leaf development by mediating the proteasome degradation of the TCP repressor TIE1. Indeed, we found that MBR1, MBR2, MBRL1 and MBRL2 interact with TIE family proteins with different specificities. We also demonstrated that MBR1 and MBRL1 have overlapping expression patterns with TEAR1 and TEAR2. Together, our results confirm that MBR/TEAR family proteins have redundant roles in control of leaf development and uncover the molecular mechanism of the wavy leaf phenotypes in VC330 and AE284 which could not be explained by interactions of MBR1 or MBR2 with PFT1/MED25.

Transcriptional repressors containing ethylene-responsive element binding factor (ERF)-associated amphiphilic repression (EAR) motifs plays essential roles in control of phytohormone signaling, stress responses and other biological processes (Ohta et al., 2001; Hiratsu et al., 2002; Szemenyei et al., 2008; Pauwels et al., 2010; Causier et al., 2012; Wei et al., 2015). A central mechanism in signal transduction of several phytohormones is the E3 ligase-mediated degradation of transcriptional repressors. The removal of repressors releases the activity of key transcription factors in response to different hormonal signals (Szemenyei et al., 2008; Pauwels et al., 2010; Jiang et al., 2013; Zhou et al., 2013). For example, following detection of auxin, jasmonic acid (JA) or strigolactone, the EAR motif-containing transcriptional repressor AUXIN (AUX)/IAA, JAZ-NINJA complex, and SUPPRESSOR OF MAX2 1 (SMAX1)-LIKE (SMXL)/D53 are targeted to the proteasome for degradation and thus activate the signaling pathways of these phytohormones (Szemenyei et al., 2008; Pauwels et al., 2010; Jiang et al., 2013; Zhou et al., 2013; Soundappan et al., 2015; Wang et al., 2015b). Recently, auxin has been shown to trigger the degradation of AUX/IAA repressors that recruit co-repressor TPL/TPRs to the MONOPTEROS (MP) transcription factor and thus alter the chromatin
state by promotion of MP binding to SWI/SNF chromatin remodeling ATPases during flower development (Wu et al., 2015). CIN-like TCP transcription factors also recruit the SWI/SNF chromatin remodeling ATPase BRM to activate ARR16 during leaf development (Efroni et al., 2013). We previously identified TIE1 as a key repressor that recruits co-repressor TPL/TPRs to CIN-like TCP transcription factors for the control of leaf development (Tao et al., 2013). In this study, we demonstrate that TIE1 is targeted for degradation by the E3 ligase TEAR1, suggesting that TEAR1 possibly causes a similar chromatin switch by promoting the release of CIN-like TCPs to recruit SWI/SNF chromatin remodeling ATPases during leaf development. The regulation of temporal and spatial TCP activity in the leaves is very important for formation of proper leaf sizes and morphologies in response to different environmental and internal signals (Ori et al., 2007; Efroni et al., 2008; Koyama et al., 2010; Shleizer-Burko et al., 2011). Our findings suggest that a chromatin state switch mediated by TEAR1, TIE1, BRM and CIN-like TCPs could provide a simple and flexible way to regulate leaf cell division and differentiation and thus form proper leaf sizes and shapes in response to the ever-changing environmental conditions.

METHODS

Plant Materials and Growth Conditions

The ecotype Columbia-0 (Col-0) of Arabidopsis thaliana was used in this study. The seeds from wild-type, mutants including SALK_152632 (tear2), GABI_587C07 (mbr2), WiscDsLox461-464G17 (mbrl1), SAIL_756_C02 (mbrl2), the CRISPR/Cas9-generated tear1 and mbr1 and jaw-5D (Tao et al., 2013), the transgenic lines including 35Spro-mTCP4 (Tao et al., 2013), VC330 and AE284 (Inigo et al., 2012), or different generations of crossed plants were placed on half-strength Murashige and Skoog (1/2MS) medium with or without 50 µg/mL kanamycin or 10 µg/mL phosphinothricin. The plates
with seeds were placed at 4°C for 2 d synchronization and were then incubated at 22°C ± 2°C under long-day conditions (16 h light and 8 h dark) with a light intensity of ~170 μmol/m²/s using bulbs (Philips F17T8/TL841 17 WATT). After 7 d to 10 d of growth, the green seedlings were transferred to soil and continually grown under the conditions described above. For the firefly luciferase complementation imaging and transient expression assays, *Nicotiana benthamiana* was grown in soil at 22°C ± 2°C under long-day conditions.

**PCR Analysis and Gene Expression Assays**

All the primers used in this study are listed in Supplemental Table 1.

For mutant analysis, TRIzol reagent (Invitrogen) was used to prepare total RNAs, and a SuperScript III kit (Invitrogen) was used for reverse transcription with the total RNA as a template according to the user’s manual. PCR or RT-PCR was performed using genomic DNA from mutants, plasmids and the diluted cDNA as the templates. The cycling conditions of PCR or RT-PCR were 94°C for 30 s, 56°C-58°C for 30 s and 72°C for 60 s-120 s.

**Generation of Binary Constructs and Transformation**

The coding sequence of *TEAR1* or *TEAR2* was amplified from Arabidopsis leaf cDNA using the primer pair TEAR1-F/R or TEAR2-F/R. The amplified fragments were cloned into pENTR/D TOPO (Invitrogen) to generate pENTR-TEAR1 or pENTR-TEAR2, respectively. pENTR-mTEAR1 or pENTR-mTEAR2 was generated using PCR-based mutagenesis with the primer pair mTEAR1-F/R or mTEAR2-F/R from pENTR-TEAR1 or pENTR-TEAR2, respectively.

To generate the *TIE1* overexpression line, the full-length coding region of *TIE1* without a stop codon was amplified from Arabidopsis cDNA using primers TIE1-F and TIE1-Rnsc and was then cloned into pENTR/D-TOPO to generate pENTR-TIE1NS. The overexpression construct 35Spro-TIE1-FLAG was generated by an LR reaction between
pENTR-TIE1NS and pK7FLAGWG2 (purchased from Ghent University). To establish the mTEAR1 overexpression lines, the 35Spro-mTEAR1 construct was generated by an LR reaction between pENTR-mTEAR1 and pK2GW7 (from Ghent University). For the overexpression of TEAR1, TEAR1 was first amplified from Arabidopsis genomic DNA using primers TEAR1 F-KpnI and TEAR1 R-SacI and then cloned into the KpnI-SacI digested pQG110 (Qin et al., 2005; Guo et al., 2015).

To examine the TEAR1 or TEAR2 and MBRI or MBRL1 expression pattern by GUS staining, 2119 bp TEAR1 or 1974 bp TEAR2 and 1914 bp MBRI or 1424 bp MBRL1 promoter regions upstream of the ATG of TEAR1 or TEAR2 and MBRI or MBRL1 were amplified from Arabidopsis genomic DNA with the primer pair TEAR1P-F/R or TEAR2P-F/R and MBRI1P-F/R or MBRL1P-F/R, respectively. Then, the products were cloned into pENTR/D-TOPO to generate pENTR-TEAR1pro or pENTR-TEAR2pro and pENTR-MBRIpro or pENTR-MBRL1pro, respectively. TEAR1pro-GUS or TEAR2pro-GUS and MBRIpro-GUS or MBRL1pro-GUS were generated by an LR reaction between pKGWFS7 and pENTR-TEAR1pro or pENTR-TEAR2pro and pENTR-MBRIpro or pENTR-MBRL1pro, respectively.

Yeast Two-Hybrid Screening and Assays

The TIE4 coding sequence was amplified using the primer pair TIE4-F/R and then cloned into the vector pENTR/D-TOPO to generate pENTR-TIE4. The bait construct DBD-TIE4 was generated by an LR reaction with pENTR-TIE4 and pDEST32 (Invitrogen). The cDNA-AD library was generated by TOPO cloning using the cDNA from a mixture of different organ tissues from Arabidopsis. Briefly, mRNA was prepared from Arabidopsis seedlings and flowers using the FastTrack™ MAG Maxi mRNA Isolation Kit (Invitrogen) and then reverse transcribed into cDNA. The cDNA products were cloned into pDONR222 (Invitrogen). The yeast strain AH109 (Clontech) was first transformed with the bait construct DBD-TIE4. Then, the cells containing the bait vector were transformed
with 10 μg of the cDNA-AD library plasmid DNA mixture in the presence of 2 mg salmon sperm DNA. Cells were resuspended in 3 mL of 2×YPAD medium and allowed to recover at 30°C for 90 min. Next, the cells were plated on SD medium lacking leucine, tryptophan, histidine and adenine (SD-Leu-Trp-His-Ade) supplemented with 3-AT for screening. Finally, the identity of the positive clones was determined by sequencing.

To further assess the interactions of TIE4 and TEARs or mTEARs, the prey constructs TEAR1-AD, TEAR2-AD, mTEAR1-AD and mTEAR2-AD were generated by LR reactions with pDEST22. The bait DBD-TIE4 plasmids were co-transformed with each prey plasmid or the blank pDEST22 into the yeast strain AH109. Media supplemented with SD-Leu-Trp-His or SD-Leu-Trp-His-Ade and 5 mM 3-amino-1, 2, 4 triazole were used for selection.

**Firefly Luciferase Complementation Imaging Assay**

To generate the vectors pCAMBIA-nLUC-GW and pCAMBIA-cLUC-GW for firefly luciferase complementation imaging assays, attR1-ccdB-attR2 was amplified from pK2GW7 (Ghent University) using primers attR1 F-KpnI and attB2 R-XhoI and cloned into the KpnI and SalI site of pCAMBIA-nLUC or pCAMBIA-cLUC (Chen et al., 2008).

To test the interaction of TIE1 and mTEAR1 *in vivo*, TIE1-nLUC was generated by an LR reaction with pENTR-TIE1 and pCAMBIA-nLUC-GW. cLUC-mTEAR1 was generated by an LR reaction between pENTR-mTEAR1 and pCAMBIA-cLUC-GW. For the TEAR1 truncation assays, *TEAR1ΔC1, mTEAR1ΔN1, TEAR1ΔC2, TEAR1ΔNC1, TEAR1ΔNC2* and *mTEAR1ΔN2* were amplified using the primer pairs *TEAR1-F/TEAR1ΔC1-R, mTEAR1ΔN1-F/TEAR1-R, TEAR1-F/TEAR1ΔC2-R, TEAR1ΔNC1-F/TEAR1ΔC1-R, TEAR1ΔNC2-F/R, and mTEAR1ΔN2-F/TEAR1-R*, respectively. The fragments were first cloned into pENTR/D TOPO and then transferred into pCAMBIA-cLUC-GW by LR reactions to generate cLUC-TEAR1ΔC1,
cLUC-mTEAR1ΔN1, cLUC-TEAR1ΔC2, cLUC-TEAR1ΔNC1, cLUC-TEAR1ΔNC2 and cLUC-mTEAR1ΔN2, respectively.

To detect the interaction between six TEAR family proteins, i.e. TEAR1, TEAR2, MBR1, MBR2, MBRL1 and MBRL2, and three TIE family members i.e. TIE1, TIE3 and TIE4, the TEAR family genes and TIE3 coding sequence was amplified using corresponding primer pairs and then cloned into the vector pENTR/D TOPO to generate pENTR vectors. The TIE3-nLUC, TIE4-nLUC and cLUC-TEAR family were generated by LR reaction with pENTR vectors and pCAMBIA-nLUC-GW or pCAMBIA-cLUC-GW.

The above constructs were transformed into *Agrobacterium tumefaciens* GV3101. The different combinations shown in Figure 1 and Supplemental Figure 6 were co-infiltrated into *N. benthamiana* leaves. The plants were placed in the dark for 24 h followed by 48 h in a growth chamber under long-day conditions (16 h light and 8 h dark). Then, the infiltrated *N. benthamiana* leaves were sprayed with luciferin (100 mM) and kept in the dark for 10 min. The leaves were observed under a low-light cooled charge-coupled device (CCD) imaging apparatus Lumazone® 1300B (Roper Bioscience).

**Pull-down Assay**

The coding sequence of TIE1 was cloned into the EcoRI and SalI site of pET-28a (+) (Novagen) to generate pET28-TIE1-His. The coding sequences of TEAR1, TEAR2, mTEAR1 and mTEAR2 were cloned into the SalI site of pMAL-c2x (NEB). To generate the vector pMAL-GW, attR1-ccdB-attR2 was amplified from pK2GW7 (Ghent University) using primers attR1 F-XbaI and attB2 R-HindIII and cloned into the XbaI and HindIII site of pMAL-c2x (NEB). For TEAR1 truncation assay, DAR1 was amplified using the primer pair DAR1-F/DAR1-R. Then, the fragment was first cloned into pENTR/D TOPO to generate pENTR-DAR1. The pMAL-TEAR1ΔC1, pMAL-mTEAR1
△N1 and pMAL-DAR1 constructs were generated by LR reactions with pMAL-GW and pENTR-TEAR1△C1, pENTR-mTEAR1△N1 and pENTR-DAR1, respectively.

The constructs were transformed into *E. coli* BL21 (DE3) competent cells for protein expression. BL21 (DE3) cells were grown in LB medium at 37°C until the OD_{600} reached 0.5. The induction of protein expression was performed at 18°C for 12 h in the presence of 0.5 mM IPTG. Bacterial lysates contained approximately 50 μg MBP-TEAR1, MBP-TEAR2, MBP-mTEAR1, MBP-mTEAR2, MBP-TEAR1△C1, MBP-mTEAR1△N1 or MBP-DAR1 fusion protein, and the control MBP proteins were mixed with lysates containing TIE1-His fusion protein. Amylose resin (30 μL, NEB) was added, and the mixtures were rotated at 4°C for 3 h. Beads were washed 5 times with the column buffer (20 mM Tris-HCl [pH7.4], 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1×C-complete protease inhibitor [Roche]). The isolated proteins were then separated with 12% SDS-PAGE and detected by immunoblot analysis with an anti-His antibody (Sigma-Aldrich, catalog number: H1029-2mL; 1:3,000 dilution) or an anti-MBP antibody (NEB, catalog number: E8032S, lot number: 0101603; 1:10,000 dilution).

**Subcellular Localization of TEAR1 and TEAR2**

The 35Spro-GFP-TEAR1 and 35Spro-GFP-TEAR2 constructs were generated by LR reactions between pK7WG2 and pENTR-TEAR1 or pENTR-TEAR2, respectively. Then, 35Spro-GFP-TEAR1 and 35Spro-GFP-TEAR2 were transformed into *A. tumefaciens* GV3101, and the Agrobacteria harboring the constructs were infiltrated into *N. benthamiana* leaves. The plants were then grown in the dark for 24 h followed by 48 h in a greenhouse under normal conditions. The transient GFP fluorescence in *N. benthamiana* leaf cells was observed under a Leica SPE confocal microscope. A DAPI (Sigma) solution was used to stain the nuclei.

For the co-localization of mTEAR1 and TIE1, TIE1-RFP was generated through a LR reaction between pENTR-TIE1 and pK7RWG2. The vectors TIE1-RFP and
GFP-mTEAR1 were co-transformed into Arabidopsis protoplasts as described previously (Wu et al., 2009). Briefly, approximately $2 \times 10^5$/mL protoplasts in 0.2 mL of MMG solution (0.4 M mannitol, 5 mM MgCl2, and 4 mM MES, at pH 5.7) were transformed with 20 µg of a mixture of plasmid DNA using the PEG method. The protoplasts were resuspended in 1 mL of W5 (1.54 mM NaCl, 1.25 mM CaCl2, 5mM KCl and 2 mM MES at pH 5.7) and incubated in a growth chamber for 16 h in the dark. GFP and RFP signals were observed under a Leica SPE confocal microscope.

**Histochemical GUS Staining**

Tissues from the TEAR1pro-GUS or TEAR2pro-GUS transgenic lines were soaked in 90% acetone solution for 20 min on ice. Then, the tissues were washed twice by phosphate buffer and incubated in GUS staining buffer containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide. Samples were vacuum infiltrated for 10 min and were stained overnight in 37°C incubator. The staining buffer was then changed to 70% ethanol for decolorizing before microscopy analysis.

**In Vitro Ubiquitination Assay**

For the *in vitro* ubiquitination assay, ubiquitination reaction mixtures (30 µL) containing recombinant wheat (*Triticum aestivum*) E1 (GI: 136632), At-UBC10 (40 ng), recombinant plant ubiquitin (R&D systems, 2 µg) and purified TEAR1 or TEAR2 (1 µg) fused with the MBP tag were mixed in a reaction buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 2 mM ATP, 2 mM DTT (Xie et al., 2002). MBP, MBP-mTEAR1 and MBP-mTEAR2 were used as the negative controls for MBP-TEAR1 and MBP-TEAR2. The reactions were incubated at 30°C for 2 h and then stopped by the addition of 5×protein loading sample buffer (258 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol and 0.5% Coomassie Brilliant Blue). Then, the reaction product was further separated with 12% SDS-PAGE and was tested by immunoblot analysis with an anti-Ub antibody (Santa Cruz Biotechnology, catalog number: sc-8017, lot number: 1611; 1 : 2,000 dilution) and input
protein was detected with an anti-MBP antibody (NEB, catalog number: E8032S, lot number: 0101603; 1: 10,000 dilution).

In Vivo and In Vitro Degradation Assays

For in vivo TIE1 degradation assay, 10-d-old 35Spro-TIE1-FLAG transgenic seedlings were treated by 50 µM CHX with or without 100 µM MG132 in 1/2 MS liquid medium for a 24 h time course. Total proteins were extracted with extraction buffer (PBS, 1×Roche C-complete protease inhibitor) containing 1% Triton X-100, and the lysate was cleared by centrifugation at 20,000 g for 10 min. Protein isolates were mixed with sample buffer and were separated with 12% SDS–PAGE. The separated proteins were then blotted onto nitrocellulose membranes, blocked with non-fat dry milk and incubated with an anti-ACTIN antibody (Abmart, catalog number: M20009M, lot number: 224183; 1: 2,000 dilution) or anti-FLAG-HRP (M2, Sigma, catalog number: A8592-2MG, lot number: SLBH1183V; 1: 10,000 dilution) to assess TIE1 degradation.

For in vitro degradation assays, total protein was extracted from 10-d-old seedling samples with native extraction buffer (50 mM Tris-MES, pH 8.0, 0.5 M sucrose, 1 mM MgCl2, 10 mM EDTA, 5 mM DTT, 1 mM PMSF, and 1×C-complete protease inhibitor [Roche]). Samples were incubated at 4ºC temperature for various time periods. The extracted proteins were then blotted onto nitrocellulose membranes, blocked with non-fat dry milk and incubated with an anti-ACTIN antibody (Abmart, catalog number: M20009M, lot number: 224183; 1: 2,000 dilution) or anti-FLAG-HRP (M2, Sigma, catalog number: A8592-2MG, lot number: SLBH1183V; 1: 10,000 dilution). Protein gel blot pictures were scanned, and the intensity of the images was quantified by ImageJ (National Institutes of Health).

TUBEs Pull-down Assay

The TUBEs pull-down assay was carried out as described previously with modifications (Hjerpe et al., 2009). Briefly, total plant proteins were extracted using extraction buffer...
(50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10% glycerol, 1 mM PMSF, and 1×C-complete protease inhibitor [Roche]), and the mixtures were then centrifuged at 14,000 g for 15 min at 4°C. For Ub immunoprecipitation assays, the deubiquitination inhibitor PR-619 (Tebu-bio) was added into the extraction buffer. Supernatants were pre-cleared for 30 min at 4°C with agarose beads (Tebu-bio). Samples were separated into two fresh tubes either for TUBE (tandem Ub-binding entities, Tebu-bio) or for agarose beads (control) pull-down assays at 4°C. The pulled-down proteins were detected by immunoblot analysis with anti-Ub (Santa Cruz Biotechnology, catalog number: sc-8017, lot number: 1611; 1:2000 dilution) or anti-FLAG-HRP (M2, Sigma, catalog number: A8592-2MG, lot number: SLBH1183V; 1: 10,000 dilution).

**Transient Expression Assays in the Leaves of N. benthamiana**

To generate the reporter vector 35Spro-UAS-LUC, 35Spro-UAS was amplified from the 35Spro-UAS-GUS reporter (Tao et al., 2013) and cloned into pDONR™ P4-P1R (Invitrogen) by a BP reaction. The coding region of the firefly luciferase gene LUC and the CaMV 35S terminator were cloned into pENTR/D-TOPO and pDONR™P2R-P3 (Invitrogen), respectively. These three constructs were used to generate the reporter vector 35Spro-UAS-LUC by LR reactions with pK7m34GW. The effector vectors G4BD and G4BD-TIE1 were generated as described previously (Tao et al., 2013). The coding sequence of Renilla luciferase was cloned into pENTR/D-TOPO (Invitrogen) to generate pENTR-Rluc. Then, pENTR-TEAR1 and pENTR-Rluc were used in a LR reaction with pK2GW7 to generate 35Spro-TEAR1 and 35Spro-Rluc.

The reporter and effector constructs were transformed into Agrobacterium GV3101. Then, different combinations, e.g., 35Spro-UAS-LUC and G4BD, 35Spro-UAS-LUC and G4BD-TIE1, or 35Spro-UAS-LUC, G4BD-TIE1 and 35Spro-TEAR1, were co-infiltrated with 35Spro-Rluc and pCam-P19 into the same N. benthamiana leaves. The plants were incubated in the dark for 24 h followed by 36 h in a growth chamber under normal
conditions. LUC and REN activity was measured using a Dual-Luciferase reporter kit (Promega).

**Generation of the tear1 and mbr1 Mutants Using the CRISPR/Cas9 System**

The sgRNA targets including (GTT CCT AGC GCC TCC AAA AG) in the TEAR1 and (GAC GAA CCC CAA CCA CCA AA) in MBR1 were synthesized and cloned into the Bbs I sites of AtU6-26SK vector (Feng et al., 2013). The chimeric RNA expression cassettes in AtU6-26SK between KpnI and SalI were subcloned into the KpnI and EcoRI sites of the pCambia1300 vector (Cambia, Canberra, Australia) together with the SalI and EcoRI fragment of the Cas9 expression cassette (Feng et al., 2013). Then, the CRISPR constructs were transformed into wild-type Arabidopsis to obtain the tear1 and mbr1 mutants, respectively. The homozygous tear1 and mbr1 mutant was identified by sequencing.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: TEAR1, At1g53190; TEAR2, At3g15070; MBR1, At2g15530; MBR2, At4g34040; MBRL1, At1g45180; MBRL2, At5g42940; TIE1, At4g28840; TIE3, At1g29010; TIE4, At2g34010; TCP4, At3g15030; LOX2, At3g45140; ARR16, AT2G40670; IAA3, At1g04240.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** TEAR1 and TEAR2 belong to a RING domain-containing protein family.

**Supplemental Figure 2.** TIE1 interacts with TEAR1 and TEAR2 in vivo and in vitro.

**Supplemental Figure 3.** Leaf phenotypes of the 35Spro-TIE1-Flag transgenic lines.
Supplemental Figure 4. Identification of tear1 and tear2 mutants.

Supplemental Figure 5. Expression patterns of MBR1 and MBRL1.

Supplemental Figure 6. Interactions between six TEAR family proteins and three TIE family members.

Supplemental Figure 7. Disruption of TEAR family proteins leads to defective leaves.

Supplemental Figure 8. Generation and identification of mbr1, mbr2, mbrl1 and mbrl2 single mutants and the multiple tear mutants.

Supplemental Table 1. The list of primers used in this study.

Supplemental Data Set 1. Text file of the alignment used for the phylogenetic analysis shown in Supplemental Figure 1.

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AUTHOR CONTRIBUTIONS

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FIGURE LEGENDS

**Figure 1.** TIE1 Interacts with TEAR1 and TEAR2

(A) Testing of interactions between TIE4 and TEAR1 or TEAR2 using yeast two-hybrid screening. TIE4 was also tested with mTEAR1 or mTEAR2, in which two conserved His residues in the RING domain were mutated to Tyr residues. AD, activation domain; DBD, DNA binding domain. Transformed yeasts were spotted on control medium (SD-Leu-Trp)
or selective medium (SD-Leu-Trp-His) and (SD-Leu-Trp-His-Ade) at dilutions of 10-, 100-, and 1000-fold. The empty vectors were used as controls. (B) and (C) Pull-down assays to test TIE1 interaction with TEAR1, mTEAR1, TEAR2 or mTEAR2 in vitro. The TIE1-His proteins were detected with an anti-His monoclonal antibody, and MBP proteins were used as the negative control. The two lanes of TIE1-His input were the control for the following two lanes of MBP and MBP-TEAR1 or MBP and MBP-TEAR2. (D) to (G) The firefly luciferase (LUC) complementation imaging (LCI) assays of TIE1 interaction with mTEAR1 or different truncated TEAR1 proteins in vivo. The left pictures were taken under bright field, while the right LUC images were captured using a low-light cooled CCD imaging apparatus. LUC signals were detected in the combination of TIE1-nLUC and cLUC-mTEAR1. The other combinations, including TIE1-nLUC and cLUC, nLUC and cLUC-mTEAR1, and nLUC and cLUC, were used as negative controls. Interactions between TIE1 and different truncated TEAR1 proteins in firefly luciferase complementation imaging assays are shown in (E) to (G). The truncated TEAR1 constructs are schematically represented in Supplemental Figure 2D. (H) Assessing TIE1 interactions with different truncated TEAR1 proteins by pull-down assays.

Figure 2. TEAR1 and TEAR2 Localize to Nuclei.

(A) and (B) GFP-TEAR1 and GFP-TEAR2 fusion proteins were transiently expressed in N. benthamiana leaf cells. From left to right, the fluorescence of GFP, staining of nuclei by DAPI, and merging of DAPI and GFP. Bar = 20 µm. (C) GFP-mTEAR1 and TIE1-RFP were co-expressed in Arabidopsis wild-type protoplasts. From left to right, the fluorescence of GFP, fluorescence of RFP, and the merging of GFP and RFP. Bar = 50 µm.

Figure 3. Expression Patterns of TEAR1 and TEAR2.
The expression pattern analysis of **TEAR1** based on GUS staining in **TEAR1pro-GUS-6**. **(A)** 6-d-old seedling. **(B)** 10-d-old seedling. **(C)** 15-d-old plant. **(D)** Close-up view of SAM (boxed in **C**) from 15-d-old plant. **(E)** 24-d-old plant. **(F)** Close-up view of SAM (boxed in **E**) from 24-d-old plant. **(G)** to **(K)** Close-up view of the young to old leaves from a 24-d-old plant.

**Expression pattern analysis of **TEAR2** based on GUS staining in **TEAR2pro-GUS-5**. **(L)** 6-d-old seedling. **(M)** 10-d-old seedling. **(N)** Close-up view of SAM (boxed in **M**) from 10-d-old plant. **(O)** 24-d-old seedling. **(P)** to **(T)** Close-up view of the young to old leaves from a 24-d-old plant.

Bars = 1 mm in **(A)** to **(C)**, **(E)**, **(H)**, **(I)** to **(M)**, and **(O)** to **(T)**; Bars = 0.1 mm in **(D)**, **(F)**, **(G)** and **(N)**.

**Figure 4.** TEAR1 and TEAR2 have E3 Ligase Activity.

**E3 Ub ligase activity was tested using MBP-tagged TEAR1** (A) or MBP-TEAR2 (B) in the presence or absence of E1, E2 and Ub. An anti-Ub antibody was used to detect ubiquitinated proteins (upper), and an anti-MBP was used to determine the amounts of MBP-TEAR1, MBP-TEAR2 and MBP, MBP-mTEAR1, MBP-mTEAR2 controls (lower). Molecular mass markers in kilodaltons are indicated on the left.

**Figure 5.** TIE1 was Ubiquitinated and Regulated by the Ub-Proteasome System.

**Ubiquitinated proteins were purified using the TUBEs resin.** Agarose resin was used to detect non-specific protein binding. Immunoblotting with anti-Ub (A) or anti-FLAG (B) antibodies was used to detect total ubiquitinated proteins or ubiquitinated Flag-tagged TIE1. Molecular mass markers in kilodaltons are indicated on the left. (C) Flag-tagged TIE1 protein levels in 35Spro-TIE1-Flag transgenic lines after treatment with 50 µM CHX with or without 100 µM MG132 for the indicated durations. Proteins were
detected by immunoblotting with an anti-FLAG monoclonal antibody. Relative amounts
of proteins were determined by the ACTIN levels. Similar results were obtained in three
repeated experiments.

**Figure 6.** TIE1 Protein Level is Negatively Regulated by TEAR1.

(A) and (B) The fluorescence signals were monitored in leaf halves co-transformed with
TIE1-nLUC and cLUC-mTEAR1 or mTEAR2 compared to with TIE1-nLUC and
cLUC-TEAR1 or TEAR2. (C) Assay of transcriptional repression activity of TIE1. The
ratio of LUC/REN was tested in *N. benthamiana* leaves using a GAL4/UAS-based
system. In 35Spro-UAS-LUC, the luciferase gene *LUC* was driven by a CaMV 35S
promoter without a TATA box fusion with six copies of the GAL4 binding sites (UAS);
in 35Spro-G4DBD, the GAL4 DNA binding domain was driven by a CaMV 35S
promoter; in 35Spro-G4DBD-TIE1, G4DBD fused with TIE1 was driven by a CaMV
35S promoter; in 35Spro-TEAR1, the coding sequence of *TEARI* was driven by a CaMV
35S promoter. The error bars represent the SD of three biological replicates, the asterisk
means P-value < 0.05. (D) and (E) The phenotypes of 42-d-old wild type, 35Spro-TIE1-Flag×35Spro-TEAR1, and 35Spro-TIE1-Flag (D). In the F2 population of
35Spro-TIE1-Flag×35Spro-TEAR1, although the level of *TIEI* transcripts was similar,
the TIE1 protein level was much lower in the siblings of 35Spro-TIE1-Flag with
35Spro-TEAR1 than those without 35Spro-TEAR1 (E). (F) and (G) Analysis of TIE1
protein degradation over time in 35Spro-TIE1-Flag line and 35Spro-TIE1-Flag with
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**Figure 7.** Disruption of TEAR1 Family Proteins Leads to Defective Leaves and Genetic
Interaction Between TEARs and TCPs.

(A) to (D) Leaf phenotypes of 28-d-old wild type and three independent
35Spro-mTEAR1 transgenic lines. (E) Close-up views of the leaves from 28-d-old
wild-type and two independent 35Spro-mTEAR1 transgenic plants. From left to right, the
5th and 6th leaves from the wild type and 35Spro-mTEAR1 transgenic lines. Bar = 1 mm.

(F) Phenotypes of 32-d-old wild type and two tear1 tear2 mbr1 mbr2 mbri1 mbri2
sextuple mutants. Bar = 1 mm. (G) Close-up views of the 6th and 7th leaves from
32-d-old wild-type and two tear sextuple mutants. Bar = 1 mm. (H) Phenotypes of
33-d-old wild type, VC330, jaw-5D and VC330 jaw-5D double mutant. Bar = 1 cm. (I)
Close-up views of the 5th and 6th leaves from 33-d-old wild-type, VC330, jaw-5D and
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35Spro-mTCP4 and VC330 35Spro-mTCP4 double mutant. Bar = 1 cm. (K) Close-up
views of the 5th and 6th leaves from 25-d-old wild-type, VC330, 35Spro-mTCP4 and
VC330 35Spro-mTCP4 double mutant. Bar = 1 mm. (L) Relative expression levels of the
known TCP direct target genes in the leaves of wild type, VC330 and 35Spro-mTCP4.
The expression levels of the genes in wild-type plants were set to 1.0. The error bars
represent the SD of three biological replicates. Total RNA was extracted from the mixed
leaf tissues of six wild-type, VC330 or 35Spro-mTCP4 plants. After reverse transcription,
real-time PCR was carried out three times to determine the relative expression levels of
the TCP direct target genes.

Figure 8. Working Model of TEAR1 Function.

TIE1 recruits co-repressor TPL/TPR3s to suppress CIN-like TCP activity as a
transcriptional repressor (Tao et al., 2013). TEAR1 functions as an active E3 ligase and
targets the TIE1 repressor for degradation through the Ub-26S proteasome system. The
degradation of TIE1 by TEAR1 promotes leaf development by releasing the
TIE1-mediated repression of CIN-like TCP activity. The cyan ellipse in the upper image
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