TREE1-EIN3–mediated transcriptional repression inhibits shoot growth in response to ethylene

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Ethylene is an important plant hormone that regulates plant growth, in which the master transcriptionactivator EIN3 (Ethylene Insensitive 3)-mediated transcriptional activation plays vital roles. However, the EIN3-mediated transcriptional repression in ethylene response is unknown. We report here that a Transcriptional Repressor of EIN3-dependent Ethylene-response 1 (TREE1) interacts with EIN3 to regulate transcriptional repression that leads to an inhibition of shoot growth in response to ethylene. Tissue-specific transcriptome analysis showed that most of the genes are down-regulated by ethylene in shoots, and a DNA binding motif was identified that is important for this transcriptional repression. TREE1 binds to the DNA motif to repress gene expression in an EIN3-dependent manner. Genetic validation demonstrated that repression of TREE1-targeted genes leads to an inhibition of shoot growth. Overall, this work establishes a mechanism by which transcriptional repressor TREE1 interacts with EIN3 to inhibit shoot growth via transcriptional repression in response to ethylene.

transcriptional repression | ethylene | *Arabidopsis*

Ethylene is one of the most important plant hormones that is essential for many physiological and developmental processes such as apical hook formation, shoot and root growth, root nodulation, flower senescence, abscission, and fruit ripening (1). Plants also produce ethylene to fight against wounding, pathogen attack, or stress threats such as extreme temperatures or drought (2–4). Ethylene is perceived by a family of receptors that bind to the endoplasmic reticulum (ER) membrane (5–8). In the absence of ethylene, the receptor ETR1 (ETHYLENE RESPONSE 1) interacts with CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1), a Raf-like protein that phosphorylates EIN2 (ETHYLENE INSENSITIVE 2), the key positive regulator of ethylene signaling, preventing the ethylene response (5, 9–13). In the presence of ethylene, both ethylene receptors and CTR1 are inactivated; the C terminus of EIN2 is dephosphorylated and cleaved by unknown mechanisms. The cleaved C-terminal end of EIN2 is translocated into the nucleus (14–16), where it interacts with a histone binding protein ENAP1 (EIN2 NUCLEAR ASSOCIATED PROTEIN 1) to regulate the histone acetylation at H3K14 and H3K23 to integrate transcriptional regulation that is mediated by transcription factors EIN3 (ETHYLENE-INSENSITIVE 3) and EIL1 (ETHYLENE-INSENSITIVE3-LIKE 1) (17).

The typical dynamic physiological response to the presence of ethylene is a rapid growth inhibition that depends on the master transcriptional regulator EIN3 (18). Both genetic and molecular studies have demonstrated that EIN3 and EIL1 are the positive regulators that are necessary and sufficient for the ethylene response (19–21). By analyzing the promoters of the genes that are highly up-regulated by ethylene, the EIN3 binding motif was identified and then validated by using an electrophoresis mobility shift assay (EMSA) (22–28). The binding motif was further verified by chromatin immunoprecipitation sequencing (ChIP-seq) of EIN3 over a time course of ethylene treatments and following data analysis (21). All of this research showed that EIN3 is a transcription activator and plays a key role in the ethylene response. Whereas the time course transcriptome analysis in response to ethylene revealed that most of the genes are repressed by ethylene in the early stage (before 1 h) of the ethylene response, about 50% of ethylene-altered genes are repressed in the later stage (after 1 h) of ethylene response (18, 21). More interestingly, parts of the ethylene-repressed genes are the binding targets of EIN3, the transcription activator (21), suggesting that EIN3 is potentially involved in the transcriptional repression. However, little research has been focused on the EIN3-mediated transcriptional repression in the ethylene response. We recently showed that histone deacetylases SRT1 (SIRTUIN 1) and SRT2 (SIRTUIN 2) are involved in the transcriptional repression in the ethylene response by maintaining a low level of H3K9Ac in the promoters of ethylene-repressed genes (29). Yet, how the EIN3-mediated transcriptional repression occurs and whether other factors are involved are still unknown.

In this study, by analyzing tissue-specific ethylene-regulated gene expression, we found that most of the genes are down-regulated by ethylene in shoots. By a motif search in the promoters of the ethylene-repressed genes in shoots, we identified a DNA binding motif that can be recognized by DUO1-ACTIVATED ZINC FINGER (DAZ) proteins. Further, we found that a Transcriptional Repressor of EIN3-dependent Ethylene-response 1 (TREE1) binds to the specific DNA motif to repress gene expression by both ChIP-seq and ChIP-qPCR. Importantly, TREE1 interacts with EIN3 that is required for TREE1 binding and transcriptional repression regulation on its targets in response to ethylene in shoots. Genetic validation demonstrated that the transcriptional repression of TREE1-targeted genes leads

Significance

Ethylene is an important plant hormone that is essential for many physiological and developmental processes such as apical hook formation, shoot and root growth, root nodulation, flower senescence, abscission, and fruit ripening. Transcriptional activation in the ethylene response has been well studied. However, the transcriptional repression in the ethylene response remains unclear. Here, by combined approaches of data analysis, molecular biology, and genetics, we found that a transcriptional repressor TREE1 interacts with EIN3 to regulate transcriptional repression, leading to an inhibition of shoot growth in response to ethylene. Our work provides insight into how the transcriptional activator can be tuned to repress downstream gene expression in hormone signals.

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The authors declare no competing interest.

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to an inhibition of shoot growth in response to ethylene. Overall, this work establishes a mechanism by which transcriptional repressor TREE1 interacts with EIN3 to inhibit shoot growth via transcriptional repression regulation in response to ethylene.

Results

A Unique DNA Binding Motif Is Present in the Promoters of Down-Regulated Genes by Ethylene in Shoots. To explore how different tissues respond to ethylene, we reanalyzed previously published RNA-seq data collected from the shoots and the roots of 3-d-old etiolated *Arabidopsis* Col-0 seedlings treated with air or 4 h of ethylene (30). In this paper, the shoots indicate the tissues other than roots, including the seedling hypocotyls and apical hooks. In total, 1,076 differentially expressed (DE) genes by ethylene treatment were identified from shoots and 868 DE genes were identified from roots (Fig. 1*A*). Among them, only 238 genes were differentially regulated in both tissues; 838 genes were specifically regulated by ethylene in shoots and 630 genes were specifically regulated in roots (Fig. 1*A*).

By further comparing the RNA-seq datasets, we found that about 60% of ethylene-regulated DE genes from 3-d-old etiolated whole seedlings were different from tissue-specific ethylene-regulated DE genes (*SI Appendix*, Fig. S1*A*). Gene Ontology (GO) analysis showed that ethylene-related GO terms were enriched only in the DE genes identified both in shoots and in roots (*SI Appendix*, Fig. S1*B*). Root development-related GO terms were specifically enriched in the DE genes in roots (*SI Appendix*, Fig. S1*C*). Stress response genes were specifically enriched in the DE genes in shoots (*SI Appendix*, Fig. S1*D*). We further found that about 40% of root-specific DE genes were down-regulated, and about 50% of the shared DE genes between shoots and roots were down-regulated (Fig. 1*B*). Whereas more than 70% of shoot-specific DE genes were down-regulated, many of these genes are involved in the stress responses (Fig. 1*B*). qRT-PCR evaluation and the Integrative Genomics Viewer (IGV) data further confirmed that transcriptional patterns in different tissues are tissues distinct (Fig. 1 *C–E* and *SI Appendix*, Fig. S1 *E–H*).

To investigate why more down-regulated genes by ethylene treatment appeared in shoots, we conducted motif searches in the promoter regions of ethylene up-regulated genes and ethylene down-regulated genes in both tissues. Only the EIN3 binding motif was identified from up-regulated genes, and no other significant DNA binding motifs were identified from the down-regulated genes. We then decided to narrow down the genes for motif search. Given the genes are regulated by ethylene and EIN3 is the key transcription factor that regulates gene expression in response to ethylene, we decided to identify EIN3 target genes for further analysis. By comparing the ethylene-regulated genes in different tissues with the EIN3 binding target genes (21), we identified 75, 49, and 43 EIN3-bound genes that are regulated by ethylene in shoots, roots, and in both of the two tissues, respectively (*SI Appendix*, Fig. S1*I*). Most EIN3 targets in roots or in both tissues were up-regulated by ethylene, which is in agreement with a previous finding that the expression of EIN3-bound genes was generally enhanced by ethylene. In shoots, however, more EIN3 targets were down-regulated than up-regulated (*SI Appendix*, Fig. S1*I*). ChIP-seq profile analysis revealed that the mean EIN3 binding signal in the promoters of ethylene-activated genes was significantly greater than that in ethylene-repressed genes in roots, or in the shared genes between shoot and root (*SI Appendix*, Fig. S1 *J* and *K*; EIN3 binding signal in shared DE genes, up vs. down: $P = 9.98E-07$; in root-specific DE genes, up vs. down: $P = 9.75E-04$). In shoots, however, the mean EIN3 binding signal intensity between ethylene-activated and ethylene-repressed genes had no significant difference (*SI Appendix*, Fig. S1*L*, $P = 0.243$).

We then searched for DNA binding motifs in 200-base pair sequences centered on the EIN3 binding summit in EIN3-bound ethylene up- or down-regulated genes in shoots and in roots. The known EIN3 binding motif was identified in genes that are activated by ethylene in shoots, in roots, or in both tissues (Fig. 1*F*). Only one DNA binding motif with the consensus sequence AGCTG$^{T}/_{G}{}^{C}/_{A}$ (E value = 1.7E-011) was identified in the genes that are repressed by ethylene in shoots (Fig. 1*G*), while not in roots. The motif was different from the EIN3 binding motifs that were determined and tested previously (21, 28), and the distance of the motif is close to the EIN3 binding motif (Fig. 1*H*).

The DNA Binding Motif Identified Is Involved in the Transcriptional Repression in the Ethylene Response. To evaluate the role of the DNA binding motif in the regulation of gene expression in response to ethylene in vivo, we identified a gene (*DROUGHT HYPERSENSITIVE 2*; *DRY2; AT1G58440*) that is down-regulated by ethylene in shoots, and its promoter region contains the DNA binding motif (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). We then constructed a reporter gene driven by the native promoter (*pDRY2^{wt}: DRY2*), by the promoter with mutations in the DNA binding motif (*pDRY^{mut}: DRY2*), or by the promoter with a deletion of the DNA binding motif (*pDRY2^{del}: DRY2*) (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). We then introduced these constructs into the *dry2* mutant (salk_022763; *SI Appendix*, Fig. S2 *B* and *C*). By examining *DRY2* gene expression in T2 plants treated with or without ethylene, we found that *DRY2* gene expression was repressed by ethylene when the gene was driven by the wild-type promoter. The expression was not altered by ethylene when the gene was driven by the promoter in which the DNA binding motif was mutated or deleted (Fig. 2*B*). These results strongly indicate that the DNA binding motif is involved in the transcriptional repression in response to ethylene in vivo.

EIN3 Interacts with TREE1 and Its Homolog DAZ3 Both In Vitro and In Vivo. To identify the protein(s) that potentially binds to the DNA binding motif, we conducted a deep search using AGCTG$^{T}/_{G}{}^{C}/_{A}$ motif to search against the database Tomtom (meme-suite.org/tools/tomtom) (31) or literatures (32, 33). A DNA binding motif bound by transcription factor DAZ1 (also known as DUO1-ACTIVATED ZINC FINGER 1 or ZAT2) and a previously functionally uncharacterized protein AT4G35610 (Transcriptional Repressor of EIN3-dependent Ethylene-response 1, TREE1) (32, 33) showed a significant similarity to the motif we identified (Fig. 2*C*, E value = 7.35E-01; *P* value = 6.51E-03). Both DAZ1 and TREE1 belong to the C2H2 family. By phylogenetic tree analysis, we found that DAZ3 is the closest homolog of TREE1; DAZ2 is the closest homolog of DAZ1 (*SI Appendix*, Fig. S3*A*). Further analyses showed that both TREE1 and DAZs contain two ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motifs that are the most predominant form of transcriptional repression motif in plants (*SI Appendix*, Fig. S3*B*) (34, 35).

Given the motif was identified from the promoter regions of EIN3 binding targets, and it is proximate to the EIN3 binding motif, we speculated that the protein candidates can potentially interact with EIN3. Therefore, we decided to detect the physical interaction between EIN3 and TREE1 or between EIN3 and the DAZ proteins (*SI Appendix*, Fig. S3*B*) by a yeast two-hybrid assay. A strong interaction was detected between EIN3 and TREE1, and a weak interaction was detected between DAZ3 and EIN3 (Fig. 3*A*). But, no interaction was detected between EIN3 and DAZ1 or DAZ2 (Fig. 3*A*). Furthermore, we found that the deletion of EAR motifs impaired the interaction between EIN3 with TREE1 or DAZ3 (Fig. 3*A*). We next confirmed the interaction between EIN3 and TREE1 or DAZ3 by an in vitro pull-down assay using recombinant proteins purified from *Escherichia coli* or *Nicotiana benthamiana* (Fig. 3 *B* and *C*).

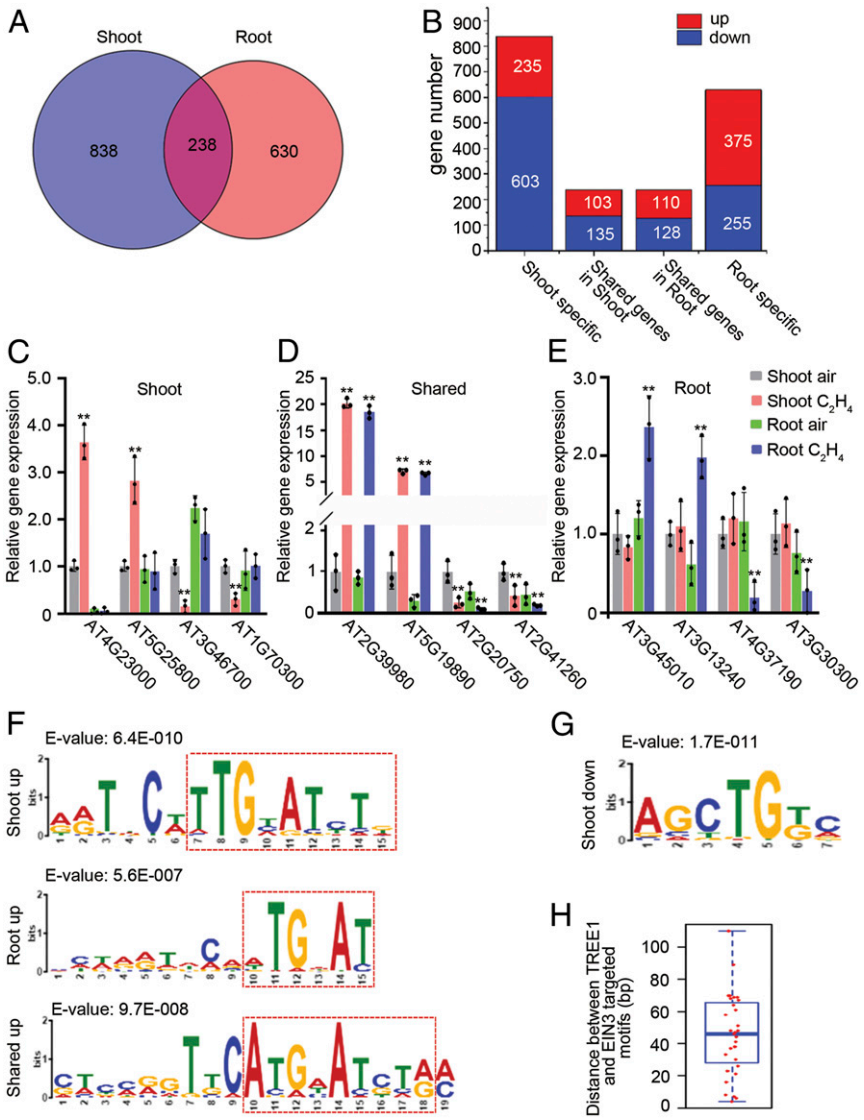

Fig. 1. Shoots and roots have distinct transcriptional responses to ethylene. (*A*) Comparison of genes that significantly differentially expressed in shoots and in roots in response to ethylene (q value ≤0.05; |Log2 fold change| ≥ 1). (*B*) Bar graph to show the numbers of genes that are up- and down-regulated by ethylene in shoots, in roots, and shared between shoots and roots. (*C–E*) qRT-PCR assays to show selected genes that are regulated by ethylene in specific tissues. The *Arabidopsis ACTIN2* gene expression serves as a reference for normalization. ** indicates *P* value <0.05 compared with the same sample treated with air. Error bars indicate the SD (n = 3). (*F*) DNA binding motifs identified from the promoters of ethylene-activated shoot-specific genes, ethylene-activated genes between shoots and roots, and ethylene-activated root-specific genes that surround EIN3 binding regions. The canonical EIN3 binding motif sequence is: (A/T)(T/C)G(A/C/T)A(T/C/G)(C/G)T(T/G) (21). Dashed boxes highlight the motifs with a similarity to the known EIN3 binding motif. (*G*) DNA binding motifs identified from the promoter regions that surrounding the EIN3 binding motif in the shoot-specific ethylene-repressed genes. E values are indicated. Motifs were found by the MEME-ChIP (meme-suite.org/tools/meme-chip) using 200-base pair sequences centered on the EIN3 binding summit in EIN3-bound ethylene up- or down-regulated genes in shoots and in roots. (*H*) Boxplot shows the distance between the motif we identified and the known EIN3 binding motif in the genes that contained both motifs.

Finally, an immunoprecipitation assay using the extracts from *TREE1-mCherry-FLAG* or *DAZ3-YFP-HA* transgenic plants treated with or without 4 h of ethylene also demonstrated the interaction between EIN3 and TREE1 or DAZ3 in vivo, specifically in the presence of ethylene (Fig. 3 *D* and *E*). Notably, mutations in EAR motifs abolished the interaction between EIN3 and TREE1 or DAZ3 (*SI Appendix*, Fig. S3 *C–E*).

TREE1 Is a Transcriptional Repressor and EIN3 Enhances the TREE1-Mediated Transcriptional Repression. In order to examine the binding activity of TREE1 to TREE1 binding motif, we first conducted EMSA. Strong binding of TREE1 to TREE1 binding motif or the *DRY2* promoter region that contains the TREE1 binding motif was detected, whereas no binding activity was detected when the motifs were mutated (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S4*A*). We then named the binding motif as TREE1 binding motif. We next decided to evaluate the function of TREE1 in the transcriptional regulation. We conducted a transient coexpression assay in *N. benthamiana* using *Agrobacterium* that expressed a strain with luciferase driven by a minimum 35S promoter region that contained five EIN3 binding motifs and six TREE1 motifs, and a second strain for the expression of GUS, EIN3, or TREE1 (Fig. 4 *C* and *D* and *SI Appendix*, Fig. S4*A*). Compared to the control with the presence of GUS protein, the luciferase signal was significantly elevated with the presence of EIN3 (Fig. 4 *E–G* and *SI Appendix*, Fig.

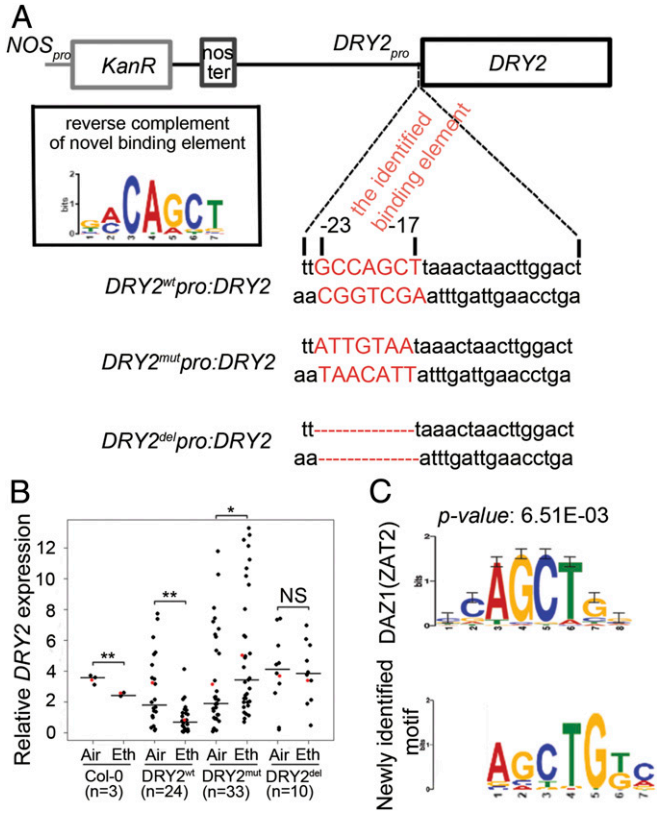

Fig. 2. The motif identified is involved in the transcriptional repression in the ethylene response. (*A*) Diagram to illustrate the *DRY2* expression constructs that are driven by the wild-type *DRY2* promoter (*Upper*), *DRY2* promoter with mutation (*Middle*), or *DRY2* promoter with deletion of the DNA binding motif (*Lower*). The reverse complement sequence of the motif is shown (see also *SI Appendix*, Fig. S2*A*). The motif sequences identified are shown in red. (*B*) qRT-PCR assay to examine the *DRY2* gene expression in response to ethylene in the Col-0 and transgenic seedlings that bear the wild-type copy (*DRY2*^{wt}), mutated copy (*DRY2*^{mut}), or deletion (*DRY2*^{del}) of the motif in the *DRY2* promoter region (T2 plants in *dry2* background). The total number of plants examined for each independent transgenic line is indicated at the *Bottom* of the graph columns. *DRY2*^{wt} represents p*DRY2*^{wt}*:DRY2/dry2*, *DRY2*^{mut} represents p*DRY2*^{mut}*:DRY2/dry2*, and *DRY2*^{del} represents p*DRY2*^{del}*:DRY2/dry2*. Bars indicate the median value; red dots indicate the mean value; black dots indicate the relative gene expression value of *DRY2* in the indicated conditions in each independent transgenic line. * indicates *P* value <0.05, ** indicates *P* value <0.01 and NS indicates not significant (*P* > 0.4) by one-tailed Mann–Whitney *U* test. Eth indicates ethylene treatment. (*C*) The comparison of the identified DNA motif with the DAZ1 (ZAT2) binding motif; the *P* value indicates the similarity between the two motifs.

S4*B*). In contrast, the expression of luciferase was suppressed in the presence of TREE1 (Fig. 4 *E*–*G* and *SI Appendix*, Fig. S4*B*). Notably, the expression of luciferase was even lower when both TREE1 and EIN3 were present compared to when only TREE1 was expressed (Fig. 4 *E*–*G* and *SI Appendix*, Fig. S4*B*). When the TREE1 binding motif was mutated, the TREE1-mediated transcriptional repression was not detected (Fig. 4 *H*–*J* and *SI Appendix*, Fig. S4*C*). All together, these data suggest that TREE1 is a transcriptional repressor, and EIN3 enhances TREE1-mediated transcriptional repression in plant cells.

TREE1 Mediates Transcriptional Repression in Response to Ethylene to Inhibit Shoot Growth. To further validate the function of TREE1 in the ethylene response, we first examined the *TREE1* gene expression by qRT-PCR. We found that *TREE1* mRNA levels were elevated by ethylene in shoots, but not in roots (*SI Appendix*, Fig. S5*A*). We then obtained *TREE1* T-DNA mutants (*tree1-1* and *tree1-2*) (*SI Appendix*, Fig. S5*B*); these plants did not display an obvious ethylene-responsive phenotype (*SI Appendix*, Fig. S5*C*). Given DAZ3 is the closest homolog of TREE1 and it locates on the same chromosome, we therefore mutated *DAZ3* in the Col-0 or in the *tree1-1* mutant using CRISPR-Cas9 gene editing to generate *daz3* single mutant and *daz3tree1-1* double mutant (36) (*SI Appendix*, Fig. S5 *D* and *E*). Similar to the *tree1-1* single mutant, the *daz3* single mutant did not display an obvious ethylene-responsive phenotype (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S5*F*). But, the *daz3tree1-1* double mutant displayed an ethylene insensitive phenotype in shoots (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S5*F*). The ethylene insensitive phenotype in shoots was further confirmed by knocking down *DAZ3* (*DAZ3RNAi*) in the *tree1-1* mutant (*DAZ3RNAi tree1-1*) (*SI Appendix*, Fig. S5 *G*–*J*). Next, we generated the *TREE1* and the *DAZ3* gain-of-function plants (*TREE1ox* and *DAZ3ox*). We found that in the absence of ethylene, the *TREE1ox* plants displayed a dwarfed phenotype (Fig. 5*C* and *SI Appendix*, Fig. S5*K*). In the presence of ethylene, the plants displayed a more severe ethylene-responsive phenotype (Fig. 5*C*). But, no obvious ethylene-responsive phenotype was observed in the *DAZ3ox* plants (Fig. 5*C* and *SI Appendix*, Fig. S5*K*), suggesting that TREE1 plays a dominant role in the process. To further confirm the function of TREE1 in the ethylene response at a molecular level, we conducted transcriptome analyses in shoots. Compared to the Col-0, the ethylene-induced repression of gene expression was largely eliminated in the *daz3tree1-1* double mutant (Fig. 5*D* and *SI Appendix*, Fig. S5*L* and Table S1). In the *TREE1ox* plants, however, most of gene expression was repressed, even in the absence of ethylene (Fig. 5*E* and *SI Appendix*, Table S1); near 50% of the genes that were repressed by ethylene in Col-0 were also repressed in the *TREE1ox* plants without ethylene treatment (Fig. 5*F*). The qRT-PCR analysis in *SI Appendix*, Fig. S5*M* further confirmed these results. Next, we examined the TREE1 binding targets by ChIP-seq in the shoots of *TREE1p:TREE1-GFP*/Col-0 transgenic seedlings by using GFP antibody (*SI Appendix*, Table S1). Over 50% of the TREE1 regulated genes were found to be bound by TREE1, under either air or ethylene treatment (*SI Appendix*, Fig. S5*N*). Near 50% of ethylene-repressed genes in shoots were binding targets of TREE1 (*SI Appendix*, Fig. S5*O*). Notably, the binding signals of TREE1 were elevated in the presence of ethylene compared to that without ethylene treatment (Fig. 5*G*). The ChIP-qPCR assay in the shoots of *TREE1ox* with or without 4 h of ethylene treatment further confirmed the ChIP-seq result (*SI Appendix*, Fig. S5*P*). Additionally, a DNA binding motif that is similar to the TREE1 binding motif (Fig. 1*G*) was found in the top 200 TREE1 binding peak regions (*SI Appendix*, Fig. S5*Q*), and the TREE1 binding to this motif was further confirmed by an EMSA assay (*SI Appendix*, Fig. S5*R*). Taken together, these results strongly suggest that TREE1 is involved in the ethylene response as a transcriptional repressor.

EAR motifs are known to mediate transcriptional repression (35, 37, 38); TREE1 has two of these motifs (*SI Appendix*, Fig. S3*B*), and the deletion/mutation of EAR motifs impaired the interaction between TREE1 and EIN3 (Fig. 3*A* and *SI Appendix*, Fig. S3 *C*–*E*), suggesting that EAR motifs play an important role in the ethylene-mediated transcriptional repression. To evaluate the function of EAR motifs in TREE1, we generated the plants that expressed *TREE1* without EAR motifs (*TREE1ΔEARox*) (Fig. 5*H* and *SI Appendix*, Fig. S5*S*). Among all of the transgenic plants, no single *TREE1ΔEARox* line showed an obvious phenotype (Fig. 5*I*). A further qRT-PCR assay in shoots showed that the ethylene-mediated transcriptional repression in Col-0, or the enhanced ethylene-mediated transcriptional repression in the *TREE1ox* shoots, was almost undetectable in the *TREE1ΔEARox*

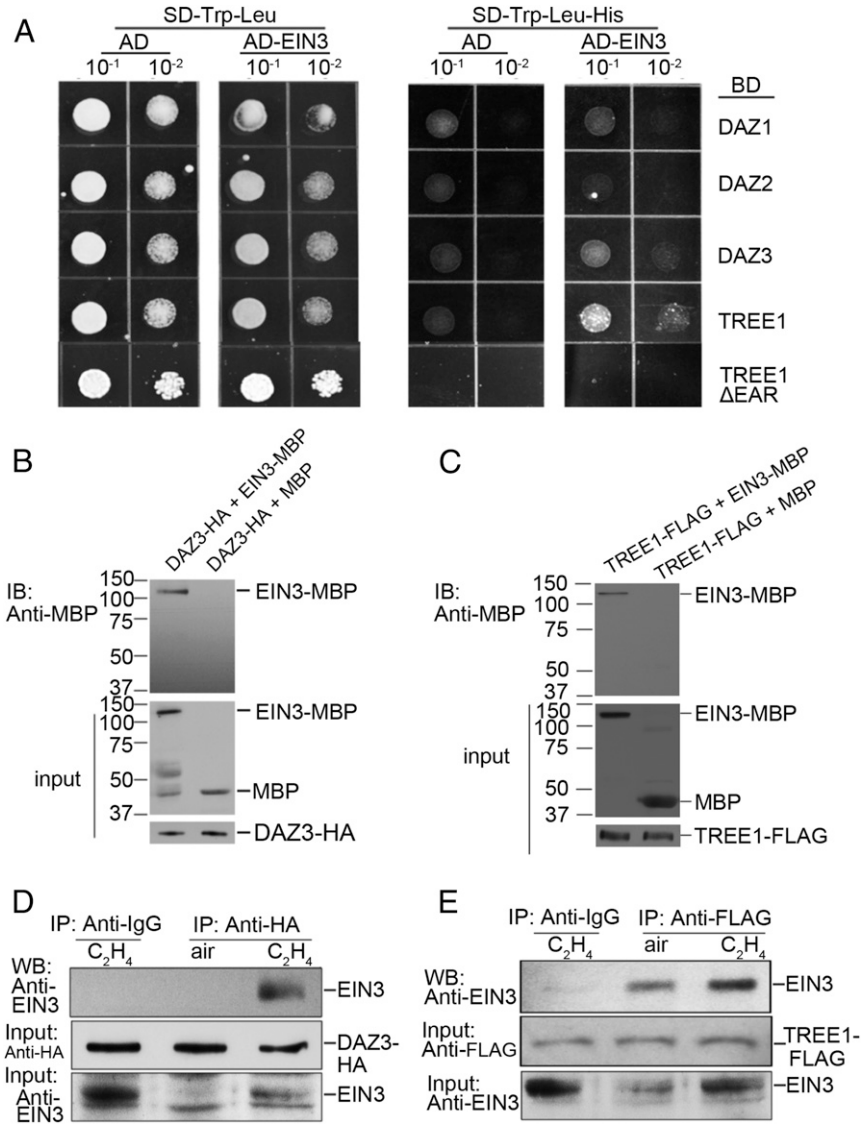

Fig. 3. The TREE1-mediated transcriptional repression in shoots occurs through an interaction with EIN3. (*A*) Yeast two-hybrid assay to examine the interactions between EIN3 with DAZ1, DAZ2, DAZ3, TREE1, and TREE1ΔEAR. (*B* and *C*) Pull-down assays to examine the interaction between DAZ3 or TREE1 and EIN3. Reactions were performed using recombinant EIN3-MBP and total plant extracts from *N. benthamiana* transiently expressed HA-tagged DAZ3 or FLAG-tagged TREE1. (*D* and *E*) In vivo coimmunoprecipitation assays to examine the interaction between DAZ3 or TREE1 and EIN3. The total proteins extracted from 3-d-old etiolated *DAZ3ox* or *TREE1ox* seedlings treated with air or 4 h of ethylene were immunoprecipitated with anti-HA or anti-FLAG and analyzed by Western blot for the indicated proteins.

shoots (Fig. 5*J*), demonstrating that EARs are important for the function of TREE1 in ethylene-mediated transcriptional repression.

EIN3 Is Required for TREE1-Mediated Transcriptional Repression in Response to Ethylene. To further explore the connection between TREE1 and EIN3, we first crossed *TREE1ox* and *ein3-1eil1-1* plants to generate *TREE1ox*/*ein3-1eil1-1* plants. The hyperethylene-sensitive phenotype in the shoots of *TREE1ox* was recovered in the *TREE1ox*/*ein3-1eil1-1* (Fig. 6*A* and *SI Appendix*, Fig. S6 *A* and *B*). However, the TREE1 protein levels were not regulated by EIN3 and EIL1 (Fig. 6*B*). We next examined the expression of TREE1 binding genes in the shoots from both the *ein3-1eil1-1* mutant and the *EIN3* gain-of-function (*EIN3ox*) plants. The ethylene-mediated transcriptional repression detected in Col-0 shoots was not detectable in *ein3-1eil1-1* shoots, whereas it was significantly enhanced in *EIN3ox* shoots (Fig. 6*C*). In addition, ethylene-mediated transcriptional repression in the target genes was not detected in *TREE1ox*/*ein3-1eil1-1* shoots (Fig. 6*C*). These results indicate that EIN3 is involved in the TREE1-mediated transcriptional repression at a molecular level.

Next, we examined how EIN3 influences TREE1 binding by ChIP-qPCR in the shoots of *TREE1ox* and *TREE1ox*/*ein3-1eil1-1* with or without 4 h of ethylene treatment (*SI Appendix*, Fig. S6 *C–H*). Compared to without the ethylene treatment, the TREE1 binding activity was elevated by ethylene treatment in *TREE1ox* shoots (Fig. 6*D*). The binding activity was reduced in *TREE1ox*/*ein3-1eil1-1* shoots compared to that in Col-0 shoots, and the ethylene-induced elevation of TREE1 binding that was detected in *TREE1ox* shoots was abolished in *TREE1ox*/*ein3-1eil1-1* shoots (Fig. 6*D*), showing that EIN3 enhances TREE1 binding, specifically in the presence of ethylene. Given that TREE1 binding is enhanced by ethylene treatment when EIN3 protein is accumulated (Fig. 5*G*), we decided to examine the

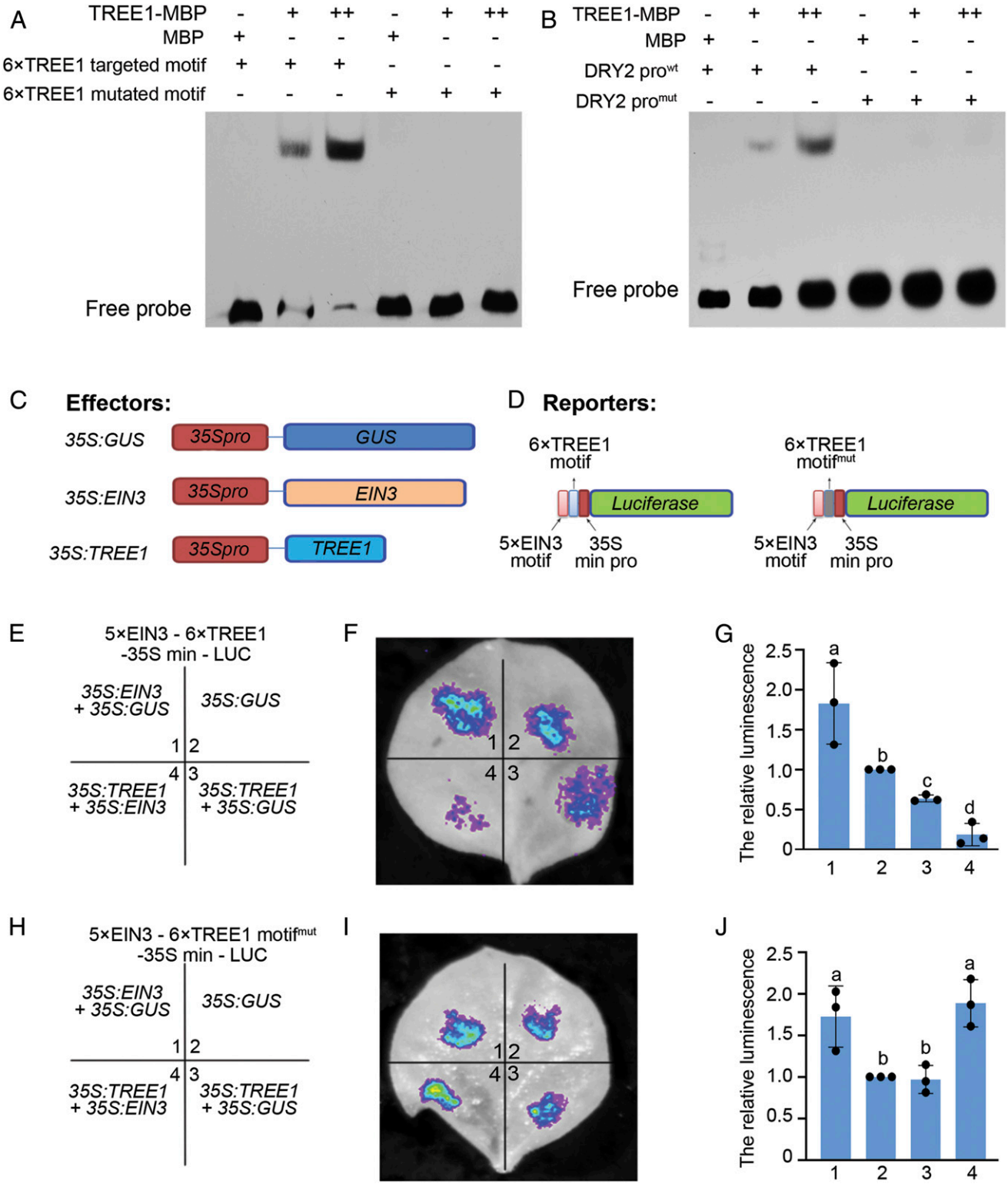

Fig. 4. TREE1 mediates inhibition of shoot growth by transcriptional repression regulation in response to ethylene. (*A*) EMSA assay to examine the binding of TREE1 to the TREE1 binding motif. (*B*) EMSA assay to examine the binding of TREE1 to the promoter of *DRY2* containing the TREE1 binding motif. Biotin-labeled probes were incubated with TREE1-MBP protein. TREE1-MBP protein-bound probes were separated from free probes by an acrylamide gel. (*C* and *D*) Schematic diagrams of *C* the effector and (*D*) the reporter constructs. (*E*–*G*) Luciferase assays examining the function of TREE1 in transcriptional regulation were conducted by the infiltration of *Agrobacterium* carrying indicated constructs into *N. benthamiana* plants. (*E*) Schematic diagrams showing the combinations of effectors and reporters. (*F*) Images of plants sprayed with 500 μM luciferin and placed in the dark for 5 min are shown. (*G*) Quantitation of luciferase intensity from three biological replications is shown in plots (error bars are SD). (*H*–*J*) Luciferase assays examining the function of TREE1 in transcriptional regulation with mutated TREE1 binding motif. (*H*) Schematic diagrams showing the combinations of effectors and reporter. (*I*) Images of plants sprayed with 500 μM luciferin and placed in the dark for 5 min are shown. (*J*) Quantitation of luciferase intensity from three biological replications are shown in plots (error bars are SD).

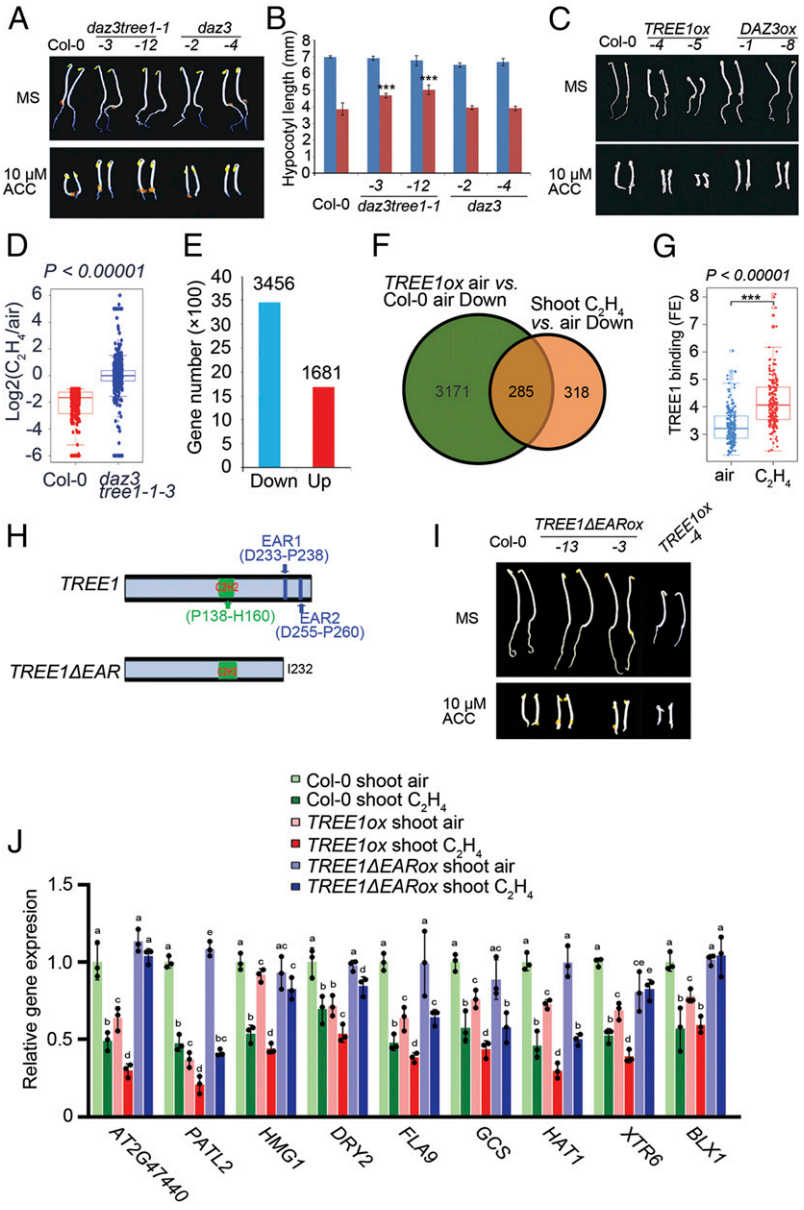

Fig. 5. The TREE1 EAR domain is essential for transcriptional repression of shoot- specific ethylene-responsive genes. (*A*) The seedling phenotype of *daz3_CRISPR/tree1-1* (*daz3tree1-1*). Three-day-old etiolated seedlings were grown on the MS medium with or without 10 μM ACC before being photographed. (*B*) The measurement of hypocotyls in the plants indicated in the figure treated with or without 10 μM ACC. *** indicates *P* value <0.001 when compare to the Col-0 control with the same treatment. (*C*) The seedling phenotype of *DAZ3ox* and *TREE1ox* plants. Three-day-old etiolated seedlings were grown on the medium with or without the presence of 10 μM ACC before being photographed. (*D*) Boxplot showing the comparison of the expression of ethylene down-regulated genes in the shoots of Col-0 and *daz3tree1-1-3* mutant. The Log2 transformed fold changes of each ethylene down-regulated gene in shoots was used for analysis. (*E*) Bar graph of up- and down-regulated genes in *TREE1ox-5* in air vs. Col-0 shoots in air. (*F*) Venn diagram showing the overlapped genes that are regulated by ethylene in Col-0 shoots and *TREE1ox-5*. (*G*) Boxplot showing the comparison of TREE1 binding fold enrichment (FE) in ethylene down-regulated genes in shoots with and without ethylene treatments. (*H*) Diagrams showing the constructs that express *TREE1*, *TREE1ΔEAR*, and *EAR*. TREE1 contains two EAR domains (EAR1 and EAR2) and the ZnF_C2H2 domain (labeled as C2H2). (*I*) The seedling phenotype of *TREE1ΔEARox* and *TREE1ox* plants, which were generated using the 35S promoter in Col-0 background. Three-day-old etiolated seedlings were grown on the medium with or without the presence of 10 μM ACC before being photographed. (*J*) qRT-PCR assay to examine gene expression in the gain of function of *TREE1* and *TREE1ΔEAR* (*TREE1ox-5* and *TREE1ΔEARox*, respectively) seedlings treated with or without ethylene. Shoot tissues were used for qRT-PCR assay. The *Arabidopsis ACTIN2* gene expression serves as a reference for normalization. Error bars indicate the SD ($n = 3$). Different letters indicate statistically significant difference with $P \leq 0.05$ by one-tailed unpaired *t* test.

influence of EIN3 on TREE1 binding by an EMSA assay. We found that the TREE1 binding activity was significantly enhanced by EIN3 and the activity was correlated with the amount of EIN3 proteins (*SI Appendix*, Fig. S6*I*), further confirming a positive regulation of EIN3 on the TREE1 binding activity. Together, all these results indicate that EIN3 is required for the TREE1-mediated transcriptional repression.

Plants with Mutations in Two of the TREE1 Targets Have Dwarfed Hypocotyl Phenotypes. We hypothesized that in the presence of ethylene, the complex of TREE1-EIN3 represses gene expression, leading to an inhibition of shoot growth. To test our hypothesis, we obtained T-DNA insertion mutants for two TREE1 binding targets that are also bound by EIN3 and their gene expression was repressed by ethylene. Plants with mutations in either

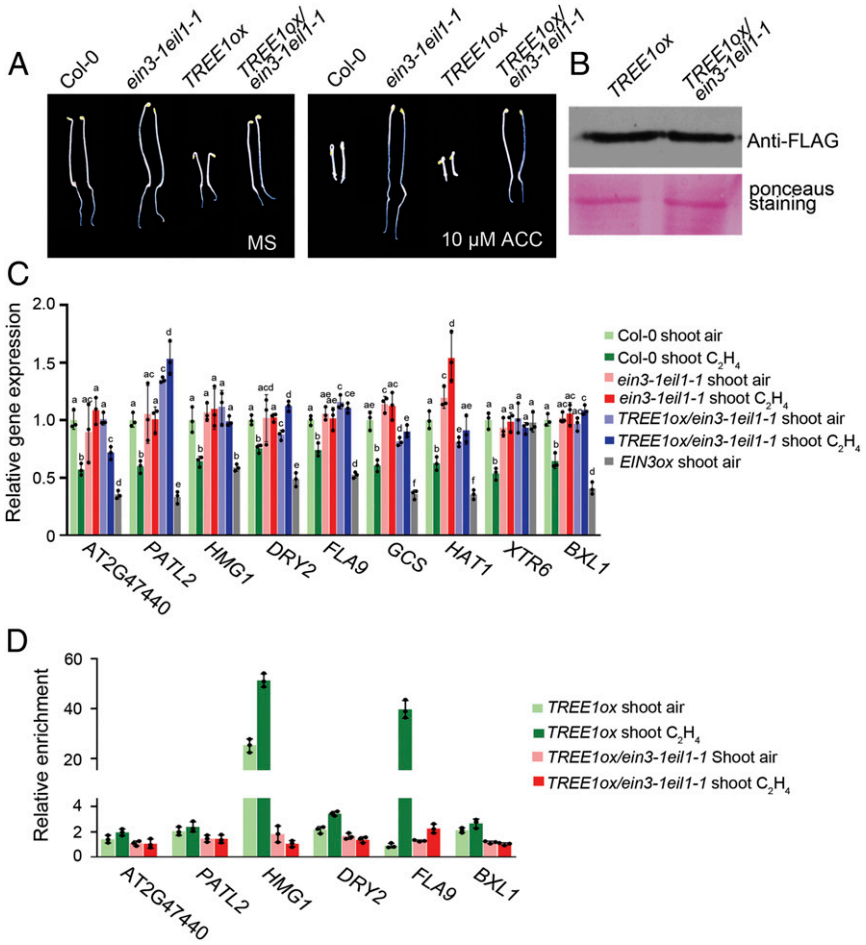

Fig. 6. EIN3 is required for TREE1-mediated transcriptional repression of shoot-specific ethylene-responsive genes. (*A*) The seedling phenotype of *TREE1ox/ein3-1eil1-1* plants. Three-day-old etiolated seedlings were grown on medium with or without 10 μM ACC before being photographed. (*B*) Western blot for TREE1 protein levels in the indicated plants. (*C*) qRT-PCR assay to examine the gene expression in *EIN3ox*, *ein3-1eil1-1*, *TREE1ox/ein3-1eil1-1*, and *EIN3ox* seedlings treated with or without ethylene. Only shoot tissues were used for qRT-PCR assay. Error bars indicate the SD ($n = 3$). The *Arabidopsis ACTIN2* gene expression serves as a reference for normalization. Different letters indicate statistically significant difference with $P \leq 0.05$ by one-tailed unpaired *t* test. (*D*) ChIP-qPCR assay of the binding of TREE1 to the promoters of ethylene-repressed genes in the *TREE1ox* and *TREE1ox/ein3-1eil1-1* seedlings treated with or without ethylene. Shoot tissue was used for ChIP-qPCR assay. Error bars indicate the SD ($n = 3$). The regions that do not contain the TREE1 binding motif served as negative controls for calculation.

of these genes, *HMG1* (*HYDROXY METHYLGLUTARYL COA REDUCTASE 1*) and *DRY2*, had similar dwarfed hypocotyl phenotypes in the absence of ethylene (Fig. 7*A* and *SI Appendix*, Figs. S2 *B* and *C* and S7 *A*–*C*). We then generated their gain-of-function plants, and we found that the *HMG1ox* and *DRY2ox* plants were less sensitive to ethylene in shoots than that of Col-0 plants (Fig. 7 *B*–*E* and *SI Appendix*, Fig. S7 *D* and *E*). Most interestingly, the adult plants were dwarfed, and they had a serious fertility problem (*SI Appendix*, Fig. S7*B*). Taken together these data provide additional genetic evidence that TREE1 mediates transcriptional repression during the ethylene response to inhibit shoot growth.

Discussion

Transcriptional repression in many plant hormones such as auxin, jasmonic acid, and gibberellic acid is mediated by transcription factors that are regulated by repressors. Upon exposure to the relevant hormone, the repressors are targeted for degradation through the 26S proteasome-mediated degradation pathway, leading to a liberation of the transcriptional factors, which activate downstream target genes (39–44). EIN3, the key transcription factor in the ethylene signaling, is not subjected to the regulation of other repressors that are regulated by the 26S proteasome-mediated degradation pathway. However, EIN3 itself is degraded by the 26S proteasome-mediated degradation pathway in the absence of ethylene (20, 45). When there is ethylene, EIN3 is stabilized, and the stabilized proteins trigger a transcription activation regulation (20, 45). Although different studies have clearly demonstrated that EIN3 is a transcription activator (19, 20, 45), transcriptome data showed that a subset of EIN3 binding target genes is repressed by ethylene (21). We recently demonstrated that histone deacetylases SRT1 and SRT2 are partially involved in the ethylene response by repressing the expression of a subset of ethylene-responsive genes through maintaining a low level of H3K9Ac (29). Yet, the transcriptional repression in the ethylene response is still largely unknown.

In this study, we provide multiple lines of evidence showing that transcriptional repression plays critical roles in the ethylene-mediated growth inhibition in shoots. First, we identified a TREE1 binding motif in the promoter regions of the genes specifically down-regulated by ethylene in shoots (Fig. 1). Second, we showed that the DNA binding motif is important for transcriptional repression, and TREE1 targets to the DNA motif we identified for transcriptional repression (Figs. 2 and 4). Third,

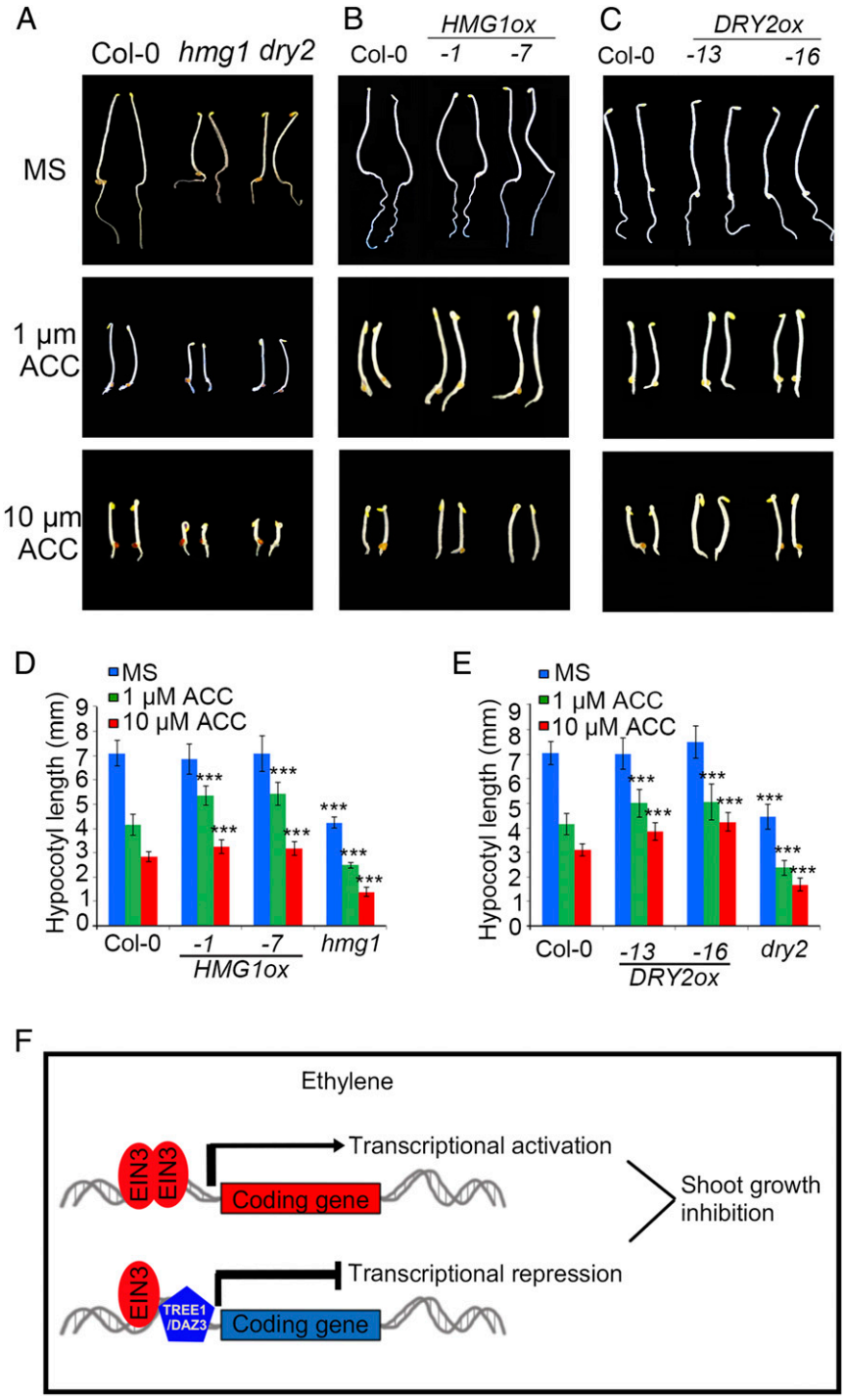

Fig. 7. TREE1-targeted genes are involved in shoot growth regulation in response to ethylene. (*A*–*C*) Photographs of representative (*A*) Col-0, *hmg1* mutant, and *dry2* mutant seedlings; (*B*) Col-0 and *HMG1ox*; and (*C*) Col-0 and *DRY2ox* seedlings. The seedlings were grown in the dark for 3 d on the medium with or without 1 μM or 10 μM ACC before being photographed. (*D* and *E*) The measurement of hypocotyls in the plants indicated in the figure treated with or without ACC. *** indicates *P* value <0.0001 when compared to the Col-0 control with the same treatment. (*F*) Model for the TREE1-mediated transcriptional repression in shoots. In the presence of ethylene, EIN3 alone targets the promoters of a subset of genes to activate transcription. In the promoters of a different subset of genes, the transcriptional repressor TREE1 interacts with EIN3 and transcription is repressed.

our genetics and molecular evidence demonstrate that TREE1 is involved in the ethylene response in shoots by targeting ethylene-responsive genes for transcriptional repression and that the EAR motifs in TREE1 are important for the transcriptional repression (Fig. 5). Fourth, we found that TREE1 and its homologous protein DAZ3 interact with EIN3 both in vivo and in vitro to repress expression of targeted genes (Fig. 3). Further genetic and molecular evidence showed that the EIN3 is required for the transcriptional repression by TREE1 in the ethylene response in shoots (Fig. 6). Finally, we provide genetics evidence showing

that the repression of TREE1 target genes confers the plants dwarfed shoot phenotype (Fig. 7). Overall, this research revealed that a tissue-specific negative regulatory circuit, by which TREE1/DAZ3 interacts with EIN3 to suppress gene expression, is one of the molecular mechanisms to inhibit shoot growth in response to ethylene (Fig. 7*F*).

We revealed that in the presence of ethylene, TREE1 interacts with EIN3 to inhibit shoot growth via transcriptional repression regulation, such as through down-regulation of *DRY2* and *HMG1* genes (Fig. 7). Interestingly, previous studies have shown that both *dry2* and *hmg1* mutants display short hypocotyls and short roots in dark-grown seedlings (46, 47) (Fig. 7), which mimics a partial ethylene-responsive phenotype. As DRY2 is a squalene epoxidase enzyme, the *dry2* mutant is able to inhibit the triterpenoid biosynthetic pathway, which is typically necessary for normal plant development, and in turn leads to the accumulation of squalene (46). Along with DRY2, HMG1 also plays a critical role in the biosynthesis of triterpenes (47, 48). Extensin-like proteins, such as AtLRX3 (LEUCINE-RICH REPEAT/EXTENSIN 3), whose expressions are regulated by HMG1 also regulates the development of epidermis (49–51). These reports suggest a possible mechanism in which the ethylene-regulated inhibition of shoot growth is partially due to the regulation of DRY2 and HMG1. In addition, we analyzed the promoter sequence of *TREE1* and identified 34 motifs, such as AE-box, CCGTCC-box, CGTCA-motif, G-box, I-box, TGACG-motif, W-box, WUN-motif, etc., indicating that *TREE1* gene expression could be regulated by many different transcription factors. However, no EIN3 binding motif was found in the promoter regions of *TREE1*. It is possible that one of the EIN3 downstream ethylene-inducible transcription factors binds to the *TREE1* promoter in a tissue-specific manner, which will be an interesting question for the next study.

EAR motif was defined by the consensus sequences of either LxLxL or DLNxxP, and it is the most predominant form of transcriptional repression motif so far identified in plants (34, 35). Many EAR-containing proteins have been found to have important functions in various developmental and physiological processes by negatively regulated gene expression (52). Mutations in the EAR motif of IAA3 (INDOLE-3-ACETIC ACID INDUCIBLE 3), IAA6 (INDOLE-3-ACETIC ACID 6), and IAA19 (INDOLE-3-ACETIC ACID INDUCIBLE 19) resulted in loss of their repression activity (53, 54). Interestingly, some Aux/IAAs contain two EAR motifs, such as IAA7, 14, 16, and 17 (52). The first EAR motif in IAA7 plays a dominant role in the transcriptional repression, while the second EAR motif plays only a minor role (55). TREE1 and DAZ3 also contain two EAR motifs, and these two motifs are essential for transcriptional repression in the ethylene response. Whether these two EAR motifs in TREE1 and DAZ3 play distinctive functions in the ethylene response will be a future interest.

The TREE1 binding motif was identified from the promoter regions of ethylene-repressed genes that are bound by EIN3 in shoots. We also provide genetics evidence showing that EIN3 is required for TREE1-mediated transcriptional repression. This result raises the question of how EIN3 targets are determined for transcriptional repression or activation in response to ethylene. It has been shown that EIN3 can form a homodimer in vitro (28). It is possible that EIN3 dimerizes in the promoter regions that contain only the EIN3 binding motif and not the TREE1 binding motif, and that this dimerization induces transcriptional activation. Formation of EIN3 and TREE1/DAZ3 heterodimers in the promoter regions that contain both EIN3 and TREE1 binding motifs may cause transcriptional repression. As EAR motifs have been extensively studied to be responsible for transcriptional repression (35, 56), it is plausible that the two motifs within TREE1 play a dominant roles in preventing the EIN3 transactivation. This in turn suppresses the expression of both TREE1 and EIN3 target genes. Alternatively, the TREE1-EIN3 heterodimer appears tofunction as a repressor in transcriptional repression. Further study on the detailed mechanisms will provide more insights. It is well known that histone deacetylation induces a more compact chromatin structure, which is associated with transcriptional repression. In previous work, we showed that histone acetylation of H3K14 and H3K23 is elevated by ethylene (17, 57, 58), while not H3K9. However, the levels of H3K9Ac are significantly lower in ethylene-repressed genes than in ethylene-activated genes, even without ethylene treatment. This signature in chromatin potentially contributes to the determination of transcription activation or repression as well. Yet, how the H3K9Ac levels are determined in the first place is unknown. ERF7 (ETHYLENE RESPONSE FACTOR 7), an EAR motif-containing transcription repressor is associated with histone deacetylase HDA19 (HISTONE DEACETYLASE 19) and is known to regulate ABA and abiotic stress responses (59). We speculate that TREE1/DAZ3, which also contains EAR motifs, may interact with histone deacetylases such as SRT1 and SRT2 to regulate H3K9Ac levels; this possibility will be the subject of future research.

Materials and Methods

Plant Growth Conditions. For phenotype assay, *Arabidopsis* seeds were surface sterilized in 50% bleach with 0.01% Triton X-100 for 15 min and washed five times with sterile, doubly distilled H_2O before plating on MS medium (4.3 g MS salt, 10 g sucrose, pH 5.7, and 8 g phytoagar per liter) with or without addition of 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma), the biosynthetic precursor to ethylene. After 3 to 4 d of cold (4 °C) treatment, the plates were wrapped in foil and kept at 24 °C in an incubator before the phenotypes of seedlings were analyzed. For propagation, seedlings were transferred from plates to soil (Promix-HP) and grown to maturity at 22 °C under 16-h light/8-h dark cycles. For all gene expression assays, protein level assays, and RNA-seq and ChIP-seq, ethylene treatment of *Arabidopsis* seedlings was performed by growing seedlings on MS plates in air-tight containers in the dark for 4 h supplied with either a flow of hydrocarbon-free air (zero grade air, AirGas) or hydrocarbon-free air with 10 ppm ethylene as previously described (11). For hypocotyl length measurements, 3-d-old seedlings were scanned using an Epson Perfection V700 Photo scanner, and hypocotyls were measured using NIH Image (https://imagej.nih.gov/nih-image/).

Plasmid and Transgenic Plant Construction. For the reporters used in luciferase assays, 6× TREE1 binding motif (primer 6× TREE1 motif *SI Appendix*, Table S2) or mutated TREE1 binding motif (primer 6× mutated motif in *SI Appendix*, Table S2), 5× EIN3 binding motif (primer 5× EIN3 motif in *SI Appendix*, Table S2), and 35S minimum promoter (primer 35Sminpro in *SI Appendix*, Table S2) sequences were generated using PCR, digested by the enzyme indicated, and cloned into empty pLUC vector. For the effectors used in luciferase assays, *EIN3* and *TREE1* coding regions with stop codon were generated using 35S-EIN3 and 35S-TREE1 (*SI Appendix*, Table S2), respectively, and further digested using *Bam*HI and cloned into pBI121 vector. For the vectors used in yeast two-hybrid assay, the coding regions of *EIN3*, *DAZ1*, *DAZ2*, *DAZ3*, and *TREE1* were generated using the primers EIN3-AD, DAZ1-BD, DAZ2-BD, DAZ3-BD, and TREE1-BD (*SI Appendix*, Table S2), respectively, and further digested using *Sal*I and *Spe*I. *EIN3* was cloned into pEXPAD502 vector; the others were cloned into pDBLeu vector. For the vectors used in pull-down or coimmunoprecipitation (Co-IP) assay, the coding regions of *TREE1*, *TREE1ΔEAR*, *DAZ3*, and *EIN3* were generated using primers TREE1ox, TREE1ΔEARox, DAZ3ox, and EIN3-pVP13 (*SI Appendix*, Table S2), respectively, and further cloned into pENTR vector. *TREE1-pENTR* and *EIN3-pENTR* were further cloned into pVP13 vectors using recombination reaction between *attL* and *attR* sites (LR reaction). *TREE1-pENTR* and *TREE1ΔEAR-pENTR* were further cloned into a modified pEarleyGate 101 vector with mCherry-FLAG sequence instead of EYFP-HA (*35S:TREE1-mCherry-FLAG*, *35S:TREE1ΔEAR-mCherry-FLAG*). *DAZ3-pENTR* was further cloned into pEarleyGate 101 vector (*35S:DAZ3-YFP-HA*). For *TREE1ox*, *TREE1ΔEARox*, and *DAZ3ox* plants, *35S:TREE1-mCherry-FLAG*, *35S:TREE1-ΔEAR-mCherry-FLAG*, and *35S:DAZ3-YFP-HA* were introduced into Col-0. For the vector used to generate *pTREE1:TREE1-GFP*, sequence was generated using TREE1 native (*SI Appendix*, Table S2), PCR products were digested by *Sbf*1 and *Bam*HI and cloned into pBI121 vector. For the vector used to

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generate *HMG1ox* plants, the coding sequence of *HMG1* was generated using *HMG1ox* (*SI Appendix*, Table S2), PCR products were digested by *Xba*I and *Bam*HI, and cloned into pBI121 vector. For the vector used to generate *DRY2ox* plants, the coding sequence of *DRY2* was generated using DRY2ox (*SI Appendix*, Table S2), PCR products were digested by *Kpn*I and *Sal*I, and cloned into pCHF3 vector. For CRISPR- Cas9 vectors used to generate *daz3tree1-1* double mutant, they were generated using gRNA primers (*SI Appendix*, Table S2) (36).

RNA Extraction and Real-Time PCR. Total RNA was extracted using an RNeasy Plant Kit (Qiagen) from 3-d-old etiolated seedlings treated for 4 h with air or ethylene gas as described above. First-strand cDNA was synthesized using SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen). Real-time PCR was performed with the LightCycler 480 SYBR Green I Master (Roche) following the manufacturer's instructions. PCR reactions were performed in triplicate. Expression levels were normalized to *ACT2*. Primers used for qPCR are listed in *SI Appendix*, Table S3. Each qRT-PCR was biologically repeated at least three times.

RNA-Seq Processing and Analysis. Three-day-old etiolated seedlings treated with air or 4 h of ethylene were harvested, and total RNA was extracted using Plant RNA Purification Reagent (Invitrogen) as described previously (14). Total RNA (4 μg) was used to prepare RNA-seq libraries using TruSeq RNA Library Prep Kit (Illumina) (60). Multiplexed libraries were sequenced on an Illumina Hi-Seq 2000. RNA-seq clean reads were aligned to TAIR10 genome release using TopHat version 2.0.9 (61) with default parameters. Differentially expressed genes were identified using Cufflinks version 2.0.1 following the workflow with default parameters described previously (62). The genes showing a $P < 0.05$ and reads per kilobase of transcript, per million mapped reads (RPKM) value larger than 1 were considered as significantly differentially expressed genes (63). Gene ontology term enrichment was performed over the sets of differentially expressed genes with the web-based tools AgriGO using default parameters with cutoff q value ≤0.05 (64). Pearson correlation coefficient was calculated using R (version 3.3.3).

Motif Analysis. Motifs were found by MEME-ChIP (meme-suite.org/tools/meme-chip) with default settings using 200-base pair sequences centered on the EIN3 binding summit in EIN3-bound ethylene up- or down-regulated genes in shoots and in roots.

TREE1 binding motif was determined by deep searching using $AGCTG^{T}/_{G}{}^{C}/_{A}$ against motifs in the database Tomtom (meme-suite.org/tools/tomtom) and by a literature search (31). In detail, the motif similarity is calculated by Pearson correlation coefficient between the aligned columns of the query and target motif. The E value and *P* value is used to measure the similarity between the query and target motif. As defined by Tomtom, matches must have an E value of 1 or smaller.

Yeast Two-Hybrid Assay. The yeast two-hybrid assay was performed using the ProQuest Two-Hybrid System (Invitrogen) following a previously published method (14, 17). Briefly, pBD-DAZ1, pBD-DAZ2, pBD-DAZ3, pBD-TREE1, and pAD-EIN3 were cotransformed into the yeast strain AH109. The transformants were grown on SD/−Trp −Leu medium or SD/−Trp −Leu −His dropout medium. Growth on SD/−Trp −Leu −His with dropout medium indicates interaction between corresponding proteins.

Western Blot. Proteins were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto a nitrocellulose membrane. The membrane was probed with the indicated primary antibodies and then with secondary goat anti-rabbit (Bio-Rad 170-6515) or goat anti-mouse (Bio-Rad 170-6516) antibodies conjugated with horseradish peroxidase. The signals were detected by a chemiluminescence reaction using the SuperSignal kit (Pierce). Polyclonal anti-EIN3 antibodies (20) were used at a dilution of 1:2,000. Monoclonal anti-FLAG (Cell Signaling) was used at a dilution of 1:2,000.

Immunoprecipitation Assays. Total proteins extracted from Col-0 were incubated with tobacco TREE1-mCherry-FLAG protein purified using anti-FLAG (Cell Signaling). Samples were incubated overnight at 4 °C and washed three times before analysis by Western blot.

***N. benthamiana* Transient Expression Assay.** Transient expression in *N. benthamiana* was performed by infiltrating 4-wk-old *N. benthamiana* plants with *Agrobacterium* containing different constructs. Leaf tissue was collected 3 d later for protein analysis. For the luciferase assay, *Agrobacterium* expressing the reporter and *Agrobacterium* harboring constructs containing the *35S::EIN3*, *35S::TREE1*, or *35S::GUS* were injected into *N. benthamiana* plants. After 3 d, the leaves were sprayed with 500 μM luciferin (Promega) and placed in the dark for 5 min. Luciferase activity was observed using the NightOWL LB 983 In Vivo Imaging System (Berthold).

ChIP-Seq and ChIP-qPCR. Briefly, 3-d-old etiolated seedlings treated with air or ethylene were harvested and cross-linked in 1% formaldehyde, and the chromatin was isolated. The anti-green fluorescent protein antibodies (Thermo Fisher Scientific) were added to the sonicated chromatin followed by incubation overnight to precipitate bound DNA fragments. DNA was eluted and amplified by primers corresponding to genes of interest. Primers used for ChIP-qPCR are listed in *SI Appendix*, Table S4. Each ChIP-qPCR was biologically repeated at least three times.

Chromatin-immunoprecipitated DNA was sequenced using an Illumina HiSeq 2000 platform according to standard operating procedures. Initial quality-control analysis was performed using FastQC. Single-end 51-bp reads were first mapped to the *Arabidopsis* genome (TAIR10) (65), using bowtie software (version 0.7.1) (66) with default parameters. Peaks significantly enriched in ChIP-seq tags were identified using MACS (version 1.4.0) (67). The nearest gene was assigned if there were more than one gene within 5 kbp of the peak regions. Genes present in either biological replicate were retained as binding targets. R (version 3.2.2) scripts were used to generate Venn diagrams.

EMSA. To detect the binding of TREE1 protein to the *DRY2* promoter, or to the TREE1 binding motif identified by ChIP-seq or ethylene down-regulated genes in shoots, EMSA was performed as described by instructions of LightShift Chemiluminescent EMSA kit (Pierce) with recombinant TREE1 and EIN3 protein produced in *E. coli* BL21 cells. In brief, the recombinant TREE1 or/and EIN3 protein was incubated with a biotin-labeled, double-stranded DNA oligonucleotide that covers the region containing the TREE1 binding sequence ($AGCTG^{T}/_{G}$) in *DRY2* promoter, or a biotin-labeled double-stranded six times TREE1 motif sequences. For control EMSA, nucleotide substitutions were introduced into the TREE1 binding site to produce the control probe. DNA binding reactions were carried out at room temperature for 20 min and the separation of protein-DNA complexes from the free DNA probes was done by nondenaturing polyacrylamide gel electrophoresis.

Data Availability. RNA-seq and ChIP-seq data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) (30, 68–70) (GSE83573, GSE120653, GSE122000, and GSE157179).

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