© 2021. Published by The Company of Biologists Ltd | Development (2021) 148, dev199635. doi:10.1242/dev.199635

CORRECTION

Correction: ETHYLENE INSENSITIVE 3 suppresses plant *de novo* root regeneration from leaf explants and mediates age-regulated regeneration decline

Hong Li, Lulu Yao, Lili Sun and Ziqiang Zhu

There was an error in *Development* (2020) **147**, dev179457 (10.1242/dev.179457).

The incorrect images were used for *WOX5pro:*GUS/*ein3 eil1* in B5+AAC (replicate 3) in Fig. S4B and for *WOX5pro:*GUS/*ein3 eil1* 12 d (all 3 replicates) in Fig. S7B.

The supplementary material has been updated with the correct images. The authors apologise to readers for this error, which does not impact the results or conclusions of the paper.

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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

ETHYLENE INSENSITIVE 3 suppresses plant *de novo* root regeneration from leaf explants and mediates age-regulated regeneration decline

Hong Li*, Lulu Yao*, Lili Sun* and Ziqiang Zhu‡

ABSTRACT

Powerful regeneration ability enables plant survival when plants are wounded. For example, adventitious roots can regenerate from the cutting site in detached *Arabidopsis thaliana* leaf explants, even in the absence of any exogenous plant hormone treatment. This process is known as *de novo* root regeneration (DNRR). Although the developmental program underlying DNRR is known, the precise regulatory mechanisms underlying DNRR are not completely understood. Here, we show that ethylene treatment or genetic activation of transcription factor ETHYLENE INSENSITIVE 3 (EIN3) strongly suppresses DNRR rates, while a mutant lacking EIN3 and its homolog EIL1 (*ein3 eil1*) displays a higher DNRR capacity. Previous reports have shown that the sequential induction of *WUSCHEL RELATED HOMEOBOX 11* (*WOX11*)/*WOX12* and *WOX5*/*WOX7* expression is required for the establishment of DNRR. We found that EIN3 directly targets *WOX11* and *WOX5* promoter regions to suppress their transcription. Furthermore, older plants show enhanced EIN3 activity, and repressed expression of *WOX11* and *WOX5*. Taken together, these results illustrate that plant aging at least partially takes advantage of EIN3 as a negative regulator to suppress DNRR through inhibiting the activation of WOX genes.

KEY WORDS: EIN3, WOX11, WOX5, Transcriptional repression, Root regeneration

INTRODUCTION

Plants exhibit a remarkable regeneration ability to form entirely new organs from detached shoots, roots or leaf explants. For example, when detached leaves from several plant species, including *Arabidopsis thaliana*, are placed on B5 medium in the absence of any exogenous plant hormones, they will initiate adventitious root formation at the cutting site (Chen et al., 2014). This process is known as *de novo* root regeneration (DNRR). Wounding stimulates auxin biosynthesis and auxin flow, resulting in a high auxin concentration above the cutting site (Chen et al., 2016). A recent study illustrates the paradigm for wounding-induced auxin biosynthesis. Jasmonate contents are rapidly induced (within 10-30 min) after wounding, and then gradually decreased after 2 h. Jasmonate activates an AP2/ERF transcription factor ETHYLENE RESPONSE FACTOR 109 (ERF109) to further directly induce the expression of *ANTHRANILATE SYNTHASE α 1* (*ASA1*). *ASA1* encodes an enzyme to catalyze the formation of anthranilate from chorismate, a key step in tryptophan biosynthesis pathway (Zhang et al., 2019). Tryptophan is a major precursor for auxin biosynthesis. These cells with high auxin concentrations are known as regeneration-competent cells. Auxin then induces the expression of *WUSCHEL RELATED HOMEOBOX 11* (*WOX11*) and *WOX12* to stimulate cell fate transition from regeneration-competent cells to root founder cells (Liu et al., 2014). After that, root founder cells divide and undergo cell fate transition into root primordium cells, accompanied by the induction of *WOX5* and *WOX7* (Hu and Xu, 2016). It has been shown that WOX11 and WOX12 directly activate *WOX5*/*WOX7* (Hu and Xu, 2016). Finally, root apical meristems and further adventitious roots are patterned from the root primordium cells described above.

Wounding also triggers the rapid biosynthesis of the plant gaseous hormone ethylene (Tsuchisaka and Theologis, 2004; Li et al., 2018). Ethylene participates in a variety of plant developmental events, including cell elongation, flowering time and senescence. The ethylene signal transduction pathway has been well established in the past decade from a series of genetic studies in *Arabidopsis*. After ethylene perception, ethylene inactivates its receptors and then deactivates CONSTITUTIVE ETHYLENE RESPONSE 1 (CTR1) (Kieber et al., 1993). *CTR1* encodes an Raf-like serine/threonine kinase that phosphorylates the COOH end (CEND) of ETHYLENE INSENSITIVE 2 (EIN2) (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). With the inactivation of CTR1, the phosphorylation status of EIN2 CEND is absent. The dephosphorylated EIN2 CEND is then cleaved by an unknown mechanism and either shuttled into the nucleus to abrogate EIN3-BINDING F BOX PROTEIN 1 (EBF1) and EBF2 E3 ligase activity (Qiao et al., 2012; Wen et al., 2012) or tethered with *EBF1*/*EBF2* mRNA into the cytoplasmic processing body (P-body) to suppress their translation (Li et al., 2015; Merchante et al., 2015). With these two strategies, EBF1/EBF2 activities are strongly inhibited and, consequently, their target protein transcription factors ETHYLENE INSENSITIVE 3 (EIN3) and its closest homolog EIN3 LIKE 1 (EIL1) are stabilized at a high level to trigger ethylene responses (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). The ethylene-stabilized transcription factors EIN3 and EIL1 control almost all the ethylene-responsive transcriptome (An et al., 2010; Chang et al., 2013).

As wounding triggers ethylene biosynthesis, we wondered whether ethylene is involved in DNRR. Our results showed that EIN3 directly represses the induction of *WOX11* and *WOX5* to suppress the DNRR process. Furthermore, EIN3 activity is gradually enhanced but rooting rates are reduced as plants age, suggesting that EIN3 conveys plant age information in the regulation of regeneration capacity.

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Handling Editor: Ykä Helariutta
Received 24 April 2019; Accepted 30 March 2020

Development (2020) 147, dev179457. doi:10.1242/dev.179457

RESULTS

Ethylene suppresses DNRR through EIN3 and EIL1

To evaluate the role of ethylene in DNRR, we initially tested whether the presence of ethylene would affect the rooting rate. It has been reported that DNRR rate in wild type (Col-0) is higher when detached leaves are kept in darkness compared with under light (Chen et al., 2016). In order to avoid the DNRR decrease under light, we therefore performed our analysis in darkness. We supplemented the B5 medium with the ethylene biosynthesis precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (B5+ACC) to mimic ethylene treatment. We found that 10 µM ACC treatment blocked root regeneration in Col-0 leaf explants (Fig. 1A,B). In fact, as little as 0.1 µM ACC was sufficient to inhibit root regeneration (Fig. S1). Rooting in the *ctr1-1* mutant (constitutive ethylene response mutant) was completely abolished (Fig. 1A), but ethylene-insensitive mutants (*ein2-5* or *ein3 eil1*) were able to regenerate roots even more rapidly than Col-0 (Fig. 1A,B). Both *ein2-5* and *ein3 eil1* produced more roots per leaf explant than did wild type (Fig. 1A,C), suggesting that ethylene negatively regulates the DNRR process.

Next, we used an estrogen-inducible system (*iEIN3-FLAG*/*ein3 eil1 ebf1 ebf2*) to induce *EIN3* expression in the *ein3 eil1 ebf1 ebf2* quadruple mutant background (An et al., 2010), and tested whether the induction of *EIN3* was sufficient to suppress DNRR. Without an inducer, more than 50% of *iEIN3-FLAG*/*ein3 eil1 ebf1 ebf2* leaf explants generated roots after 6 days on B5 medium (Fig. 2A,B). However, after supplying estradiol to induce EIN3-FLAG fusion protein expression (Fig. 2C), the rooting rates were severely reduced (Fig. 2A,B). These results demonstrate that EIN3 protein accumulation is sufficient to repress the DNRR process. Taken together, these results indicate that ethylene suppresses DNRR through the ethylene-responsive transcription factor EIN3.

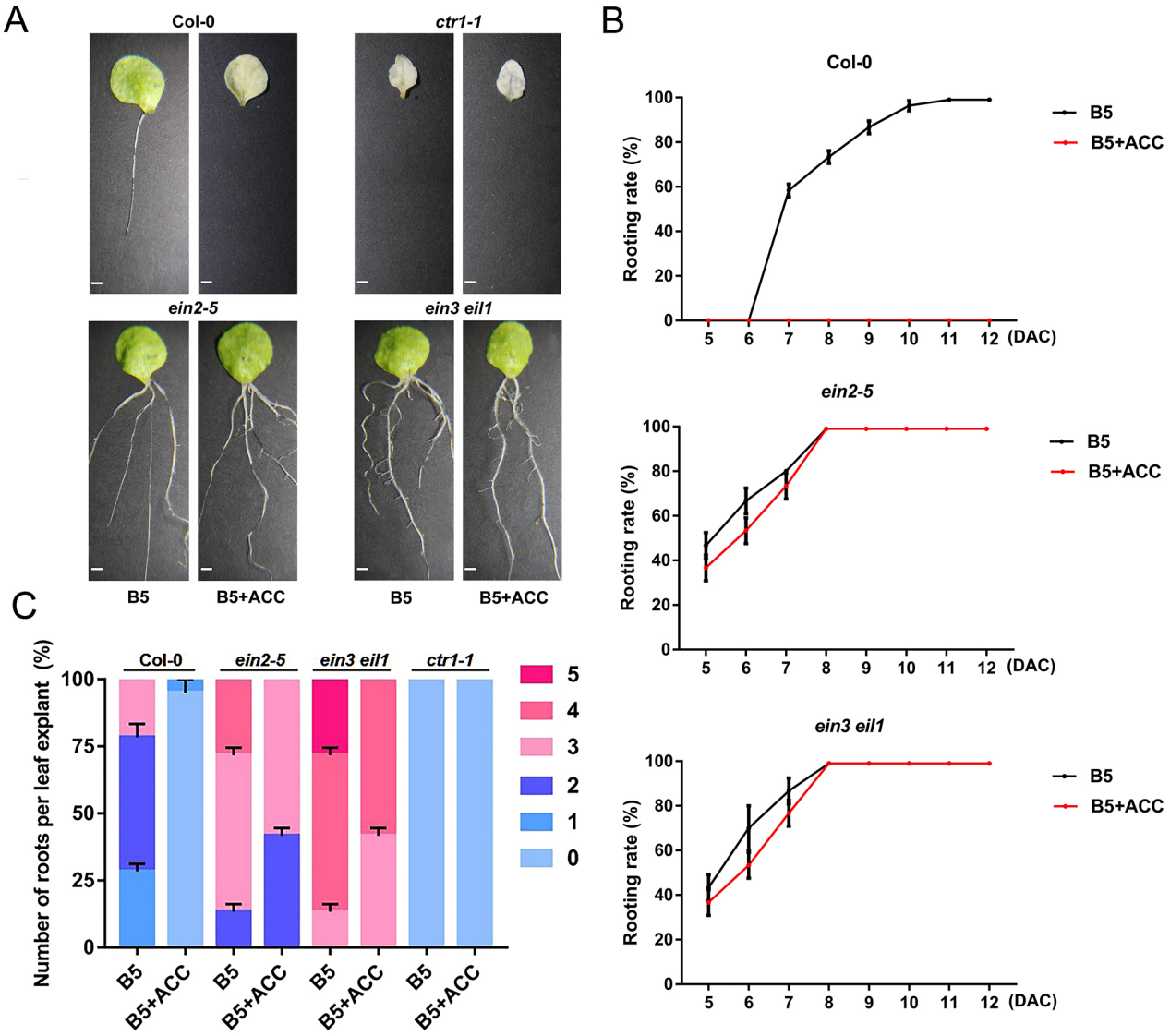

Fig. 1. Ethylene suppresses *de novo* root regeneration. (A,B) Root regeneration phenotypes. After growth on 1/2 MS medium for 12 days, the first pair of true leaves of the indicated genotypes were detached and placed on B5 medium or B5 medium containing 10 µM ACC (B5+ACC) for different numbers of days after culture (DAC). Representative images show the rooting phenotypes at 12 DAC (A). Scale bars: 1 mm. Statistical analysis of rooting rates in individual genotypes at different DAC are presented in B. Data are mean±s.d., *n*=3. (C) Quantitative analysis of the adventitious root numbers per 12 DAC leaf explant grown on B5 medium or B5 medium containing 10 µM ACC (B5+ACC). Each sample includes at least 20 explants, and the number of roots per leaf explant was counted and calculated as a percentage. The average of three independent replicates is shown ±s.d.

Development (2020) 147, dev179457. doi:10.1242/dev.179457

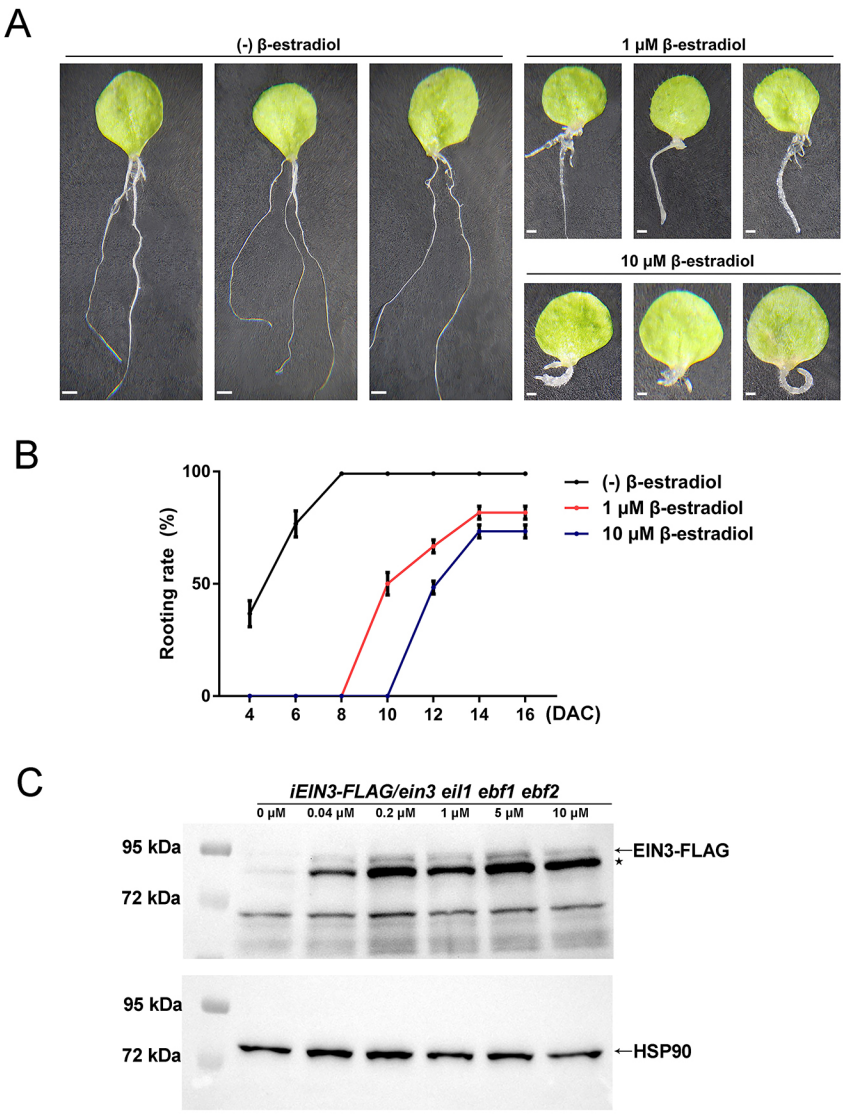

Fig. 2. EIN3 is sufficient for repressing *de novo* root regeneration. (A) Representative individual 12 day after culture (DAC) leaf explants (*iEIN3-FLAG/ein3 eil1 ebf1 ebf2*) on B5 medium containing 0, 1 or 10 μM β-estradiol. Scale bars: 1 mm. (B) Rooting rates of leaf explants (*iEIN3-FLAG/ein3 eil1 ebf1 ebf2*) on B5 medium containing 0, 1 or 10 μM β-estradiol. Data are mean±s.d., *n*=3 in each repeat. (C) Immunoblot analysis of EIN3 in *iEIN3-FLAG/ein3 eil1 ebf1 ebf2* plants. After culturing on B5 medium containing the indicated concentrations of β-estradiol for 12 days, the leaf explants were harvested for protein extraction. Protein extracts were separated on SDS-PAGE, transferred onto nitrocellulose membrane and blotted using anti-FLAG or anti-HSP90 (loading control) antibodies. Protein extracts from 12 DAC Col-0 leaf explants served as negative controls. Asterisk indicates a non-specific band.

Auxin could not rescue the ethylene-triggered DNRR defect

Wound-induced auxin accumulation is the initial step for establishing DNRR. Therefore, we examined whether ethylene affected auxin accumulation. DII-VENUS is a widely used auxin sensor for describing cellular auxin dynamics (Brunoud et al., 2012). The VENUS signal intensity is negatively correlated with auxin level in cells. Compared with normal conditions, ethylene treatment substantially enhanced VENUS expression in detached leaf explants (Fig. S2A), i.e. ethylene repressed the wound-induced auxin accumulation at the cutting site. To further confirm this observation, we monitored GUS (glucuronidase) expression in the auxin-responsive reporter line *DR5*:GUS. In the presence of ethylene, *DR5*:GUS was still expressed in cells above the cutting site, although it was expressed at lower levels than in normal conditions (Fig. S2B). These results suggest that ethylene limits auxin activity in regeneration-competent cells.

Exogenous auxin treatment has been shown to promote DNRR (Chen et al., 2014). Therefore, we compared the rooting rate after supplying indole-3-acetic acid (IAA) at various concentrations simultaneously with ethylene. Low concentration (0.1 μM) IAA alone promoted rooting rates in both Col-0 and *ein3 eil1* mutants (Fig. S3A,B), whereas a high concentration (1 or 10 μM) of IAA triggered callus formation in the cutting site in both genotypes. However, none of these IAA treatments could trigger root regeneration in *ctr1-1* explants (Fig. S3A). Consistently, IAA could not rescue the ethylene-triggered rooting defects in Col-0 (Fig. S3A,B). These results imply that, although ethylene negatively affects auxin accumulation during DNRR, it is not sufficient to block rooting.

EIN3 directly suppresses *WOX11* and *WOX5* transcription

Next, we checked WOX gene expression during DNRR. As expected, ethylene strongly induced EIN3 activity in leaf explants (Fig. 3A), as determined by monitoring *EBS*:GUS reporter expression. The synthetic promoter of *EBS*:GUS contains five copies of EIN3-binding sites (EBS), fused with the minimal 35S promoter to drive the GUS expression (Stepanova et al., 2007). However, ethylene treatment diminished the induction of both $WOX11_{pro}$:GUS and $WOX5_{pro}$:GUS (Fig. 3A). Promoter analyses revealed that there were multiple consensus EIN3-binding sites (ATNCAN) in the promoter regions of both *WOX11* and *WOX5* (Fig. 3B). Therefore, we hypothesized that EIN3 might directly associate with *WOX* promoters to suppress transcription. Electrophoretic mobility shift assay (EMSA) results showed that the EIN3 DNA-binding domain (amino acids 141-352) was able to

Development (2020) 147, dev179457. doi:10.1242/dev.179457

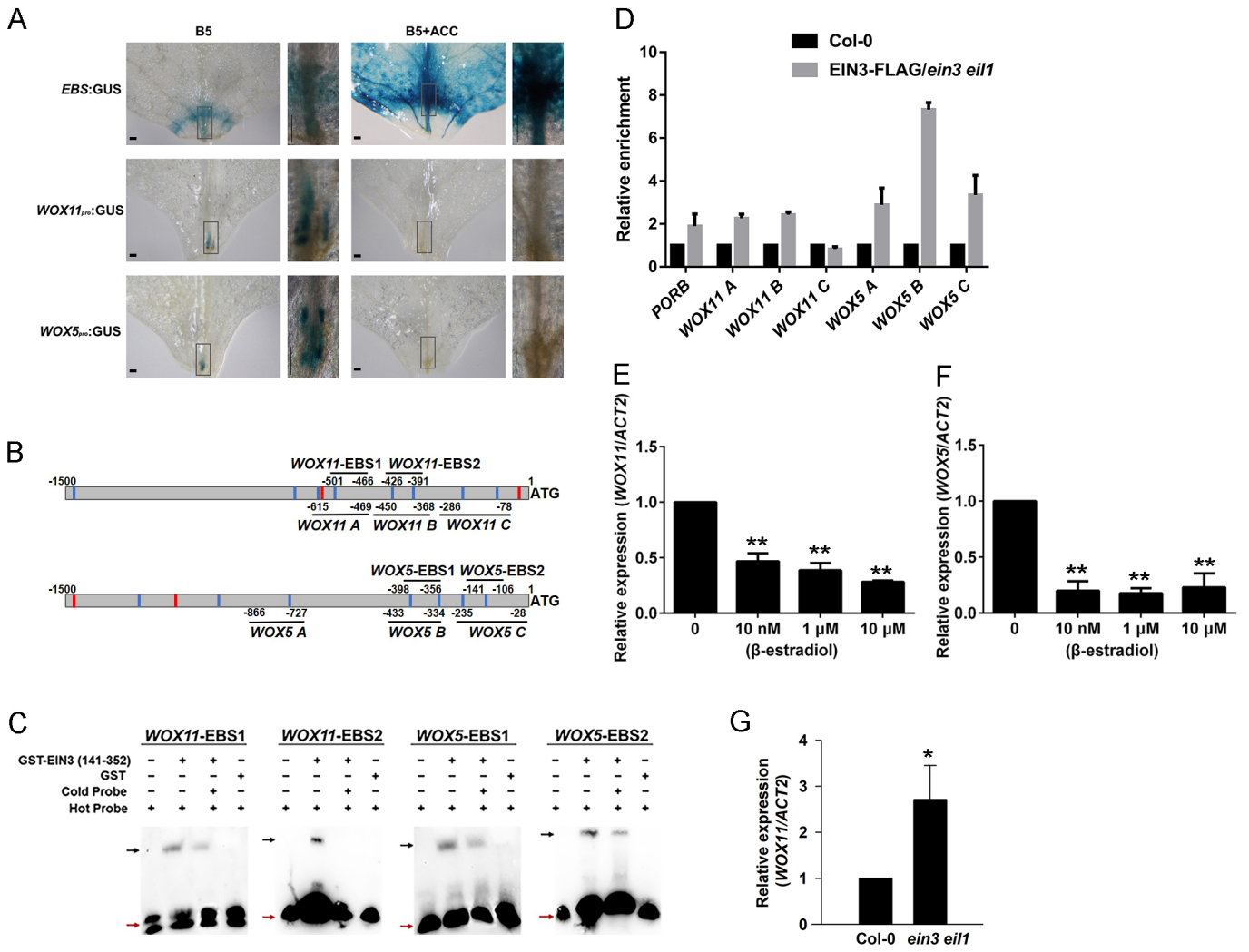

Fig. 3. EIN3 directly suppresses *WOX11* and *WOX5* transcription. (A) GUS staining of leaf explants 4 days after culture (DAC) from *EBS*:GUS, *WOX11*$_{pro}$:GUS and *WOX5*$_{pro}$:GUS, cultured on B5 medium or B5 medium containing 10 µM ACC (B5+ACC). A more detailed view of each boxed region is shown on the right. Scale bars: 0.1 mm. (B) Schematic diagram of the *WOX11* or *WOX5* promoter region. Short blue lines represent putative EIN3-binding sites (ATNCAN); short red lines represent auxin response elements (AuxREs, TGTCTC). Numbers indicate distance to start codon (ATG). (C) EMSA to analyze the DNA binding activity of EIN3 to *WOX11* or the *WOX5* promoter. The purified GST-EIN3 (141-352) proteins were incubated with *WOX11*-EBS1, *WOX11*-EBS2, *WOX5*-EBS1 and *WOX5*-EBS2 probes labeled with biotin. An excess of unlabeled probes was added to compete with biotin-labeled probes. The positions of retarded bands and free labeled probes are indicated with a black arrow or a red arrow, respectively. Hot probe, biotin-labeled EBS probe; cold probe, non-labeled for competition (200-fold of hot probe). Probe locations are depicted in B. (D) ChIP-qPCR assays showing *in vivo* association between EIN3 and *WOX11* or the *WOX5* promoter. Crosslinked chromatins extracted from EIN3-FLAG/*ein3 eil1* or Col-0 (negative control) were immunoprecipitated with anti-FLAG antibody. Eluted DNA was used as a template to amplify a fragment covering putative EIN3-binding sites by q-PCR. Detailed fragment information is shown in B. ChIP signal is displayed as a percentage of total input DNA. The EIN3-binding region in the promoter of *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE B* (*PORB*) served as a positive control. (E,F) qRT-PCR analysis of *WOX11* (E) and *WOX5* (F) transcript levels in 7-day-old light-grown *iEIN3-FLAG*/*ein3 eil1 ebf1 ebf2* seedlings treated with β-estradiol at various concentrations for 8 h. *Actin 2* was used as the internal control. Data are mean±s.d., *n*=3. Student's *t*-test (**P<0.01) was used to analyze statistical significance. (G) *WOX11* expression in leaf explants from 12-day-old light-grown Col-0 or *ein3 eil1* placed on B5 medium for 2 days. The cutting sites of leaf explants (about 0.25 cm^2) were collected for analysis of gene expression. *Actin 2* was used as the internal control. Data are mean±s.d., *n*=3. Student's *t*-test (*P<0.05) was used to analyze statistical significance.

bind to two separate fragments in either the *WOX11* or *WOX5* promoter (Fig. 3C). In *in vivo* chromatin immunoprecipitation PCR (ChIP-PCR) analyses, EIN3 directly associated with *WOX11* and *WOX5* promoters (Fig. 3D), although the binding capacity differed among promoter different regions. To directly test the transcriptional regulation on *WOX11*/*WOX5* by EIN3, we again took advantage of the *iEIN3-FLAG*/*ein3 eil1 ebf1 ebf2* system, which did not express any EIN3 protein in the absence of inducer. After estradiol induction, we found that both *WOX11* (Fig. 3E) and *WOX5* (Fig. 3F) transcript levels were downregulated, indicating that the accumulation of EIN3 repressed *WOX11* and *WOX5* transcription. Consistently, in the absence of EIN3/EIL1 (*ein3 eil1* double mutants), *WOX11* expression was higher (Fig. 3G). We also compared either *WOX11*$_{pro}$:GUS or *WOX5*$_{pro}$:GUS expression patterns after ethylene treatment in both Col-0 and *ein3 eil1* background, and found that *GUS* expression was not reduced in *WOX11*$_{pro}$:GUS/*ein3 eil1* or *WOX5*$_{pro}$:GUS/*ein3 eil1* by ethylene (Fig. S4). These results together demonstrate that EIN3 physically binds to *WOX11* and *WOX5* promoters to suppress their expression.

Development (2020) 147, dev179457. doi:10.1242/dev.179457

To further illustrate whether WOX genes act downstream of ethylene signaling, we characterized the DNRR phenotypes in *WOX11* overexpression lines (*35S$_{pro}$:WOX11*) under ethylene treatment. Although Col-0 leaf explants could not generate any roots even under very low concentrations of ACC (0.1 µM ACC), *35S$_{pro}$:WOX11* at least sustained 1 µM of ACC (Fig. 4A,B). Next, we took advantage of a dexamethasone (DEX) activation system to activate WOX11-GLUCOCORTICOID (GR) fusion proteins (*35S$_{pro}$*:3×FLAG-WOX11-GR) for further testing whether activation of *WOX11* was sufficient to trigger root regeneration. Our results showed that, without DEX, both Col-0 and *35S$_{pro}$*:3×FLAG-WOX11-GR leaf explants could not regenerate roots in the presence of ACC (Fig. S5); however, supplying DEX successfully rescued the rooting defects in *35S$_{pro}$*:3×FLAG-WOX11-GR (Fig. S5). These results suggest that overexpression of *WOX11* is able to partially rescue the EIN3-triggered DNRR decrease. We also examined the rooting phenotypes in *WOX5* overexpression lines (*35S$_{pro}$:WOX5*) and observed similar rooting patterns to those we described in *35S$_{pro}$:WOX11* (Fig. 4C,D). *WOX* overexpression (*35S$_{pro}$:WOX11* and *35S$_{pro}$:WOX5*) (Fig. 4) or *WOX11* activation (*35S$_{pro}$*:3×FLAG-WOX11-GR) lines (Fig. S5) still exhibited rooting rate decline in response to the increase of ACC concentrations. Recent studies have shown that auxin is required for fully activating both WOX11 and WOX5 (Chen et al., 2016; Hu and Xu, 2016). The rooting rates in either *35S$_{pro}$:WOX11* or *35S$_{pro}$:WOX5* explants are reduced in the presence of an auxin biosynthesis inhibitor or auxin transport inhibitor, respectively (Chen et al., 2016; Hu and Xu, 2016). We reveal that high concentrations of ACC cause the repression of the auxin response (Fig. S2), which might be the cause of the decline in rooting rates in *WOX* activation lines. Nonetheless, all these WOX activation lines largely rescue the ethylene-induced DNRR defects. Taken together, we show that

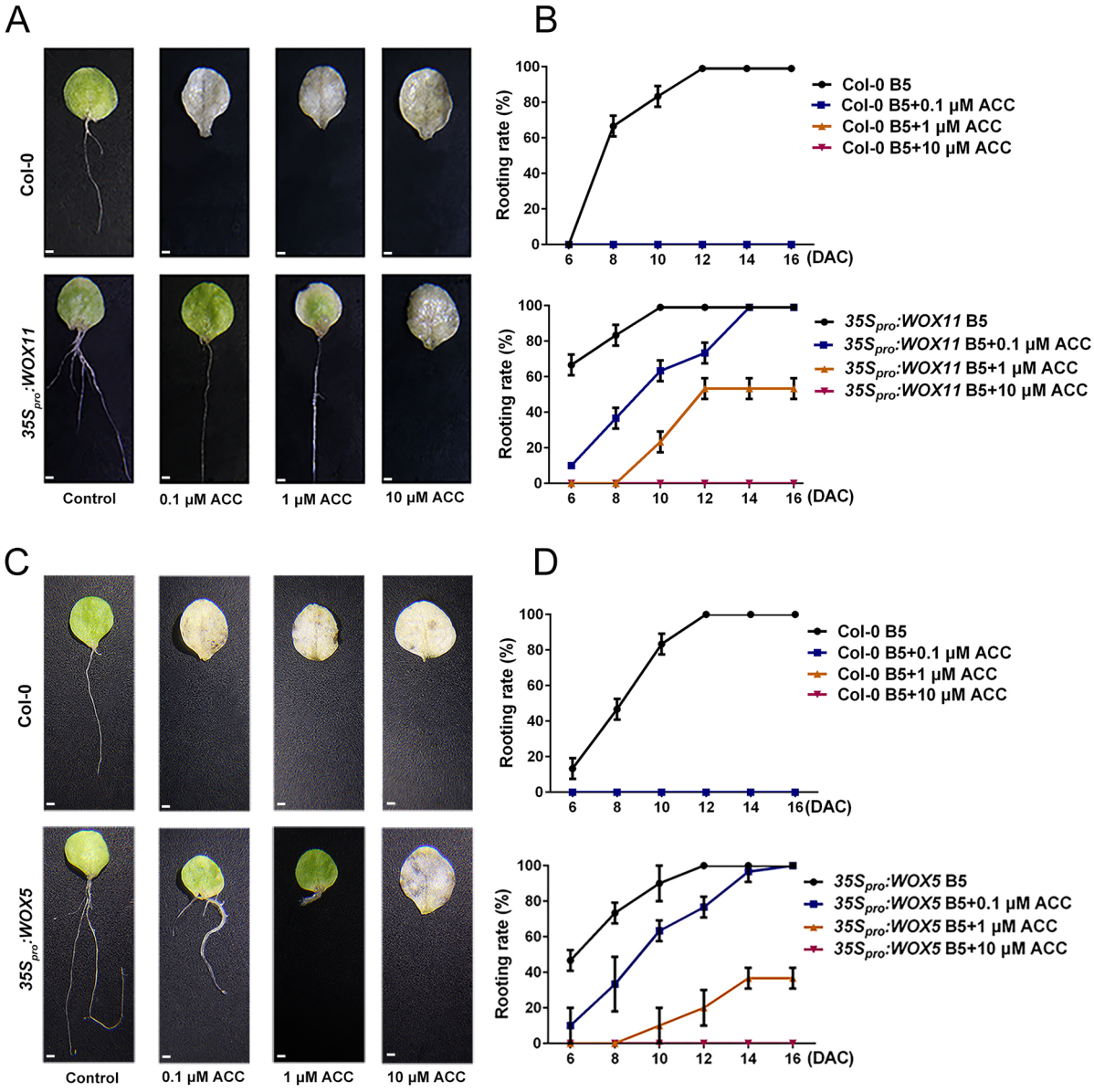

Fig. 4. Overexpression of *WOX11* or *WOX5* partially rescues the EIN3-triggered DNRR decrease. (A,C) Representative images of 12 days after culture leaf explants from Col-0 and *35S$_{pro}$:WOX11* (A) or *35S$_{pro}$:WOX5* (C) cultured on B5 medium containing different concentrations of ACC. Scale bars: 1 mm. (B,D) Quantitative analyses of rooting rates under different treatments in Col-0 and *35S$_{pro}$:WOX11* (B) or *35S$_{pro}$:WOX5* (D) explants. Data are mean±s.d., *n*=3.

Development (2020) 147, dev179457. doi:10.1242/dev.179457

ethylene inhibits DNRR through the EIN3-mediated transcriptional repression on *WOX11*/*WOX5*.

Plant age induces *EIN3* transcription and represses *WOX11*/*WOX5* expression

It has been reported that EIN3 is correlated with plant aging (Li et al., 2013), and that the DNRR process is blocked in older plants. Therefore, we speculated that EIN3 might be involved in the decline in regeneration triggered by plant aging. The qRT-PCR results showed that *EIN3* transcription was gradually upregulated during the juvenile to adult switch (from 12-day-old seedlings to 30-day-old plants) (Fig. S6). Consistently, *EBS*:GUS reporter assays showed that EIN3 activity was enhanced in older plants (Fig. 5A). In contrast, the expression of both $WOX11_{pro}$:GUS and $WOX5_{pro}$:GUS was reduced in older plants (Fig. 5B,C). The correlation of enhanced EIN3 activity and decreased WOX expression suggests that EIN3 conveys the plant age information to suppress WOX activation. We then checked *WOX11* expression levels in detached Col-0 and *ein3 eil1* at different ages and found that *WOX11* transcription levels were consistently decreased in Col-0 with aging (Fig. 6A). However, *WOX11* expression was also gradually reduced in *ein3 eil1* (Fig. 6A). Nevertheless, *WOX11* transcription was still higher in *ein3 eil1* when compared with Col-0. The *WOX11* expression levels in 30-day-old *ein3 ei1 l* plants were still comparable with Col-0 at its seedling stage (12 days old) (Fig. 6A). We also took advantage of the GUS reporter lines to dissect the *WOX11* and *WOX5* transcription in the Col-0 or *ein3 eil1* background. Consistent with qRT-PCR results, the age-responsive *GUS* expression decay was attenuated in the $WOX11_{pro}$:GUS/*ein3 eil1* (Fig. S7A) or $WOX5_{pro}$:GUS/*ein3 eil1* plants (Fig. S7B). These results indicate that, as plants age, the activation of *WOX11* or *WOX5* is suppressed at least partially through the induction of EIN3 activity.

Consistent with these gene expression patterns, the rooting rate in Col-0 was reduced in 20-day-old plants, and no rooting occurred in 30-day-old plants (Fig. 6B,C). However, *ein3 eil1* double mutant plants displayed a relatively high rooting rate even at 30 days (Fig. 6B,C). Consistent with the decrease in *WOX11* and *WOX5* expression (Fig. 6A and Fig. S7), we also noticed a moderate DNRR decline in *ein3 eil1* with plant age (Fig. 6C). In conclusion, as plants age, the expression of *WOX11* and *WOX5* reduces, partially due to the increase in EIN3 activity, and this hinders root regeneration.

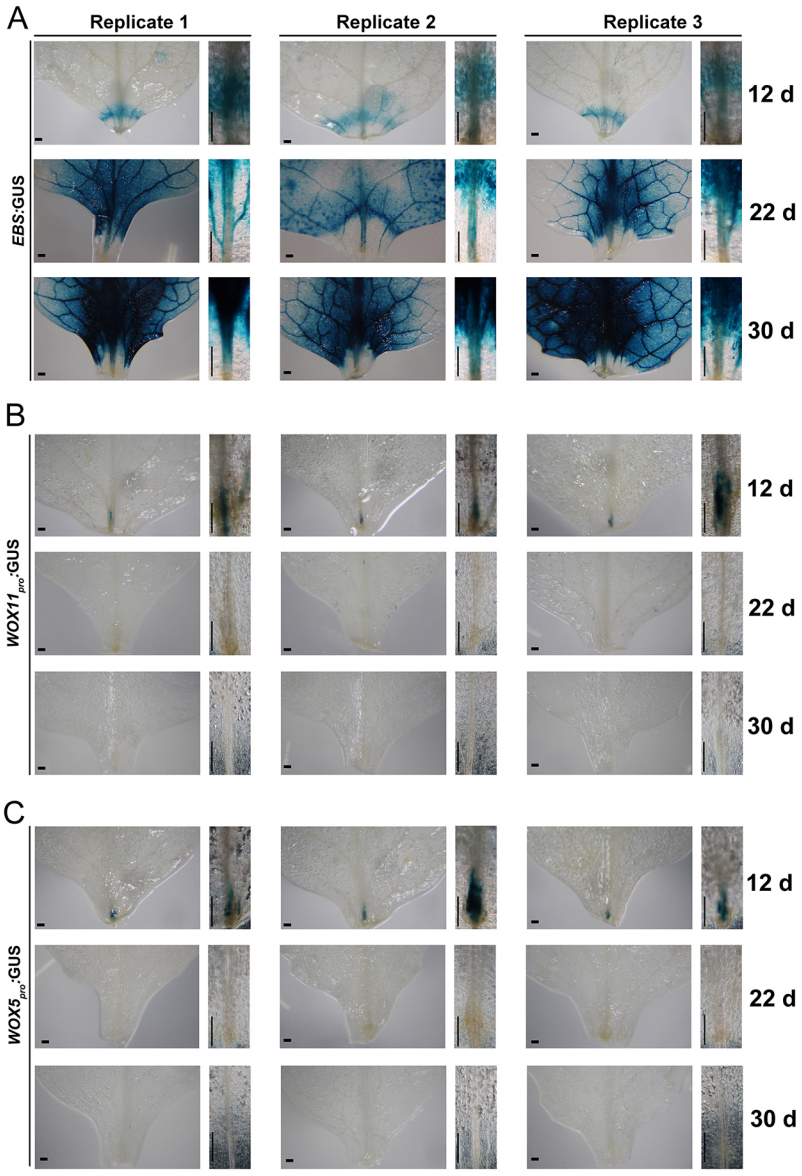

Fig. 5. GUS reporter analysis. Leaf explants detached from 12, 22 or 30-day-old *EBS*:GUS (A), $WOX11_{pro}$:GUS (B) or $WOX5_{pro}$:GUS (C) plants were placed on B5 medium for 4 days and then stained to detect GUS expression. A more-detailed view of each enlarged region is shown on the right. Three arbitrarily selected images are shown as replicates (replicate 1/2/3) for each treatment. Scale bars: 0.1 mm.

Development (2020) 147, dev179457. doi:10.1242/dev.179457

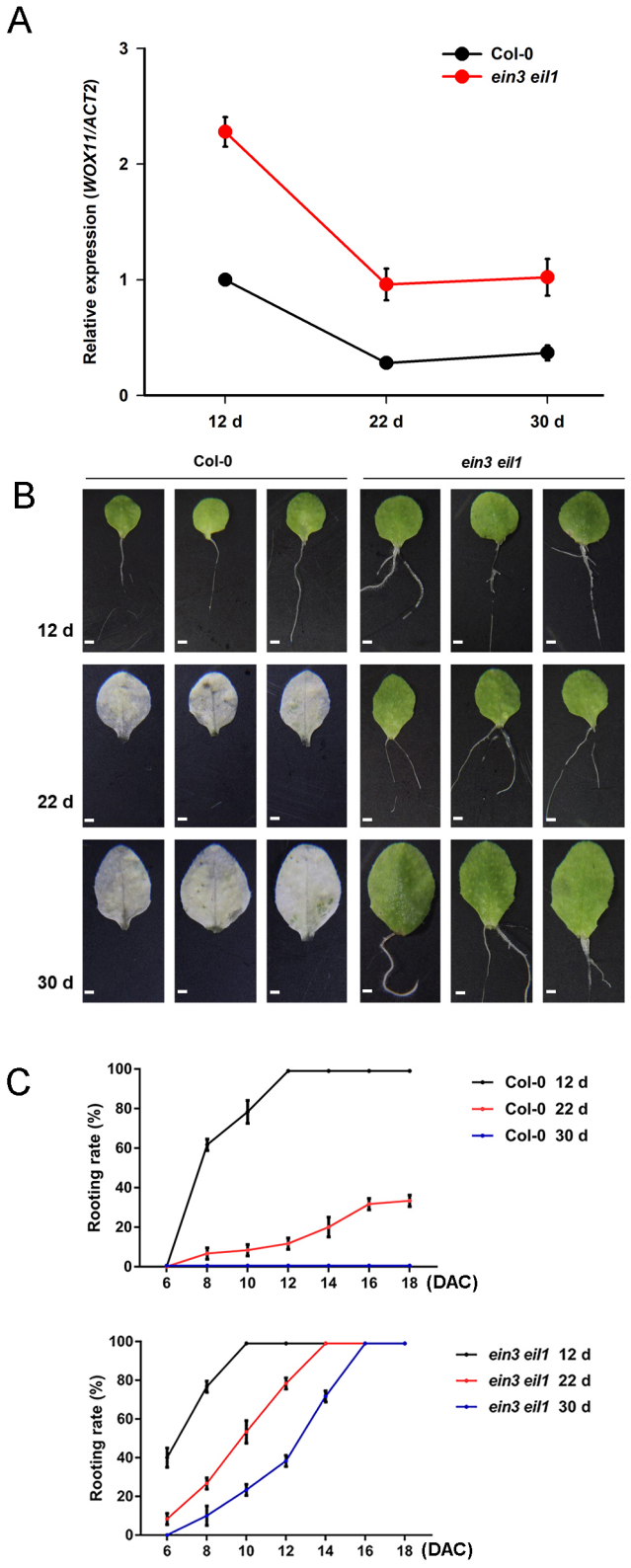

Fig. 6. EIN3 is required for age-mediated regeneration decay. (A) qRT-PCR analysis of *WOX11* transcription levels. Leaf explants detached from 12, 22 or 30-day-old Col-0 or *ein3 eil1* plants were placed on B5 medium for 2 days, then the wounding areas above the cutting sites in each sample (about 0.25 cm^2) were manually collected for RNA extraction and downstream gene expression analysis. Data are mean±s.d., *n*=3. (B) Leaf explants detached from 12, 22 or 30-day-old Col-0 or *ein3 eil1* were placed on B5 medium for 12 days. Representative images showed the typical root regeneration results. Scale bars: 1 mm. (C) Rooting rate analysis of leaf explants from 12, 22 or 30-day-old Col-0 or *ein3 eil1* on B5 medium at different days after culture. Data are mean±s.d., *n*=3.

DISCUSSION

Ethylene is responsible for the plant wounding and aging response, both are related to the DNRR process. Wounding-induced auxin accumulation around the cutting site is the prerequisite for DNRR initiation, while aging inhibits DNRR. Our present study shows that the ethylene-stabilized transcription factor EIN3 directly suppresses the activation of key cell fate-determining genes (*WOX11* and *WOX5*) to inhibit adventitious root formation from detached leaf explants (Fig. 7). Ethylene also reduces auxin accumulation (Fig. S2), which further slows down root regeneration.

Our physiological observations indicate that EIN3 activity is negatively correlated with DNRR rates through either exogenous ethylene treatment or genetic approaches (Figs S1, S2). Meanwhile, we also find that ethylene not only represses DNRR but also causes chlorophyll degradation if roots cannot be regenerated (Fig. 1A, Fig. S1 and Fig. 4). This phenomenon prompts us to consider whether there is another interpretation of our data, i.e. that ethylene triggers chlorophyll degradation first and then causes the DNRR decline. According to the following lines of evidence, we conclude that, although it is true that ethylene results in chlorophyll degradation, the repression of *WOX11* and *WOX5* is the major reason for inhibiting DNRR. Comparing the rooting rates in Col-0, *ein2-5* and *ein3 eil1* under normal condition (B5 medium, without ethylene) (Fig. 1A,B), although both leaf explants are green, *ein2-5* and *ein3 eil1* mutants exhibit higher rooting rates. These results indicate that green leaf explants in different genetic backgrounds already display distinct rooting rates in an EIN3-dependent manner. Under the same concentrations of ACC treatment (0.1 or 1 µM of ACC), although Col-0 leaf explants are pale and rootless, $35S_{pro}$:*WOX11* or $35S_{pro}$:*WOX5* explants still regenerate roots (Fig. 4A,C). If ethylene-triggered chlorophyll degradation is a prerequisite for repressing DNRR, it is unlikely that overexpression of *WOX11* or *WOX5* could overcome this negative effect. The DEX-inducible activation of *WOX11* further substantiates this explanation (Fig. S5). In the presence of ethylene, the expression levels of both *WOX11* and *WOX5* are reduced in an EIN3-dependent manner (Fig. 3A,G and Fig. S4). This could not be simplified to being a consequence of chlorophyll degradation.

Nonetheless, we speculate that ethylene has a stimulation effect on chlorophyll degradation, but after explants form adventitious roots, this effect is counteracted. It was recently reported that EIN3 directly upregulates expression of three chlorophyll catabolic genes (*NYE1*, *NYC1* and *PAO*) to promote degreening during leaf senescence (Qiu et al., 2015). Thus, we assume that, in the presence of ethylene, EIN3 simultaneously triggers two parallel events (chlorophyll degradation and DNRR repression). The detailed antagonistic mechanisms between root formation and chlorophyll degradation are worthy of future study.

As a necessary transcription factor in ethylene signaling, EIN3 targets several genes to negatively control various growth and developmental events. For example, EIN3 directly binds to the promoter of *SALICYLIC ACID INDUCTION DEFICIENT 2* (*SID2*) (Chen et al., 2009) or *C-REPEAT BINDING FACTOR* (*CBF*) genes (Shi et al., 2012) to negatively control pathogen-induced salicylic acid biosynthesis or plant freezing tolerance, respectively. Our results showed that EIN3 also targets two cell fate-determining genes to suppress DNRR. Although these studies suggest that negative regulation by EIN3 is a key event in signal transduction, the

Development (2020) 147, dev179457. doi:10.1242/dev.179457

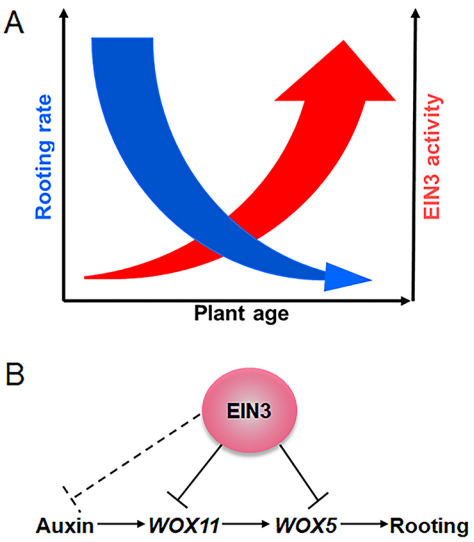

Fig. 7. Working model. (A) With increasing plant age, EIN3 activity is gradually induced and, consequently, rooting rate during *de novo* root regeneration is reduced. (B) Auxin stimulates the sequential activation of *WOX11* and *WOX5* to initiate root regeneration in detached leaf explants. However, EIN3 directly suppresses the transcription of *WOX11* and *WOX5* to inhibit root regeneration. Ethylene also suppresses auxin accumulation, most likely through EIN3 (as depicted by the dotted lines). Blunt ends mean repression; arrowheads indicate promotion.

detailed molecular basis is still unclear. Taking the EIN3-WOX promoter interaction as an example, there are several auxin-responsive elements (AuxREs) in WOX promoters (Fig. 3B) (Liu et al., 2014), and some of these AuxREs are adjacent to, or surrounded by, EIN3-binding sites. In future studies, it will be interesting to determine whether EIN3 competes with an AUXIN RESPONSE FACTOR (ARF) to occupy these AuxREs and suppress WOX activation. In addition, we have previously reported that EIN3 physically interacts with HISTONE DEACETYLASE 6 (HDA6) (Zhu et al., 2011). Thus, it is also possible that EIN3 recruits HDA6 as a co-repressor to alter the chromatin status, thereby suppressing target gene transcription.

In our experiments, ethylene partially suppressed auxin accumulation at the cutting site (Fig. S2). It is unknown why ethylene inhibited auxin accumulation but did not promote auxin biosynthesis, as commonly occurs in the typical triple response (Stepanova et al., 2005). Wound-induced auxin biosynthesis is initially triggered by jasmonate (Zhang et al., 2019). As ethylene antagonizes a proportion of jasmonate responses through competition between EIN3 and MYC2 (a jasmonate-responsive bHLH transcription factor) (Song et al., 2014), we speculate that ethylene might use the same strategy to suppress jasmonate signaling and then repress auxin accumulation during DNRR. It is intriguing to test whether EIN3 suppresses ERF109 activity to inhibit *ASA1* transcription in future. On the other hand, although jasmonate signaling is required for auxin biosynthesis and DNRR, long-term jasmonate treatment or constitutively activation of *ERF109* also suppresses DNRR (Zhang et al., 2019), which is reminiscent of ethylene treatment in our work. In fact, strong activation of ethylene signaling (in *ebf1 ebf2* double mutants) results in embryonic lethality, which is caused by the overaccumulation of EIN3 and EIL1 transcription factors (Guo and Ecker, 2003; An et al., 2010). Plants also use both jasmonate and ethylene signaling to trigger leaf cell death as a defense against necrotrophic fungi (Bouchez et al., 2007; Zhang and Xing, 2008). These results suggest that both jasmonate and ethylene trigger plant death but not survival.

As plants age, EIN3 activity is enhanced (Fig. S6 and Fig. 5A), whereas the activation of *WOX11* and *WOX5* in detached leaf explants is reduced (Figs 5B-C, 6A and Fig. S7). However, *WOX11* and *WOX5* activation is also attenuated in *ein3 eil1* mutants (Fig. 6A and Fig. S7), suggesting that an EIN3-independent repression route also exists. Nevertheless, *WOX11* expression levels in adult *ein3 eil1* mutants are still comparable with juvenile Col-0 explants (Fig. 6A and Fig. S7A). These results demonstrate that, although EIN3 is not sufficient for plant age-related *WOX* repression during DNRR, EIN3 is required for this process. When EIN3 reaches a threshold, it will completely block the regeneration pathway. In other words, EIN3 acts as a timekeeper of plant age and determines cell fate accordingly.

MATERIALS AND METHODS

Plant materials and root regeneration procedures

All the seeds described in this article were in the Columbia (Col-0) ecotype. The seeds of *ein3 eil1* (An et al., 2010), *ctr1-1* (Kieber et al., 1993), *ein2-5* (Alonso et al., 1999), *iEIN3-FLAG/ein3 eil1 ebf1 ebf2* (An et al., 2010), *EBS*:GUS (Stepanova et al., 2007), $35S_{pro}$:*WOX11*, $WOX11_{pro}$:GUS (Liu et al., 2014), $35S_{pro}$:*WOX5*, $WOX5_{pro}$:GUS, $35S_{pro}$:3×FLAG-WOX11-GR (Hu and Xu, 2016), *DR5*:GUS (Stepanova et al., 2007), EIN3-FLAG/*ein3 eil1* (An et al., 2010) and DII-VENUS (Brunoud et al., 2012) have been described previously. For generating $WOX11_{pro}$:GUS/*ein3 eil1* or $WOX5_{pro}$:GUS/*ein3 eil1*, the *WOX11pro*:GUS or *WOX5pro*:GUS reporter lines were genetically crossed with *ein3 eil1* double mutants, respectively, and homozygous lines were obtained through genotyping. For generation of adventitious roots, seeds were initially sterilized with 10% of bleach and 0.1% Triton X-100 for 5 min and then washed five times with sterile water. Sterilized seeds were placed on half-strength Murashige and Skoog (1/2 MS) medium [2.2 g l^{-1} MS salt, 1% sucrose, 1% agar (pH 5.8)] at 22°C. After stratification at 4°C for 3 days, the plates were irradiated with white light (80-90 µmol m^{-2} s^{-1}, 16 h light/8 h dark period) at 22°C for the indicated number of days. Leaf explants (the largest pair of rosette leaves) from plants of the indicated ages were carefully detached and then placed on B5 medium [Gamborg B5 Basal Salt Mixture with 3% sucrose and 0.8% agar (pH 5.7)] with or without additional treatments. If not specified, plants were initially grown for 12 days on 1/2 MS before further detaching experiments. Plates were sealed with Parafilm and wrapped with aluminum foil and then kept at 22°C in complete darkness before recording rooting phenotypes. For calculating rooting rate, the rooted leaf explant numbers were divided by the total detached leaf explant numbers (at least 20 leaf explants for each sample) and expressed as a percentage.

GUS staining

Leaf explants from different GUS reporter lines were incubated with GUS staining solution [1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 0.1% Triton X-100, 10 mM EDTA, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 50 mM sodium phosphate buffer (pH 7.2)] for 5 h (12 h for $WOX11_{pro}$:GUS lines). After staining, leaf explants were washed with increasing concentrations of ethanol and then imaged under a dissecting microscope (Nikon) equipped with a Canon DSLR camera.

Confocal laser scanning microscopy

After germination on 1/2 MS medium for 12 days, leaf explants of a DII-VENUS seedling were cut and cultured on B5 medium with or without 10 µM ACC for 4 days. Images were captured on a laser-scanning confocal microscope (Nikon). Scanner and detector settings were optimized to avoid saturation and to maximize resolution, and kept unchanged throughout the experiment. VENUS fluorescence was excited using the 488 nm line of an argon laser and was collected from 500 to 530 nm.

Protein extraction and immunoblot analysis

Total protein was extracted from approximately 50 leaf explants in 100 µl of lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM sodium

fluoride, 25 mM β-glycerophosphate, 2 mM sodium orthovanadate, 10% glycerol, 0.1% Tween 20, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1×Complete Protease Inhibitor Cocktail]. Protein extracts were separated on SDS-PAGE, transferred onto a nitrocellulose membrane, and blotted with anti-FLAG (Sigma-Aldrich) or anti-HSP90 (Santa Cruz Biotechnology) antibody.

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). After DNA removal, 1 µg of RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Vazyme). Real-time PCR reactions were performed on a LightCycler 96 PCR machine (Roche) with SYBR Green Master Mix (Vazyme). *Actin2* was used as the internal control. Primers used are listed in Table S1.

Protein expression and purification

The coding sequence of EIN3 (amino acids 141-352) were digested with BamHI-XhoI and then inserted into pGEX-5X-1 vector (GE Healthcare) for GST fusion and transformed into *Escherichia coli* BL21 (DE3)-competent cells. Protein expression and purification were carried out following the manufacturer's instructions (GE Healthcare).

EMSA

Oligonucleotide probes (listed in Table S1) were synthesized and labeled with biotin at the 3′ end (Invitrogen). EMSA was performed using a LightShift Chemiluminescent EMSA kit (Thermo Scientific). Briefly, 20 fmol of biotin-labeled probes (hot probes) were incubated in 1×binding buffer, 2.5% glycerol, 50 mM KCl, 5 mM $MgCl_2$ and 10 mM EDTA with or without proteins at room temperature for 20 min. For unlabeled probe competition (cold probes), 4 pmol of unlabeled probe was added to the reactions.

ChIP-PCR

ChIP-PCR was carried out according to literature with minor modifications (Zhang et al., 2014). Briefly, 1.5 g of the 7-day-old seedlings were crosslinked in 1% formaldehyde. The chromatins were then isolated following standard procedures. Anti-FLAG antibody (Sigma-Aldrich) was incubated with the sonicated chromatin followed by incubation overnight to precipitate bound DNA fragments. After immobilization using Recombinant Protein A-Agarose (Santa Cruz Biotechnology), bound DNA was eluted and amplified with primers corresponding to sequences neighboring the EIN3 binding sites in the *WOX* promoter.

Acknowledgements

We thank Dr Lin Xu for *WOX11*$_{pro}$:GUS, *WOX5*$_{pro}$:GUS, *35S*$_{pro}$:*WOX11*, *35S*$_{pro}$:3×FLAG-WOX11-GR and *35S*$_{pro}$:*WOX5* seeds. We are grateful to Drs Lin Xu and Jia-Wei Wang for their valuable suggestions on this project. We also thank Drs Xinyan Zhang and Wenyang Li for their comments on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z.Z.; Validation: H.L., L.Y.; Formal analysis: H.L., L.Y., L.S.; Investigation: H.L., L.Y.; Writing - original draft: H.L., L.Y., Z.Z.; Writing - review & editing: H.L., L.Y., Z.Z.; Supervision: Z.Z.; Funding acquisition: H.L., Z.Z.

Funding

H.L. was funded by the National Natural Science Foundation of China (31800198) and the Natural Science Foundation of Jiangsu Higher Education Institutions (18KJB180010); Z.Z. was supported by the National Natural Science Foundation of China (31970256), the Fok Ying Tong Education Foundation (161023) and by open funds from the National Key Laboratory of Plant Molecular Genetics.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.179457.supplemental

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