Ethylene promotes root hair growth through coordinated EIN3/EIL1 and RHD6/RSL1 activity in Arabidopsis

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Root hairs are an extensive structure of root epidermal cells and are critical for nutrient acquisition, soil anchorage, and environmental interactions in sessile plants. The phytohormone ethylene (ET) promotes root hair growth and also mediates the effects of different signals that stimulate hair cell development. However, the molecular basis of ET-induced root hair growth remains poorly understood. Here, we show that ET-activated transcription factor ETHYLENE-INSENSITIVE 3 (EIN3) physically interacts with ROOT HAIR DEFECTIVE 6 (RHD6), a well-documented positive regulator of hair cells, and that the two factors directly coactivate the hair length-determining gene RHD6-LIKE 4 (RSL4) to promote root hair elongation. Transcriptome analysis further revealed the parallel roles of the regulator pairs EIN3/EIL1 (EIN3-LIKE 1) and RHD6/RSL1 (RHD6-LIKE 1). EIN3/EIL1 and RHD6/RSL1 coordinately enhance root hair initiation by selectively regulating a subset of core root hair genes. Thus, our work reveals a key transcriptional complex consisting of EIN3/EIL1 and RHD6/RSL1 in the control of root hair initiation and elongation, and provides a molecular framework for the integration of environmental signals and intrinsic regulators in modulating plant organ development.

Significance

Root hairs are unicellular extensions of root epidermal cells that help plants increase water and nutrient uptake and improve soil anchorage, both of which are crucial for the globally recognized goal of yield improvement with reduced fertilizer use. Previous studies have implicated numerous genes and phytohormones in the control of root hair development. This work uncovers the molecular mechanism of ethylene (ET)-promoted root hair growth and identifies a transcriptional complex consisting of EIN3/EIL1 and RHD6/RSL1 as the key regulator of root hair initiation and elongation. As ET mediates the effects of various root hair stimuli, this work also elucidates a convergent signaling network that integrates diverse environmental cues and intrinsic signals to modulate plant organ development.


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acid (ACC) or a loss-of-function mutation in CTR1 leads to much longer root hairs (23, 24). In contrast, the completely abolished ET response in the ein2 mutant leads to a short-hair phenotype and reduced sensitivity to other hair growth stimuli, such as auxin, strigolactones, and low boron (23, 25, 26).

Although the roles of ET in promoting root hair growth and mediating other hair growth signals are well established, the underlying molecular mechanism remains unknown. Our findings show that EIN3/EIL1 are necessary and sufficient for ET-induced root hair elongation. EIN3 and RHD6 interact and cooperatively promote the hair elongation factor RSL4. Moreover, the functions of EIN3/EIL1 and RHD6/RSL1 are coordinated during root hair initiation. These findings elucidate the molecular mechanism of ET action during root hair initiation and elongation, and provide insight into the coordination of environmental and developmental signals during plant organ development.

Results

EIN3/EIL1 Are Critical for ET-Promoted Root Hair Elongation. The ein3 eil1 mutants were previously found to have reduced root hair density and compromised hair growth induction by jasmonic acid, suggesting that EIN3/EIL1 positively regulate root hair development (27). Phenotypic analysis indicated that EIN3 and EIL1 appeared to function partially redundantly in regulating root hair length (Fig. S1). To investigate the role of EIN3/EIL1 in mediating ET-induced root hair growth, we examined root hair length in young Arabidopsis wild-type Col-0 and ein3 eil1 seedlings upon ACC treatment. In Col-0, a low concentration of ACC (20 nM) stimulated significant root hair elongation, with further elongation observed under a higher concentration (100 nM). The ein3 eil1 root hairs were shorter than those of Col-0 and were completely unresponsive to ACC induction, suggesting that EIN3/EIL1 are necessary for ET-induced root hair elongation (Fig. 1A and B).

We also tested the sufficiency of EIN3 protein in promoting Arabidopsis root hair elongation. In the ein3 eil1 mutant background, root hair length significantly increased with accumulated EIN3-FLAG fusion protein induced by 5 nM and 10 nM β-estrogen application (Fig. 1C and D). These results show that EIN3/EIL1 are essential positive regulators mediating ET-promoted root hair elongation.

ET-Promoted Root Hair Growth Requires RSL4/RSL2. Arabidopsis root hair formation is regulated by a series of transcription factors (2, 4). We therefore assessed the interplay between EIN3/EIL1 and these transcription factors during root hair development. RHD6/RSL1 and RSL4/RSL2 positively regulate root hair differentiation downstream of GL2 (7, 12, 23). In fact, there are no visible root hairs in the rhd6 rsl1 and rsl4 rsl2 mutants (9, 12). To determine whether ET promotes hair growth through these factors, we assessed the response of these mutants to ACC treatment. In rhd6 rsl1, root...
hairs grew to about 400 \mu m in length with ACC treatment (Fig. 2), suggesting rescue of the root hair formation defect. The expression pattern of RHD6-GFP fusion protein driven by the native RHD6 promoter remained largely unchanged during ACC incubation (Fig. S2A), thereby excluding the regulation of RHD6 expression by ET. Furthermore, RHD6 overexpression under the constitutive 35S promoter in the ein3 eil1 mutant background resulted in longer hair growth, suggesting that RHD6 may function downstream of EIN3 in hair growth (Fig. S2B and C).

**RSL4 is a Direct Target of EIN3.** To determine whether EIN3/EIL1 regulate RSL genes, we measured RSL transcript levels in ein3 eil1 and in transgenic plants with inducible EIN3 overexpression. Exogenous ET treatment increased RSL4 and RSL5 transcript levels, but only the increase of RSL4 was completely dependent on EIN3/EIL1 (Fig. 3A). In plants with inducible EIN3 overexpression, RSL4 mRNA levels increased as the induction time increased (Fig. 3B). RSL5 levels were very low in untreated plants but dramatically increased after ET treatment and induction of EIN3 overexpression (Fig. 3A and B). RSL4 was previously reported to positively regulate RSL5 (11); thus, the effect of ET and EIN3 induction on RSL4 transcription likely promoted RSL5 expression. In contrast, RSL2 and RSL3 transcript levels were either slightly reduced or largely unchanged upon exogenous ET application or EIN3 induction (Fig. 3A and B).

The changes in RSL4 mRNA abundance indicated that RSL4 may be a direct target of EIN3. Analysis of the promoter region of RSL4 uncovered a putative EIN3-binding site (EBS, 5'-ATGTAT-3', starting at -853 upstream of the RSL4 gene). In vitro electrophoretic mobility shift assays (EMSAs) confirmed the specific binding of Escherichia coli-purified EIN3 protein (DNA-binding region, 141–352 aa) to the RSL4 EBS but not to the mutated EBS (5'-GGAGCC-3') (Fig. 3C). Accordingly, EIN3 induction of RSL4 was significantly impaired in plants when the promoter EBS motif was mutated (Fig. S3A). We also conducted an in vivo chromatin immunoprecipitation (ChIP) assay and verified the binding of EIN3-FLAG fusion protein to the RSL4 EBS-containing region in Arabidopsis roots using anti-FLAG antibody (Fig. 3D). Consistently, RSL4 was identified as an ET-responsive EIN3 target gene in a ChIP-sequencing assay using endogenous anti-EIN3 antibody (28). Furthermore, RSL4 overexpression restored root hair elongation in ein3 eil1. (E) Representative root hairs from Col-0, ein3 eil1, RSL4ox, and RSL4ox ein3 eil1. (Scale bar: 200 \mu m.) (F) Quantification of root hair length in E. Data are means ± SD (n = 10 roots). R4ox, RSL4ox. One-way ANOVA with a post hoc Tukey HSD test (**p < 0.01, *p < 0.05) was used.
root cell protoplasts and found that transient expression of EIN3 together with RHD6 activated RSL4 transcription more effectively than expression of either EIN3 or RHD6 alone (Fig. 4E). Based on these results, we conclude that EIN3 and RHD6 associate with each other and coactivate RSL4 transcription.

**ET Promotes Root Hair Initiation Through EIN3/EIL1 and RHD6/RSL1.** Besides promoting root hair elongation, RHD6/RSL1 are known regulators of root hair initiation (8, 9). ET also promotes root hair initiation under hairless conditions (10, 30, 31). We carefully observed the surface changes of rhd6 rsl1 and rsl4 rsl2 in response to ACC and found that ACC led to bulge formation in both mutants, a marker event of successful hair initiation (Fig. 5 A and B). When higher ACC concentrations were used to treat rhd6 rsl1, the number of root hairs and hair length both increased (Fig. 2).

Nevertheless, large portions of the root epidermal regions of rhd6 rsl1 remained hairless (Fig. 5 A and B), suggesting that the effect of ET on root hair initiation partially depends on the presence of RHD6/RSL1. In rsl4 rsl2, all H positions formed bulges upon ACC treatment (Fig. 5 A and B). However, the lack of RSL4/RSL2 led to tip-growth failure of the hair bulges, and no length-measurable hair was observed (Figs. 2 and 5). We further examined hair initiation in ein3 ein4 rhd6 rsl1 and ein3 ein4 rsl4 rsl2. Neither bulge formation nor hair growth was found even in the presence of ACC, illustrating the importance of EIN3/EIL1 and RHD6/RSL1 in ET-induced root hair initiation.

**Identification of Genes Coregulated by EIN3/EIL1 and RHD6/RSL1 in Root Hair Initiation.** For genome-wide analysis of EIN3/EIL1 and RHD6/RSL1 interaction, transcriptome profiles of Col-0, ein3 ein4, rhd6 rsl1, and ein3 ein4 rhd6 rsl1 roots were obtained by RNA sequencing. A total of 956 differentially expressed genes (DEGs) were identified in ein3 ein4 rhd6 rsl1 vs. Col-0. Biological processes involved in root hair development were statistically overrepresented among the 956 DEGs, including cell wall organization, cell tip growth response to stimulus, and root epidermis differentiation (Fig. S4A). Moreover, the majority of the 956 genes had a greater fold change in ein3 ein4 rhd6 rsl1 vs. Col-0 than in either double mutant versus Col-0 (Fig. S4B), suggesting that gene expression coregulation by EIN3/EIL1 and RHD6/RSL1 occurs at loci throughout the genome and not only at RSL4.

Next, we identified 187 genes induced by ET in an EIN3/EIL1-dependent manner in rhd6 rsl1 (ET-promoted genes). Compared with other published genes related to root epidermis morphology, strikingly, 43 of 154 core H genes (10) were included (Fig. 5C), but none of the 54 N genes was found, strongly indicating that EIN3/EIL1 and RHD6/RSL1 positively regulate hair formation. In rhd6 rsl1, the expression level of 43 H genes induced by ET was still lower than that in Col-0 without ET. Such an expression trend was consistent with the finding that ET only partially restored the hair growth defect in rhd6 rsl1 (Figs. 2 and 5 A and B), suggesting that the coordinated activity of EIN3/EIL1 and RHD6/RSL1 is needed to fully activate root hair initiation. In light of the coactivation of the hair elongation factor RSL4 by EIN3/EIL1 and RHD6/RSL1, we found 25 of the 43 H genes to be RSL4-regulated genes (12, 32) (Fig. 5C). The remaining 18 genes not influenced by RSL4 (Fig. 5C) were considered to be potential initiation stage factors. Furthermore, coexpression analysis of the
187 genes revealed compact clustering of previously published root hair genes, including core root epidermal genes (10), RSL4-dependent genes (12, 32), and H cell-enriched genes (33) (Fig. S5). Although nine genes within the compact cluster were not found in previous studies, they may nevertheless participate in root hair development considering the similarity of their expression patterns to those of other root hair genes. These nine genes and the 18 non–RSL4-regulated H genes comprised a candidate pool of 27 putative downstream target genes of RHD6/RSL1 and EIN3/EIL1 involved in root hair initiation (Table S1). More analysis details are provided in SI Identification of Genes Coregulated by EIN3/EIL1 and RHD6/RSL1 in Root Hair Initiation.

EIN3/EIL1 and RHD6/RSL1 Mediate Diverse Root Hair Stimuli. Transcriptome profiling revealed a more general role of EIN3/EIL1 and RHD6/RSL1 as gene expression coregulators during root hair formation. RSL4, the target gene coactivated by EIN3 and RHD6, is required for several root hair-inducing signals, including nutrient deficiency and hormone application (12, 14, 15). We therefore assessed whether EIN3/EIL1 and RHD6/RSL1 mediate the effects of these stimuli. Four root hair stimuli, application of auxin or cytokinin and depletion of phosphorus or nitrogen, were used to assess the response in rhd6 rsl1, ein3 eil1, and ein3 eil1 rhd6 rsl1. The rhd6 rsl1 was responsive to both hormone treatments but not to nutrient deficiency (Fig. 6 A–D and Fig. S7), suggesting that RHD6/RSL1 are essential for mediating the effects of nutrient depletion but not hormone application. The ein3 eil1 was responsive to all four stimuli, although root hair length in the mutant was relatively shorter than that of wild type under all conditions (Fig. 6 A–D). The ein3 eil1 rhd6 rsl1 quadruple mutant was insensitive to all four stimuli and had no visible root hairs (Fig. 6 A–D and Fig. S7), highlighting the importance of EIN3/EIL1 and RHD6/RSL1 coordination in response to these treatments.

Discussion

Research over the past few decades has clearly established the importance of ET in regulating plant root hair development. However, the underlying molecular mechanisms were poorly understood. In this study, we found that ET promotes root hair formation at both the initiation and elongation stages. Moreover, the master regulators EIN3/EIL1 mediate the effects of ET at both stages. EIN3 directly binds the promoter region of the hair-length-determining gene RSL4 and activates its transcription to promote root hair elongation. EIN3 also physically interacts with RHD6, another essential regulator of root hair development upstream of RSL4. EIN3/EIL1 and RHD6/RSL1 function in parallel and synergistically as positive regulators of root hair initiation and elongation. Based on these findings, we propose the following model for ET-induced root hair growth. In wild-type roots where EIN3/EIL1 levels are low, RHD6/RSL1 are mainly responsible for the induction of RSL4 and root hair initiation genes to maintain normal root hair growth. Upon ET treatment, EIN3/EIL1 accumulate and complex with RHD6/RSL1 to synergistically activate the expression of hair initiation genes as well as RSL4 to potently increase hair growth. In rhd6 rsl1, ET-induced EIN3/EIL1 act independently to partially rescue the hair initiation and elongation defects (Fig. 6 E).

ET, a widely documented stress hormone, helps plants adapt to various environmental challenges. In the proposed model, the ET signal is integrated with the internal root hair development pathway, with EIN3/EIL1 conferring stress responsiveness to the EIN3–RHD6 transcription complex. Meanwhile, due to its strict expression pattern in H cells, RHD6 confers spatiotemporal specificity to the complex. In this way, different internal and external signals converge at key nodes through associated transcription factors that provide flexibility and adaptability to ever-changing environments. Consistent with this hypothesis, the simultaneous loss of both EIN3/EIL1 and RHD6/RSL1 led to virtual insensitivity to various root hair-inducing signals, underscoring the central role of the EIN3/EIL1–RHD6/RSL1 transcription complex in signaling integration. Notably, ET signaling is known to act upstream of auxin biosynthesis in the root tip on cell elongation and polar root hair initiation (34–38). The ein3 eil1 rhd6 rsl1 remains hairless in the presence of auxin applications (Fig. 6 A and B), revealing that ET does not promote root hair initiation and elongation simply through increasing auxin biosynthesis. Other effects on promoting hair formation by ET–auxin interplay, including transport and signaling, still need further investigation. Similar to EIN3, ARF5 is also a direct regulator of RSL4 in mediating auxin-promoted polar growth (14). Combining findings in this study, RSL4 promoter is suggested to be a direct, central point of convergence for auxin signaling via ARF5 and for ET signaling via EIN3, and that it is regulated by the major hair cell differentiation regulator RHD6.

In addition to RSL4, EIN3/EIL1 and RHD6/RSL1 coregulate a subset of genes that likely contribute to root hair initiation and elongation. Of the 27 candidate root hair initiation genes identified
in this study, some have been reported to participate in root hair formation. For example, overexpression of ROOT HAIR SPECIFIC 3 (RSH3) leads to spiral, bent, and branched hair morphologies (39). LEUCINE-RICH REPEAT/EXTENSIN 1 ( LRX1) encodes a chimeric leucine-rich repeat/extension protein, and the lrx1 mutant exhibits aberrant root hair formation, including aborted, swollen, and branched hairs (40). Lotus japonicus (Lj) RHL1-LIKE 3 (LRL3), encoding a bHLH subfamily XI protein, rescues the hairless defect of Ljrh1 mutants (41).

As master regulators, EIN3/EIL1 associate with a group of transcriptional regulators in hormone cross-talk. All EIN3-associated proteins reported thus far repress their biological function (27, 42, 43). Intriguingly, this study uncovered a class of EIN3-associated transcription factors whose enrichment in H cells through how this enhancement is achieved is unclear. Several possibilities can be considered. First, RHD6 may act as a positive regulator that enhances EIN3 transcription activity. Although RHD6 directly regulates RSL4 expression, no DNA-binding ability has been demonstrated. RHD6 may be recruited by other DNA-binding factors, such as EIN3, to the RSL4 promoter. Second, RHD6 may directly bind to a specific DNA sequence in the RSL4 promoter, while EIN3 binds to the EBS motif. In turn, the association between the two classes of transcription factors could mutually and greatly enhance their respective DNA-binding ability. Third, RHD6 may adopt a de-repression mechanism by competing with EIN3-associating repressors in H cells, and RHD6 interaction could release EIN3 from an otherwise repressed state. Further investigation of these alternative mechanisms is needed to fully understand the action and importance of the EIN3–RHD6 complex.

Materials and Methods

Plant Materials

The Arabidopsis mutant ein3 eil1 and transgenic plant ein3 ein3 eil1 ebf1 ebf2 and ein3 ein3 eil1 were described previously (43, 44). The rsh1 rsh2 double mutants and transgenic pRDH6::GFP::PFRID6 and RSL4::GFP plants were gifts from Liam Dolan, University of Oxford, Oxford, United Kingdom. Details about plant growth conditions and treatments are described in SI Materials and Methods. Plant transformation, root hair length measurement, gene expression, CHIP-qPCR, EMSA, yeast two-hybrid assay, pull-down analysis, LC, dual-luciferase reporter assay, and sequencing analyses were carried out according to protocols described in SI Materials and Methods.

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