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Ethylene signaling increases reactive oxygen species accumulation to drive root hair initiation in *Arabidopsis thaliana*

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ABSTRACT

Root hair initiation is a highly regulated aspect of root development. The plant hormone, ethylene, and its precursor, 1-amino-cyclopropane-1-carboxylic acid (ACC), induce formation and elongation of root hairs. We asked whether elevated ethylene induced root hair formation by increasing reactive oxygen species (ROS) synthesis in hair cells. Using confocal microscopy paired with redox biosensors and dyes, we demonstrated that treatments that elevate ethylene levels led to increased ROS accumulation in hair cells prior to root hair formation. In two ethylene-insensitive mutants, *etr1-3* and *ein3/eil1*, there was no increase in root hair number or ROS accumulation. Conversely, *etr1-7,* a constitutive ethylene signaling receptor mutant, has increased root hair formation and ROS accumulation similar to ethylene-treated wild type seedlings. The *rhd2-6* mutant, with a defect in the gene encoding a ROS synthesizing Respiratory Burst Oxidase Homolog C (RBOHC), showed impaired ethylene-dependent ROS synthesis and root hair formation and decreased RBOH enzyme activity compared to Col-0. To identify additional proteins that drive ROS induced root hair formation, we examined a time course root transcriptomic dataset examining Col-0 grown in the presence of ACC and identified *PRX44* and other positively regulated transcripts that encode class III peroxidases (PRXs). The *prx44-2* mutant has decreased root hair initiation and ROS accumulation when treated with ACC compared to Col-0 and *pPRX44*::GFP fluorescence is increased in response to ACC treatment. Together, these results support a model in which ethylene increases ROS accumulation through RBOHC and PRX44 to drive root hair formation.

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SIGNIFICANCE STATEMENT

Root hairs are essential for water and nutrient acquisition and anchorage in soil. The hormone ethylene increases both root hair initiation and elongation. Short-term treatment with ethylene and its precursor, ACC, increased reactive oxygen species (ROS) accumulation in trichoblast cells prior to root hair initiation. Ethylene signaling through the ETR1 receptor and transcription factors, EIN3/EIL1, increased ROS and root hair initiation. Genetic and biochemical approaches identified ROS producing enzymes that are regulated by ethylene signaling and required for root hair initiation. Ethylene signaling increased activity of RBOHC and elevated transcript abundance of a class III peroxidase, PRX44, to drive root hair initiation. Our findings demonstrate that ethylene-induced root hair initiation is dependent on changes in ROS homeostasis.

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INTRODUCTION

The initiation of root hairs is genetically programmed and environmentally sensitive, making them an ideal model for studying single cell development in plants. Root hairs are single-cell extensions that differentiate from longitudinal epidermal cell files, known as trichoblasts (1, 2). These structures rapidly respond to environmental changes and act to increase root surface area to allow for efficient water and nutrient uptake (3), while also anchoring plants in soil to reduce erosion (4). In Arabidopsis, the root epidermis consists of an alternating pattern of trichoblasts, which form root hairs, and atrichoblasts, which are non-hair forming cells (2, 5). Root hair formation is dictated by cell positioning; epidermal cells overlying two cortical cells will become hair forming cells, while those overlying one cortical cell will become non-hair cells (2, 6). Root hair development is separated into two processes: root hair initiation and root hair elongation (7). Prior to initiation, RHO Of Plants (ROP) proteins accumulate at the future site of initiation (8), this is followed by actin polymerization along which vesicles move to deposit membrane allowing for polarized tip growth (2). During tip growth, tip focused reactive oxygen species (ROS) (9, 10) and Ca^{2+} gradients (11) drive exocytosis of the cell wall and membrane materials driving subsequent root hair elongation. These processes are separable as mutants with impaired root hair initiation or elongation have been identified (12, 13)

Genetic screens in *Arabidopsis thaliana* have provided a wealth of insight into the proteins that drive root hair development (14-16). For example, the mutants *transparent testa glabra (ttg)*, *glabra2 (gl2)*, and *werewolf (wer)* have root hairs that form from both trichoblast and atrichoblast cells (16-18). Many of the protein products of these mutants have been mapped to transcriptional cascades that drive root hair differentiation (19). In non-hair cells, a transcriptional complex comprised of three transcription factors (TFs), WER, GLABRA 3 (GL3), or its functionally redundant ENHANCER OF GLABRA (EGL3), and TTG1, function as a transcriptional activator of the GL2 protein, which leads to repression of root hair initiation (2, 20). In trichoblasts, *WER* expression is repressed, which allows for the formation of an alternate transcriptional complex comprised of CAPRICE (CPC) or the functionally redundant proteins ENHANCER OF TRY AND CPC1 (ETC1), ETC3, or TRYPTICHON (TRY) (21). When this pathway is active, GL2 is not expressed and root hair initiation proceeds (2).

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Another genetic screen identified the *root hair defective* (*rhd*) mutants which have impaired root hair initiation, elongation or structure (12, 13). In one of these mutants, *rhd2,* the mutation was mapped to the *RBOHC* (respiratory burst oxidase homolog C/NADPH oxidase) gene (9). The *rhd2* mutant was identified for altered root hair elongation (12), but was recently reported to also have impaired root hair formation (10). RBOHs are integral plasma membrane proteins that produce superoxide, which can be dismutated to hydrogen peroxide via superoxide dismutase (SOD) or other non-enzymatic mechanisms (22). H_2O_2 can then enter into the cell through aquaporins (23), where it can act as a signaling molecule to drive cellular processes. Signaling induced ROS regulates protein function by reversibly oxidizing cysteine residues to sulfenic acids (Cys-SOH) (24).

There are 10 RBOH family members (RBOHA-RBOHJ) in Arabidopsis and each play distinct roles in organ development and stress response (22). RBOHs can be regulated transcriptionally or enzymatically by a number of mechanisms, including calcium binding, phosphorylation, and phosphatidic acid binding (25-27). RBOH-induced ROS production is also regulated by hormone signaling, as many plant hormones generate ROS as a mechanism to drive growth and developmental processes (22, 27-29). For example, abscisic acid (ABA), a hormone involved in abiotic stress response, has been shown to induce RBOH-derived ROS production to prevent water loss in leaves (29). Auxin induces root hair elongation through a localized increase in ROS (30), suggesting that RBOH may drive hormone induced root hair formation.

The plant hormone ethylene enhances root hair initiation and elongation. Treatment with ethylene, or its precursor 1-amino-cyclopropane-1-carboxylic acid (ACC), leads to proliferation of root hairs, with substantial increases in length (31). The ethylene induction of root hairs occurs through the canonical ethylene signaling pathway, which is initiated when ethylene binds to one of the five receptors, ETR1, ERS1, ETR2, ERS2, or EIN4 (32, 33). When ethylene is absent, the receptors are in the “on state” leading to activation of the CTR1 Raf-like kinase (34); this turns the pathway off via phosphorylation and subsequent degradation of the EIN2 transmembrane protein (35, 36). When ethylene binds to its receptors they shift to the “off state” which prevents activation of CTR1. This allows for cleavage and translocation of the EIN2 C-terminus into the nucleus (36, 37) where it stabilizes the EIN3/EIL1 TFs leading to ethylene responsive gene expression. Previous work has shown that ethylene regulates root hair elongation through the EIN3

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120 transcription factor (38). EIN3 physically interacts with RHD6, a known positive regulator of root hair
121 development, to form a transcriptional complex that binds to and induces expression *RSL4*. This
122 transcriptional regulation results in increased root hair length (38). However, the mechanistic events that
123 drive ethylene-induced root hair initiation have not been fully described, and the role of ROS as a
124 downstream molecule in ethylene-induced root hair development has not been reported.

125 We asked whether ethylene acts to increase ROS levels to drive root hair initiation. Using
126 fluorescent dyes and biosensors that report ROS levels, we examined ROS accumulation after ACC
127 treatment in trichoblast cells in the differentiation zone. We asked whether this response is dependent on
128 ETR1 receptor activity and EIN3 TF activity. We also examined the role of RBOHC and class III peroxidase
129 enzymes in ethylene-dependent ROS synthesis. Together these experiments demonstrated that ROS is a
130 signaling molecule in ethylene induced root hair development and identified several enzymes that
131 participate in producing ethylene-induced ROS.

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RESULTS

Root hair number was increased in ACC treated roots

To understand the role of ethylene signaling in root hair initiation we examined the effects of short-term treatments with the ethylene precursor, ACC, on the number and position of root hairs in 5 day old seedlings. Root hairs were visualized in wild type (Col-0) seedlings grown in the presence of 1 μM ACC for 4 hours (Figure 1A). The root tip was divided into three 500 μm zones starting from the root tip and he number of root hairs formed in each zone was quantified Figure 1B). In zone 1, root hairs did not form in either untreated or ACC-treated seedlings. In zone 2, there were very few root hairs in untreated roots, however, ACC-treatment increased the number of root hairs by 10-fold. There was also a 2-fold increase in root hair number in zone 3 of ACC-treated seedlings compared to untreated controls. These data suggest that ACC-induced root hair initiation begins between 500 and 1000 μM from the root tip. Therefore, to understand the mechanisms driving the process of root hair initiation our experiments focused on this region.

ACC treatment also resulted in a shorter primary root due to reduced elongation of root cells (39), therefore we asked whether the increase in root hairs seen with ACC treatment was due to shorter cell length resulting in more root hairs in zone 2. To explore this possibility, roots treated with or without ACC for 4 hours were stained with the cell wall specific dye, propidium iodide (PI). We then measured the length of 5 epidermal cells from 6-8 roots per treatment condition, measuring the length of cells at either end and in the middle of zone 2 (Figure S1). We found that there was no difference in cell length after 4 hours of ACC treatment compared to untreated controls at both ends of this zone. These results are consistent with short term and low dose ACC treatments increasing root hair number by inducing more root hair forming trichoblasts in zone 2, rather than as an indirect effect of a shorter primary root.

ROS accumulation was increased in ACC treated roots

We asked whether ethylene leads to elevated reactive oxygen species (ROS) to drive ethylene-induced root hair initiation. We examined ROS dependent gene expression along the root using the ZAT12p-ROS ratiometric biosensor (40). This reporter construct contains the promoter of the ROS sensitive

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158 transcription factor, ZAT12, driving GFP and the constitutive ubiquitin10 promoter driving mCherry. We
159 compared the fluorescent signal of ZAT12p-ROS in the presence and absence of ACC as visualized by
160 laser scanning confocal microscopy (LSCM), with GFP reported as green and mCherry reported as
161 magenta (Figure 1C). The ratio of signal of GFP to mCherry was quantified across the entire root as a
162 distance from the root tip. In ACC-treated roots, the GFP/mCherry ratio increased beginning at
163 approximately 500 μm from the root tip, which corresponded to zone 2, where root hair induction was
164 maximal upon treatment with ACC (Figure 1D).

165 We also used the hydrogen peroxide selective dye, Peroxy-Orange 1 (PO1) to ask whether there
166 were cell type-specific increases in ROS in response to ACC. PO1 is a permeable, boronate-based dye
167 that is non-fluorescent in its reduced form, but becomes fluorescent when irreversibly oxidized by H_2O_2
168 (41). In Col-0 roots, PO1 fluorescence was visualized using LSCM in zone 2 of roots treated with and
169 without ACC for 4 hours (Figure 1E). In Arabidopsis, root hairs form in alternating patterns, so that every
170 root hair forming cell (trichoblast) is adjacent to a non-hair cell (atrichoblast). We quantified ROS
171 accumulation by analyzing PO1 signal after confocal imaging, by drawing a line across the width of the root
172 that spans 5 epidermal cell files so that the PO1 signal in 3 trichoblasts and 2 atrichoblasts was quantified
173 (Figure 1F). We found that PO1 accumulation in trichoblasts of untreated seedlings was slightly, but not
174 significantly higher than atrichoblasts. In trichoblast cells in ACC treated seedlings, there was a significant
175 increase in PO1 accumulation in ACC-treated trichoblasts as compared to trichoblasts of untreated
176 seedlings and compared to atrichoblasts of both untreated and ACC treated seedlings. In contrast, there
177 were no changes in PO1 in the atrichoblasts suggesting that ACC treatment increased ROS levels in only
178 the cells that formed root hairs (Figure 1F).

179 **ROS accumulation increased prior to root hair emergence**

180 To determine whether ethylene-induced ROS accumulation drives root hair emergence, we asked
181 whether the ACC-induced ROS increases were detectable prior to the first ACC-induced root hair initiation.
182 Wild type Col-0 seedlings were treated with ACC for either 2 or 4 hours and PO1 fluorescence was
183 visualized in trichoblasts that did not have a root hair bulge (Figure 2A). These trichoblasts were chosen
184 for quantification to determine whether ACC-induced ROS increases were seen in hair forming cells prior

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to root hair emergence. Total PO1 accumulation was measured in 5 individual trichoblasts per root treated with and without ACC. Signal was quantified across the area of the entire cell and the average PO1 intensity of 24-30 individual cells was reported (Figure 2B). Atrichoblast signal was not quantified as ROS levels did not change in those cells (Figure 1F). Cells treated with ACC for 4 hours were not quantified because nearly all of the trichoblasts in Zone 2 at this time point had already begun the process of root hair initiation (Figure 2C). These data showed a 1.3-fold increase in PO1 accumulation in hair cells of seedlings treated with ACC for 2h as compared to untreated controls, when trichoblast cells that had not yet begun to initiate root hairs were examined. These data are consistent with the hypothesis that ROS acts as a driver of root hair initiation downstream of ethylene signaling.

Ethylene signaling mutants showed altered ROS accumulation patterns and root hair phenotypes

We examined ROS accumulation in response to ACC treatment in seedlings with mutations in genes encoding key ethylene signaling proteins to ask whether this response was dependent on the ethylene signaling pathway and downstream transcriptional responses. The number of root hairs and average length were previously reported in loss-of-function and gain-of-function ethylene receptor mutants, *etr1-7 and etr1-3,* and the transcription factor mutant *ein3eil1* in the presence and absence of ACC treatment (39). *etr1-7* is a LOF mutant in which the ETR1 receptor is inactive, therefore the ethylene signaling pathway is constitutively signaling and there are increased numbers of root hairs independent of ACC addition, while *etr1-3* is a GOF mutant in which the ETR1 receptor is always active leading to an inactive signaling pathway (39, 42). The double mutant *ein3eil1* has mutations in genes encoding EIN3 and EIL1 TFs (43, 44). Both *etr1-3* and *ein3eil1* have reduced root hair initiation in response to ACC treatment (39). We examined the PO1 distribution patterns in root hair cells with and without ACC treatment in these three mutants. We visualized PO1 fluorescence via LSCM and saw that the constitutive ethylene signaling mutant *etr1-7* had increased H_2O_2 in root hair cells and increased root hair initiation from these cells regardless of ACC treatment (Figure 3). The opposite response was seen in the ethylene-insensitive *etr1-3* and *ein3eil1* mutants, as they showed reduced PO1 signal and root hair initiation and no increase in H_2O_2 accumulation in the presence of ACC. Together, these results suggest that the ETR1 receptor and EIN3/EIL1 TFs are required for ethylene-induced ROS accumulation and root hair initiation.

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To determine if the changes in ROS accumulation seen in response to ACC were due to ACC's conversion to ethylene, we treated Col-0 seedlings with 0.05 ppm of ethylene gas for 4 hours followed by PO1 staining and signal quantification as described above. We observed the same ROS accumulation patterns in both treatment conditions, with PO1 fluorescence intensity values in trichoblasts of ethylene-treated seedlings showing the same magnitude increase as root treated with ACC (Figure S2). This is consistent with our results showing that ACC has no effect on root hair number or ROS accumulation in the constitutive ethylene insensitive signaling mutants, *etr1-3* and *ein3/eil1* and in the constitutive ethylene signaling receptor mutant, *etr1-7,* in the absence (or presence) of ACC

The RBOHC knockout mutant, *rhd2-6*, showed decreased ROS accumulation after ACC treatment

It has been previously reported that the respiratory burst oxidase homolog C (RBOHC) is involved in ROS production and subsequent root hair elongation (9). We have also shown that there is decreased ROS in root hairs of the *rhd2-6* mutant, which has an insertion mutation in the *RBOHC* gene (10). Therefore, we examined root hair numbers via light microscopy and PO1 accumulation patterns via LSCM in ACC-treated *rhd2-6* (*rbohc*) seedlings (Figure 4). Root hair number and root hair length in the ACC-treated *rhd2-6* mutant was significantly less than ACC-treated Col-0, suggesting that RBOHC contributes to both ethylene-induced root hair initiation and elongation (Figure 4B). We found that, in the ACC-treated *rhd2-6* mutant, the significant increase in H_2O_2 fluorescence accumulation seen in Col-0 hair cells was at lower levels in the mutant (Figure 4C-D), suggesting that RBOHC contributes to ethylene-induced ROS accumulation. To determine if other RBOHs that are expressed in root tissue contributed to ethylene-induced ROS accumulation, PO1 accumulation patterns were examined in the *rbohd/f* double mutant. No change was seen in ROS levels in hair cells of *rbohd/f*, suggesting that these RBOHs do not contribute to ethylene-induced ROS synthesis in root hair cells (Figure S3).

RBOH enzyme activity was increased in response to ethylene signaling

Elevated ethylene may either increase *RBOHC* transcript abundance or enzyme activity. We examined several transcriptomic datasets with ACC or ethylene treated roots and found a subtle change in the *RBOHC* transcripts, but not significant enough to pass the filtering on this transcriptomic analysis (39, 45) (Table S1). To determine if ethylene signaling regulated RBOH enzyme activity, a spectrophotometric

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assay using nitro blue tetrazolium (NBT) dye as an electron acceptor, was performed. NBT is reduced by superoxide to monoformazan and this reduction can be detected at 530 nm. This experiment was performed in protein extracts from 7-day old roots of Col-0 and *rhd2-6* treated with and without ACC for 4 hours. Older roots were used in this experiment to obtain an adequate amount of protein for these assays. There was a significant 2-fold increase in monoformazan production in protein extracts of Col-0 roots treated with ACC for 4 hours compared to controls (Figure 4E). The enzyme activity of roots of untreated *rbohc/rhd2-6* was 3-fold lower than roots of untreated Col-0 and the enzyme activity in the presence of ACC was significantly reduced relative to ACC treated Col-0 (Figure 4E). These data are consistent with previous results indicating that the RBOHC enzyme constitutes the majority of the superoxide production in roots (10, 22).

Transcription of *PRX44* increases with ACC treatment driving ROS accumulation and ethylene-induced root hair initiation

The *ein3/eil1* transcription factor mutant has reduced root hair initiation and a reduction in PO1 signal in trichoblasts suggesting that there is transcriptional regulation of ROS producing enzymes that drive root hair initiation. We examined a previously published microarray time course experiment in roots treated with ACC (39) to identify candidate transcriptional targets profiling the expression pattern of transcripts encoding both ROS producing enzymes and proteins linked to trichoblast cell specification. ROP2, ROPGEF3, ROPGEF4, GL2, RSL4 and RHD6, which are linked to root hair initiation, showed no transcriptional response to ACC treatment and primers that recognize RSL1 and RSL2 are not present on the microarray and could not be examined in this dataset (Table S1). A number of transcripts encoding class III PRXs changed in abundance in response to ACC, including the transcript encoding *PRX44,* which increased by 3-fold. Class III peroxidases are specific to plants and exist in large multigene families. They have been implicated in root hair tip growth, as null mutants have shorter root hairs compared to wild type (30). Recent work has shown that auxin induces expression of genes encoding four class III peroxidases (PRX), which results in an increase in both root hair length and ROS accumulation (30), however, the role of ethylene signaling in regulating class III PRX expression to modulate root hair initiation or elongation has not been reported.

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We examined control and ACC-treated seedlings harboring the *PRX44* promoter driving GFP and examined GFP expression via LSCM. Seedlings treated with ACC showed a statisictally significant >2-fold increase in GFP fluorescence, suggesting that the *PRX44* promoter is induced downstream of ethylene signaling (Figure 5A-B). We also asked whether these peroxidases participate in ACC-regulated root hair initiaion. We examined root hair numbers via light microscopy in Col-0 and *prx44-2* seedlings treated with and without ACC. There were significantly fewer root hairs in *prx44-2* controls compared to Col-0 controls and the effect of ACC on root hair initiation was reduced in the *prx44-2* mutant, although there was still a significant response to ACC (Figure 5C-D). The root hairs in the ACC-treated mutant also appeared shorter compared to Col-0, which is consistent with the phenotype that has been reported in response to auxin treatment (30). When PO1 accumulation patterns were examined via LSCM, we observed no significant increase in PO1 accumulation in hair cells of *prx44-2* seedlings treated with ACC (Figure E-F). We also examined *prx73-4*, since the transcripts encoding PRX73 has also been reported to be induced with auxin treatment to drive root hair elongation (30) and these transcripts profound increases in response to ACC treatment (Table S1). The *prx73-4* mutant showed no change in PO1 fluorescence in the presence or absence of ACC as compared to Col-0 (Figure S4) and showed no changes in root hair initiation after ACC treatment. These combined data indicate that the class III PRX44 is transcriptionally regulated by ethylene and contributes to ethylene induced ROS accumulation and root hair initiation.

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DISCUSSION

Ethylene is a key hormonal regulator of root hair initiation and elongation (34, 46). Although the proteins that drive ethylene signaling are well characterized, the downstream proteins that control the root hair developmental processes have received less study. Reactive oxygen species (ROS) are critical for both root hair initiation and elongation (9, 10, 47, 48). In root hairs, one source of ROS is the NADPH oxidase (NOX)/respiratory burst oxidase homolog C (RBOHC) that is localized to root hairs to facilitate cell wall loosening and subsequent tip focused Ca^{2+} accumulation leading to cell elongation (9). The plant hormone auxin also induces ROS to drive root hair elongation (30), so we asked if ROS is a signaling molecule in ethylene-induced root hair development. In this study, we examined the effects of ethylene signaling on root hair initiation and ROS accumulation to determine if increased ethylene drives root hair initiation through regulation of ROS producing enzymes and subsequent ROS accumulation in trichoblasts from which root hairs form.

We asked whether ethylene signaling induced root hair initiation at the root tip by examining root hair number of Col-0 and ethylene signaling mutant seedlings grown in the presence of low concentrations of the ethylene precursor, ACC (1 μM), for 4 hours. There was a significant increase in the number of trichoblasts that formed root hairs in ACC treated seedlings compared to untreated controls. This root hair induction was most pronounced in a region starting 500 μm from the root tip and ending at 1000 μm, which we refer to as zone 2. Consistent with this effect occurring through ACC conversion to ethylene, the ACC induction in root initiation is lost in ethylene insensitive mutants *etr1-3* and *ein3eil1*, consistent with our prior reports (39) other prior studies which showed that ACC-inhibited root elongation, gravitropism, and lateral root formation are lost in the ethylene insensitive *ein2-5* and *etr1-3* mutants at this dose of ACC under our growth conditions (39, 49, 50). These findings are also consistent with efficient conversion of ACC to ethylene and ACC effects on ROS synthesis and root hair initiation due to ethylene signaling responses.

We asked whether ACC treatment increased ROS levels in wild-type seedlings using both genetic and chemical ROS probes. Using a ratiometric reporter of ROS-induced gene expression, ZAT12p-ROS, we found that in ACC-treated roots, ROS dependent gene expression was increased in a region spanning from 200 to 1000 μm from the root tip. Expression was quantified in the entire root and elevated

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fluorescence was seen in a region 500-1000 µm from the root tip (zone 2) in ACC treated roots compared to untreated controls. We also used a boronate based hydrogen peroxide sensor, PO1, and found that H_2O_2 accumulation was elevated in the trichoblast cells in zone 2 and significantly increased within 2 hours after ACC treatment, prior to the initiation of root hair bulges. We also performed treatments with 0.05 ppm of ethylene gas and demonstrated that ethylene gas also increases PO1 signal in trichoblasts prior to root hair initiation. These data suggest that ROS act downstream of ethylene signaling in zone 2 of the root, and that these signals occur prior to root hair initiation.

We next asked which components of the ethylene signaling pathway were required for ethylene-induced ROS synthesis in trichoblasts of zone 2. ROS accumulation was monitored in two *etr1* mutants: a constitutive ethylene signaling *etr1-7* allele and an ethylene insensitive *etr1-3*, as well as the TF null mutant *ein3eil1.* Compared to Col-0, *etr1-7* exhibited an increase in ROS accumulation and root hair number in the presence and absence of ACC treatment, while these responses were lost in the ACC-treated ethylene insensitive *etr1-3* and *ein3eil1* mutants. These results are consistent with the ethylene receptor ETR1 and the EIN3/EIL1 master transcriptional regulators being required for ethylene-induced ROS accumulation and root hair initiation.

To determine if RBOH/NOXs were contributing to ethylene-induced ROS synthesis, we asked whether the RBOHC enzyme was required to drive ROS accumulation and root hair initiation. Based on previous work detailing the role of RBOHC in root hair initiation and growth (9, 10), we asked whether this enzyme was contributing to ethylene-induced ROS accumulation and subsequent root hair initiation and/or elongation. ACC treated *rhd2-6*, which is an *RBOHC* null mutant, had reduced ROS accumulation in trichoblasts compared to Col-0 and ACC failed to increase the number of initiated root hairs. The abundance of RBOHC transcripts showed small changes in response to ACC treatment (39), while there was more than a 2-fold increase in RBOH activity in response to ACC treatment and there was a 3-fold reduction in activity in the *rhd2-6* (*rbohc*) mutant. These data suggest that ethylene signaling induces superoxide production in roots via increases in RBOHC enzyme activity to drive root hair initiation and elongation. However, since there is still some ACC induction of root hair initiation in the *rhd2-6* mutant, these data suggest that initiation may require additional proteins.

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We examined previously published ACC and ethylene transcriptomic datasets to search for transcripts encoding root hair specification proteins and ROS producing enzymes that are ethylene regulated. Although ACC did not change abundance of transcripts encoding ROP2, ROPGEF3, ROPGEF4, GL2, RHD6, or RSL4, we identified several transcripts encoding class III peroxidase enzymes that showed substantial increase in response to ACC. *PRX44 and PRX73*, showed a significant increase in abundance after 4 hours of ACC treatment. We then examined the effects of ACC treatment on the null mutant *prx73-4* and *prx44-2* and found decreased ROS accumulation in root hair forming trichoblasts in *prx44-2* and no effect in *prx73-4* compared to Col-0. The decreased PO1 signal in *prx44-2* was accompanied by significant reductions in the number of root hairs formed in this same root region. We examined a PRX44 promoter driven GFP fusion protein (*pPRX44*::GFP) observing its expression in trichoblast cells in zone 2 and demonstrated that ACC treatment leads to significant increases in fluorescence intensity compared to untreated controls. Together these experiments implicate Class III peroxidase enzymes in root hair initiation.

These peroxidase enzymes have also been implicated in regulation of root hair elongation, as null mutants treated with auxin have shorter root hairs and less ROS compared to wild type (30). Class III peroxidases are specific to plants and exist in large multigene families. Reverse genetic studies have shown that they are involved in lignification, cell elongation, stress defense and seed germination (51). At least 73 Arabidopsis genes encode class III peroxidases, however, due to their redundancy and lack of information on their substrate specificity, their functions and mechanisms of regulation are relatively unclear. They are secreted into the cell wall or surrounding medium and are involved in a number of different reactions that involve both ROS consumption and production (52, 53). However, previous work regarding peroxidase activity in root hair elongation suggests that they act as ROS producers, as 3 PRXs have been reported to be involved in increasing the ROS gradient required for root hair elongation (30)

To summarize our findings on ethylene-dependent root hair initiation via increased ROS synthesis and to tie these findings with other studies, we present a model in Figure 6. ACC treatment induces ethylene signaling, which acts through the ETR1 receptor and the canonical signaling pathway which then increases the activity of EIN3/EIL1 TFs. EIN3/EIL1 binds to the root hair specific TF RHD6 to induce RSL4

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363 expression and root hair elongation (38). Root hair growth is controlled by several proteins that eventually
364 activate the RHD6 TF. Downstream of RHD6, the RSL4 TF is activated and it defines final root hair length
365 based on its level of expression (30, 54). Previous work has shown that EIN3 physically interacts with RHD6
366 to form a transcriptional complex that coactivate RSL4 to promote root hair elongation (38). However, in
367 our microarray dataset (39), *RSL4* transcript abundance only showed slight changes in response to ACC
368 treatment (Table S1). It has also been shown that auxin treatment results in increased RSL4 transcript
369 abundance, which binds to the promoters of RBOH and four class III peroxidase (*PRX*) genes, including
370 *PRX44*, to drive ROS synthesis in root hair cells. RSL4 activation via auxin induces class III peroxidase and
371 *RBOH* expression, which leads to ROS accumulation required to drive root hair elongation (30). We find
372 that ACC treatment increases activity of RBOHC enzymes leading to increased ROS and root hair
373 elongation, without changing the abundance of *RBOHC* transcripts. Additionally, ACC treatment leads to
374 the transcriptional regulation of class III peroxidases (39), resulting in ROS production and root hair
375 initiation. Further investigation regarding the mechanisms of ethylene-regulated ROS synthesis is required.
376 For example, one important question to be answered is how ethylene signaling regulates RBOH activity.
377 This could occur through a number of different mechanisms, such as calcium binding or phosphorylation,
378 which are known to regulate RBOH enzymes (27). It is also unknown as to whether ethylene regulates
379 class III peroxidase expression via EIN3 or an alternate transcriptional mechanism, or if ethylene also
380 regulates class III peroxidase enzyme activity.

381

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MATERIALS AND METHODS

Growth conditions and root hair quantification

All Arabidopsis mutants were in the Col-0 background. The *etr1-7*, *etr1-3* (AT1G66340), *ein3-1* (AT3G20770) *eil1-1* (AT2G27050) double and *rhd2-6* (AT5G51060) mutants have all been described previously (10, 39, 42, 55). The ZAT12p-ROS construct was generously provided by Won-Gyu Choi (40). *prx44-2* (AT4G06010) and *prx73-4* (AT5G67400) were obtained from the Arabidopsis SALK center (SALK_057222C and SALK_020724, respectively), the mutations were verified by PCR, and homozygous lines were isolated. The transcriptional reporter *pPRX44*::GFP transgenic line was described previously (56). Seeds were sterilized in 70% ethanol for approximately 5 minutes and grown on 100 x 15 mm Petri dishes containing 25 mL media. Seedlings were grown on 1x MS supplemented with 1% sucrose, vitamins (1 µg/ml thiamine, 0.5 µg/ml pyridoxine and 0.5 µg/ml nicotinic acid), 0.05% MES (w/v) and 0.8% (w/v) agar. Media pH was 5.5. Micropore tape was used to seal the top of the Petri dish and plated seeds were stratified at 4◦ C in darkness for 2 days. Plants were grown under 24h light from T5 fluorescent lights at 120-150 µmol photons m^{-2} s^{-1}. Seedlings were grown for 5 days on control media and then transferred to media containing 1 µM ACC and grown for 4 hours or other indicated times. To examine root hairs, 5-day old seedlings were imaged using bright-field on an Axio Zoom V16 stereomicroscope. Extended depth of focus was used to combine z-stack images. Root hairs were quantified using Fiji/ImageJ software in three zones (0-500 µm, 500-1000 µm, 1000-1500 µm) starting from the root tip.

Confocal imaging of dyes and reporters of ROS levels

H_2O_2 was visualized with Peroxy-Orange 1 (PO1). PO1 was dissolved in DMSO to make a 500 µM stock and was further diluted in H_2O to make a 50 µM working solution. Seedlings were incubated in PO1 for 15 minutes in the dark and were then rinsed with H_2O and mounted in H_2O for imaging. All seedlings were imaged on a Zeiss 880 laser scanning confocal microscope using a 10x objective. PO1 was excited with a 488 nm laser at 0.25% power and emission was collected between 544-695 nm. Images were analyzed using Fiji/ImageJ software. Plot profiles were taken across the epidermal cell files of maximum intensity projections using a 20-pixel line and values were averaged within each cell file.

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5-day old seedlings harboring the ZAT12p-ROS construct were mounted in H_2O and excited with 488 and 561 nm lasers at 6% and 1.2% laser power, respectively. GFP emission was collected at 521 nm and mCherry emission was collected at 593 nm. Fiji/ImageJ software was used to generate two single channel images to form individual GFP and mCherry channels. Plot profiles were taken using a 250-pixel wide line to measure fluorescence of the entire root. Measurements were taken starting at 200 µm back from the root tip and ending at 1500 µm. Ratios were generated by dividing the GFP channel by mCherry using the Image Calculator tool (Process/Image Calculator).

PI staining and *proPRX44*::GFP imaging

Cell walls were stained with 0.5 µg/mL propidium iodide (PI) dissolved in H_2O. 5 day old seedlings treated with and without ACC were incubated in PI for 4 minutes before imaging. Fluorescence was visualized using a 561 nm laser at 0.15% and emission spectra set to 561-695 nm. These settings were used for all images. Optical slices of the top section of the root to show epidermal cells and maximum intensity projections of z-stack images are shown in Figure S1. Fiji software was used to measure cell length of 3 epidermal cells per root using optical slices of the top section of the root. Cells were chosen at the bottom of zone 2 (cell 1, in the middle (cell 2), and at the top (cell 3). 4 cells of each of the 3 cell types were averaged and are shown in FS1. 6-8 roots of both control and ACC treated seedlings. Transgenic seeds harboring *pPRX44*::GFP were obtained from the lab of Dr. Jose Estevez, 5-day old seedlings were mounted in H_2O and excited with a 488 nm laser at 5% and emission was collected between 490-606 nm. Seedlings were then stained with PI.

DNA extraction and PCR for mutant genotype analysis

T-DNA insertion lines were grown for approximately 2 weeks on media described above, then transferred to soil and grown under 24h light at 50-80 µmol photons m^{-2} s^{-1}. Leaves were harvested from 6 week old plants and stored in eppendorf tubes at -20° C prior to DNA extraction. Frozen leaves were ground in DNA extraction buffer (100 mM Tris-HCl pH 8.0, 10 mM Na2EDTA, 100 mM $LiCl_2$, 1% (w/v) SDS). Samples were then washed once with isopropanol and three times with 80% (v/v) ethanol. Finally, DNA pellets were dried, resuspended in sterile H_2O and stored at -20° C. Each PCR reaction contained 1X GoTaq Polymerase, 1 µM of each primer, 1 uL of DNA and 5 µL H_2O. To confirm that mutants were homozygous

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for the desired T-DNA insertion, one reaction was performed with primers (left primer and right primer) flanking the left and right sides of in-tact genes while one reaction was performed with the right primer and LBb1.3, which is a primer specific for the left border of the T-DNA insertion. PCR products were run on 0.8 % (w/v) agarose gels containing 0.002% SERVA DNA stain G.

NADPH oxidase/RBOH enzyme assays

Protein extract was isolated from seedlings grown on a nylon filter as described previously (39). After 2 days of stratification and 7 days of growth under conditions described above, the nylon was transferred to growth medium with and without 1 µM ACC for 4 hours. Roots were then cut from seedlings and flash frozen in liquid nitrogen. Frozen samples were ground in liquid nitrogen using a mortar and pestle. RBOH extraction buffer (50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM $MgCl_2$, 10% (v/v) glycerol) was then added to a plant material/buffer ratio of 1:3 (w/v). Samples were centrifuged and supernatant was collected and desalted and concentrated using the Amicon Ultra-0.5 Centrifugal Filter devices. RBOH reaction mixture (50 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 0.1 mM nitroblue tetrazolium (NBT), 0.1 mM NADPH) was then mixed with protein extract at a 1:1 ratio. The reduction of NBT to monoformazan was monitored spectrophotometrically at 530 nm. Monoformazan concentrations were calculated using a 12.8 $mM^{-1}cm^{-1}$ extinction coefficient. This assay was adapted in sweet peppers and has previously been described (57).

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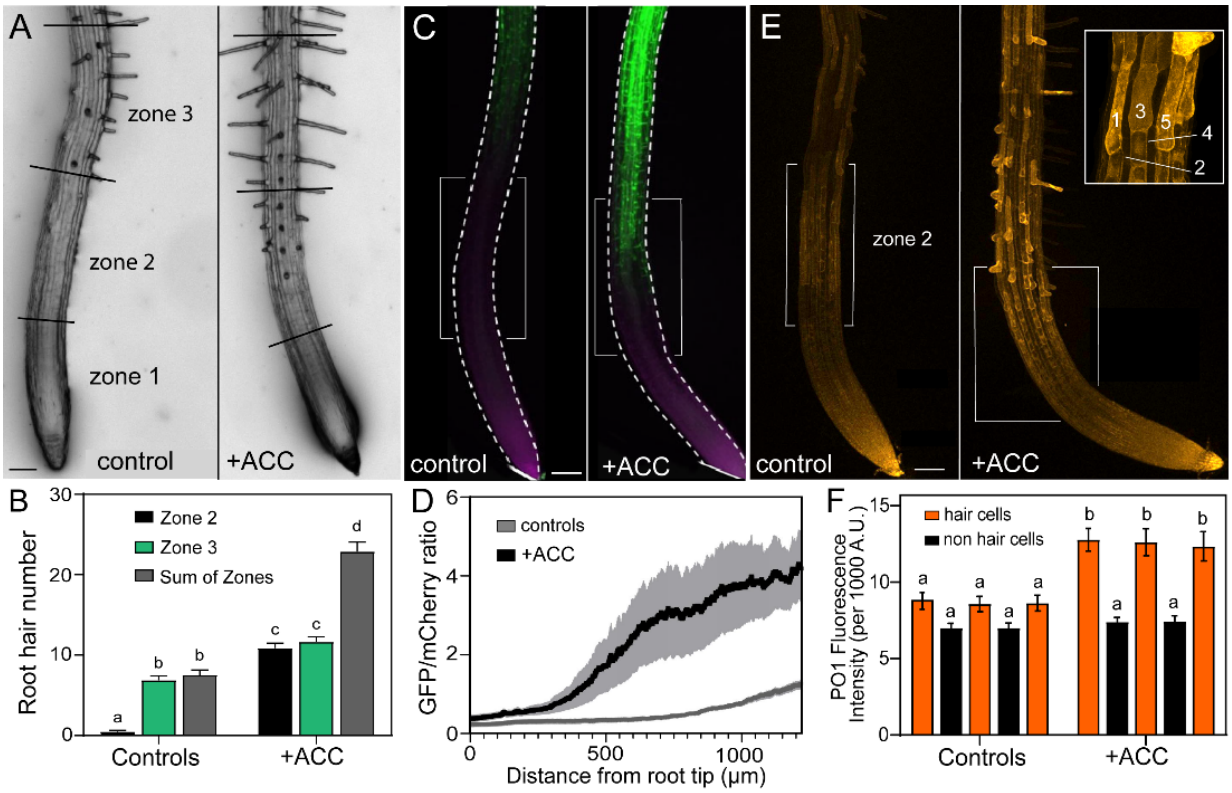

Figure 1. ACC treatment induced root hair number, ROS dependent gene expression along the root, and H_2O_2 accumulation in trichoblasts after 4 hours (A) Representative images of root tips in Col-0 treated with and without ACC for 4 hours. Zones 1, 2, and 3 represent 500 μm root sections. Scale bar for all images are 100 μm. (B) Root hair quantification of untreated and 4h ACC treated seedlings. No root hairs formed in zone 1. Data are means ± SEM of total RH in zone 2, zone 3, and all zones from 3 experiments (n=18-24 seedlings per experiment). Columns with different letters indicate statistically significant differences determined by two-way ANOVA followed by Tukey's multiple comparisons test. (C) Representative images of root tips of Col-0 containing the ZAT12p-ROS reporter treated with or without ACC for 4 hours with GFP signal in green and mCherry signal in magenta. White brackets indicate zone 2 of the root. (D) Quantification of the ratio of GFP/mCherry fluorescence intensity using a line profile along the roots of an average of 6-12 seedlings of 3 independent experiments of untreated or ACC treated seedlings. Data are means ± SEM (E) Representative roots illustrating the alternating PO1 fluorescence between trichoblast and atrichoblast epidermal cell files of cells in Col-0 roots with and without ACC treatment for 4 hours. White brackets indicate zone 2 of the root. Inset indicates hair cells (1, 3, 5) vs. non hair cells (2, 4). (F) Quantification of PO1 fluorescence intensity in trichoblasts (orange bars: 1, 3 and 5) and atrichoblasts (black bars: 2, 4). Data are means ± SEM of 3 independent experiments (n=18-24 seedlings/experiment). The error is too small to see in untreated roots. Columns with different letters indicate statistical significance compared to Col-0 untreated cells as determined by two-way ANOVA followed by Tukey's multiple comparisons test.

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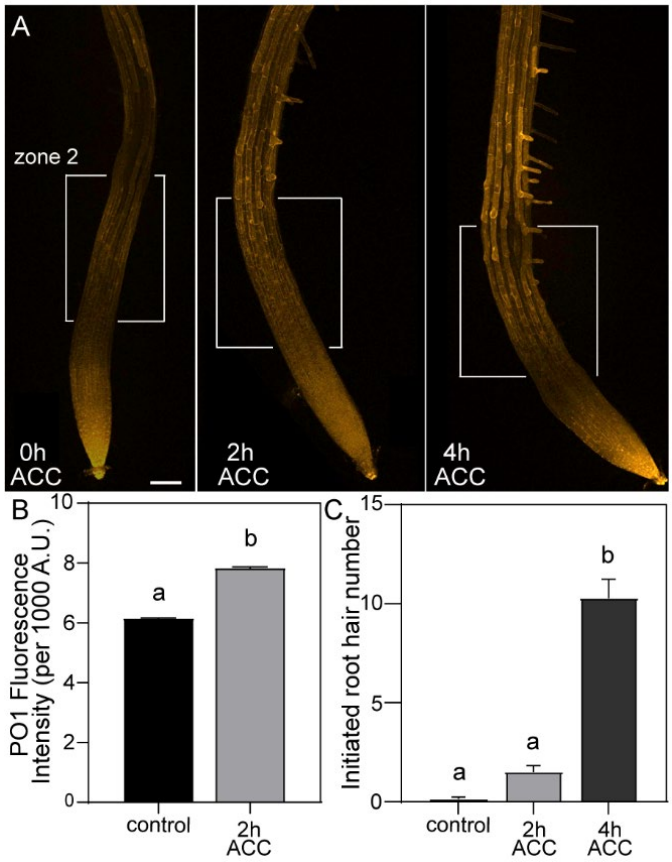

Figure 2. Ethylene-induced ROS accumulation preceded root hair initiation. (A) Representative images of epidermal PO1 fluorescence in untreated Col-0 and Col-0 treated with ACC for 2 or 4h. Scale bar is 100 µm. White brackets indicate zone 2 of the root. (B) Quantification of trichoblasts that had not yet formed root hair bulges of control and 2h ACC treated seedlings. (C) Quantification of number of initiated root hairs in zone 2 of the root (500 µm-1000 µm from root tip) in untreated and 2 and 4h ACC-treated seedlings. Data are mean ± SEM of individual cells from 3 independent experiments (n= 12-18 seedlings/experiment). Columns with different letters indicate statistical significance compared to untreated hair cells as determined by Student's t-test ($p < 0.0001$) in B. Columns with different letters indicate statistically significant differences determined by one-way ANOVA followed by Tukey's multiple comparisons test in C.

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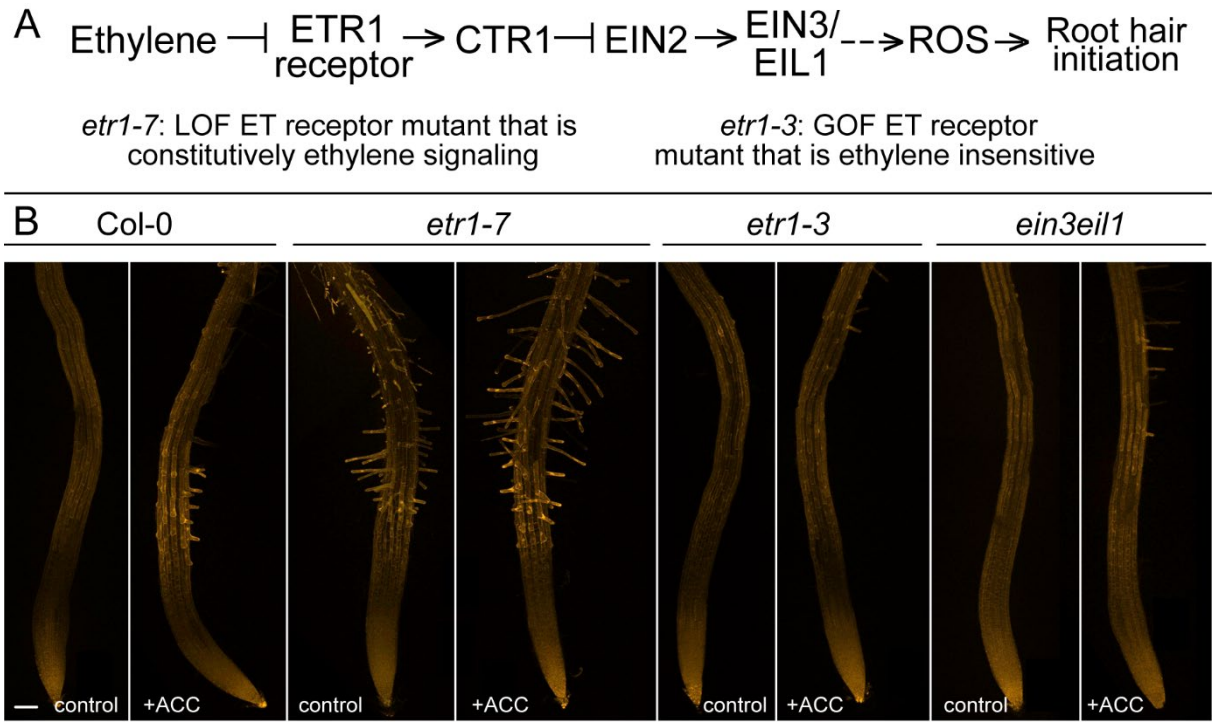

Figure 3. The ETR1 receptor and EIN3/EIL1 transcription factors are required for ethylene-induced ROS accumulation and root hair proliferation. (A) A schematic diagram of the ethylene signaling pathway and explanation of the character of the *etr* mutants. ETR1 (ethylene resistant 1) is the ethylene receptor controlling root hair formation, CTR1 (constitutive triple response 1 is a kinase, EIN2 (ethylene insensitive 2) is a signaling protein, EIN3 and EIL1 (EIN3-like 1) are transcription factors. (B) Representative images of PO1 epidermal fluorescence in Col-0, *etr1-7*, *etr1-3*, and *ein3-1eil1-1* treated with and without ACC for 4 hours. 18-20 seedlings from each genotype and treatment were imaged. Scale bar is 100 μm.

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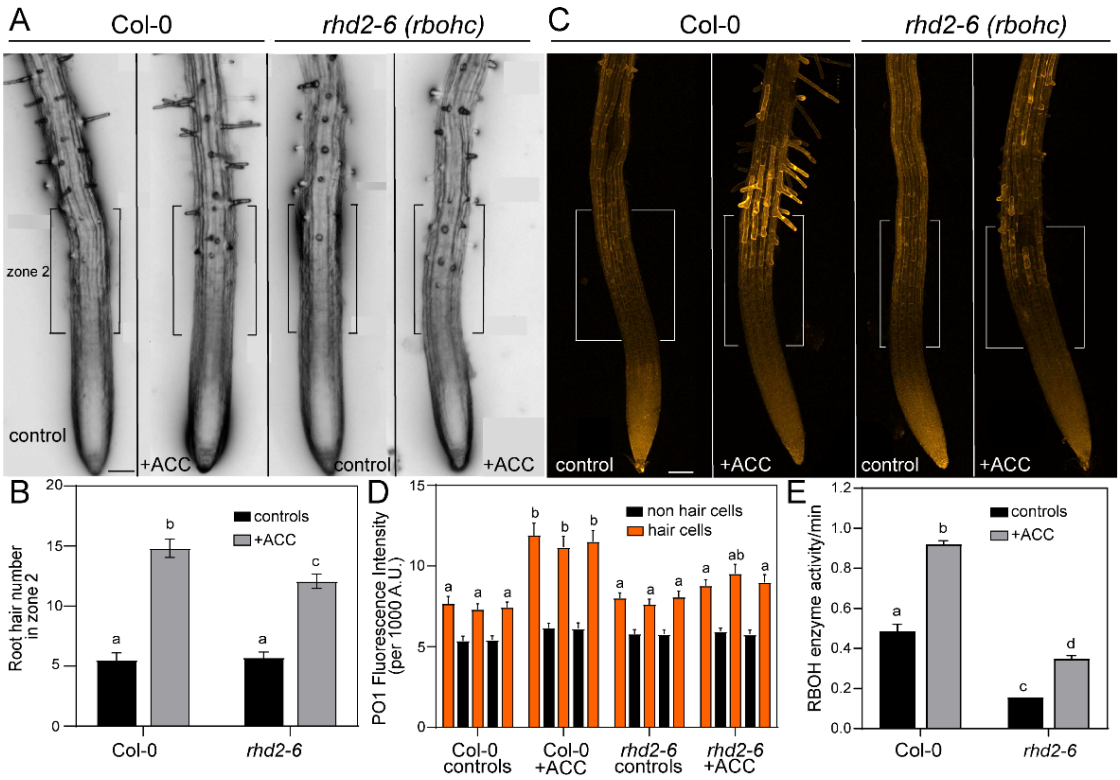

Figure 4. RBOHC activity contributes to ethylene-induced ROS accumulation. (A) Representative images of root hairs of Col-0 and *rhd2-6* treated with and without ACC for 4 hours. Black brackets indicate zone 2 of the root. (B) Root hair quantification of untreated and 4h ACC treated seedlings. Data are means ± SEM of total RH in zone 2 from 3 experiments (n=18-24 seedlings per experiment). Columns with different letters indicate statistically significant differences determined by two-way ANOVA followed by Tukey's multiple comparisons test. (C) Representative images of PO1 epidermal fluorescence in Col-0 and *rhd2-6* treated with and without ACC for 4 hours. White brackets indicate zone 2 of the root. (D) Quantification of PO1 fluorescence intensity in hair cells and non-hair cells. Data are means ± SEM of 3 experiments (n=18-24 seedlings/experiment). Columns with different letters indicate statistical significance in PO1 signal compared to other hair cells as determined by two-way ANOVA followed by Tukey's multiple comparisons test. (E) Quantification of formazan concentration per minute in roots of Col-0 and *rhd2-6* treated with and without ACC for 4 hours. Data are means ± SEM of 3 experiments. Columns with different letters indicate statistical significance as determined by two-way ANOVA followed by Tukey's multiple comparisons test. Scale bars for all images are 100 μm.

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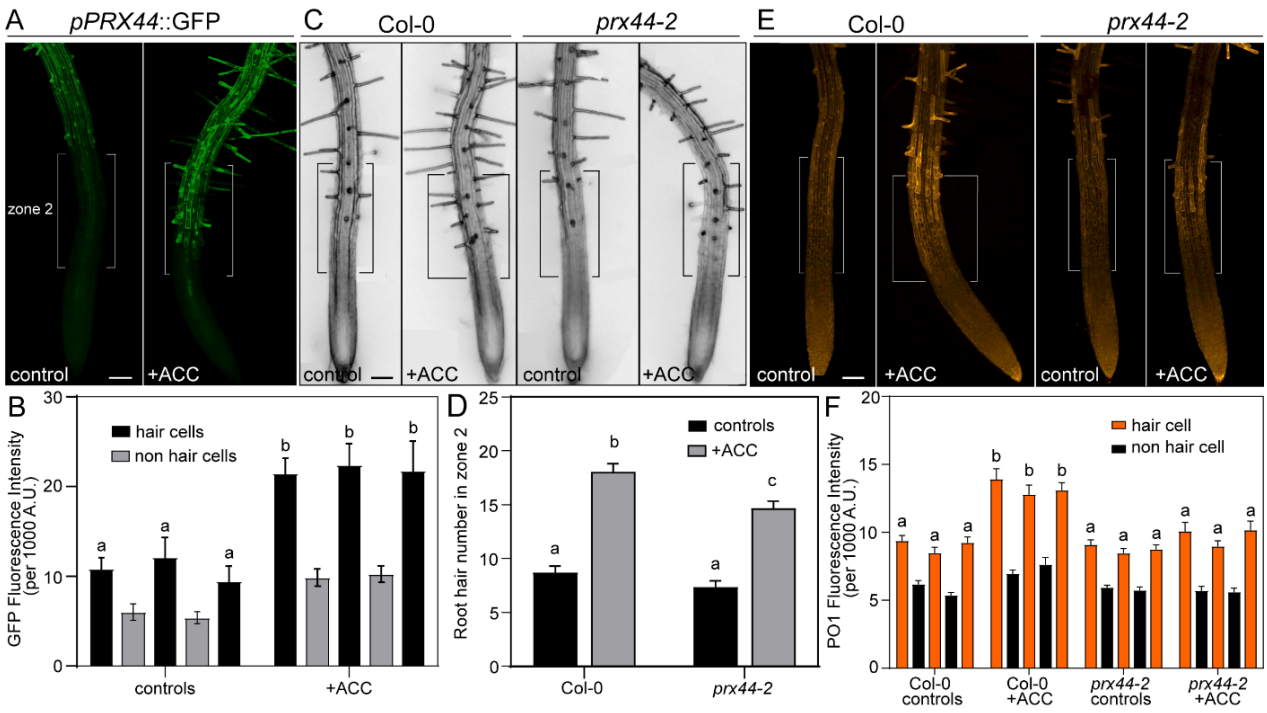

Figure 5. *pPRX44*::GFP expression increases in roots treated with ACC and PRX44 contributes to ethylene-induced root hair formation and H_2O_2 accumulation in root hair cells (A) Representative images of *pPRX44*::GFP treated with and without ACC for 4 hours. White brackets indicate zone 2 of the root. (B) Quantification of GFP signal in hair and non-hair cells of *pPRX44*::GFP treated with and without ACC. Data are means ± SEM (C) Representative images of RH number in Col-0 and *prx44-2* treated with and without ACC for 4 hours. Black brackets indicate zone 2 of the root. (D) Root hair quantification of untreated and 4h ACC treated seedlings. Data are means ± SEM. of 3 independent experiments (n=18-24 seedlings per experiment). Columns with different letters indicated statistically significant differences determined by two-way ANOVA followed by Tukey's multiple comparison test. (E) Representative images of Col-0 and *prx44-2* treated with and without ACC for 4 hours and stained with PO1. White brackets indicate zone 2 of the root. (F) Quantification of PO1 accumulation in hair and non-hair cells of Col-0 and *prx44-2* treated with and without ACC for 4 hours (n=12-18 seedlings per experiment). Data are means ± SEM of 3 experiments. Columns with different letters indicate statistical significance compared to all other hair cells as determined by two-way ANOVA followed by Tukey's multiple comparisons test. Scale bars for all images are 100 µm.

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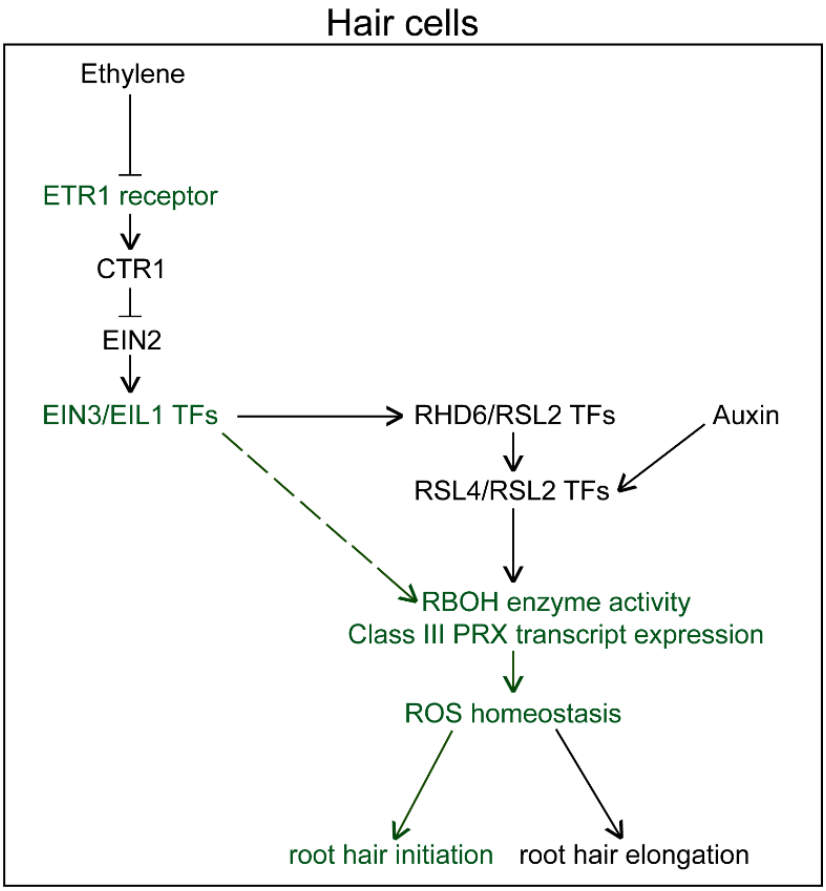

Figure 6. Summary of mechanisms by which ethylene and auxin modulate ROS and root hair formation. In hair cells, ethylene, acting through the ETR1 receptor and the canonical ethylene signaling pathway induces accumulation of EIN3/EIL1 TF proteins. Previous work has shown that EIN3/EIL1 physically interacts with RHD6/RSL2 TFs to induce expression of RSL4/RSL2 transcripts leading to root hair elongation (38). It has also been shown that RSL4/RSL2 is induced by auxin signaling and that RSL4/RSL2 binds to the promoters of RBOH and class III PRX genes to induce transcript expression and root hair elongation (30). Here, highlighted in green, we have shown that ethylene signaling through ETR1 and EIN3/EIL1 induces ROS accumulation through RBOHC enzyme activity and PRX44 transcript expression to drive root hair initiation.