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

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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) and the *prx44-2* mutant defective in a class III peroxidase, showed impaired ethylene-dependent ROS synthesis and root hair formation and decreased RBOH enzyme activity compared to Col-0. Together, these results support a model in which ethylene increases ROS accumulation through RBOHC and PRX44 to drive root hair formation.

36 **SUMMARY STATEMENT**

37 The gaseous hormone ethylene increases root hair initiation by elevating reactive oxygen species (ROS)
38 levels. Genetic and biochemical approaches identified ethylene-regulated ROS producing enzymes that
39 drive root hair initiation.

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 (Leavitt, 1904; Salazar-Henao et al., 2016). The formation of root hairs is modulated by environmental changes to increase root surface area to allow for efficient water and nutrient uptake (Bruex et al., 2012), while also anchoring plants in soil to reduce erosion (De Baets et al., 2020). In *Arabidopsis*, the root epidermis consists of an alternating pattern of trichoblasts, which form root hairs, and atrichoblasts, which are non-hair forming cells (Pemberton et al., 2001; Salazar-Henao et al., 2016). Root hair formation is dictated by cell positioning; epidermal cells overlying two cortical cells can become hair forming cells, while those overlying one cortical cell will become non-hair cells (Berger et al., 1998; Salazar-Henao et al., 2016). Root hair development is separated into two processes: root hair initiation and root hair elongation (Dolan et al., 1994), although a recent report divided this process into 10 precise stages and examined the molecular mechanisms that drive this process. During stages -7 to -1, RHO Of Plants (ROP) proteins (ROP2, ROP4, and ROP6) and ROP Guanine Nucleotide Exchange factors (ROP GEFs) that regulate ROP activity accumulate at the future site of initiation (Denninger et al., 2019; Molendijk et al., 2001), this is followed by actin polymerization along which vesicles move to deposit membrane allowing for polarized tip growth (Salazar-Henao et al., 2016). During tip growth, tip focused reactive oxygen species (ROS) (Foreman et al., 2003; Gayomba and Muday, 2020) and Ca^{2+} gradients (Carroll et al., 1998) have been identified to 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 (Masucci and Schiefelbein, 1994; Schiefelbein and Somerville, 1990).

Genetic screens in *Arabidopsis thaliana* have provided a wealth of insight into the proteins that drive root hair development (Lee and Schiefelbein, 1999; Masucci and Schiefelbein, 1996; Rerie et al., 1994). For example, the mutants *transparent testa glabra* (*ttg*), *glabra2* (*gl2*), and *werewolf* (*wer*) have root hairs that form from both trichoblast and atrichoblast cells (Di Cristina et al., 1996; Galway et al., 1994; Lee and Schiefelbein, 1999). Many of the protein products of these mutants have been mapped to

transcriptional cascades that drive root hair differentiation (Shibata and Sugimoto, 2019) and have been shown to localize prior to root hair initiation (Denninger et al., 2019). 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 (Grebe, 2012; Salazar-Henao et al., 2016). 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*) (Schiefelbein et al., 2014). When this pathway is active, *GL2* is not expressed and root hair initiation proceeds (Salazar-Henao et al., 2016).

Another genetic screen identified the *root hair defective* (*rhd*) mutants which have impaired root hair initiation, elongation or structure (Masucci and Schiefelbein, 1994; Schiefelbein and Somerville, 1990). In one of these mutants, *rhd2*, the mutation was mapped to the *RBOHC* (respiratory burst oxidase homolog C/NADPH oxidase) gene (Foreman et al., 2003). The *rhd2* mutant was identified for altered root hair elongation (Schiefelbein and Somerville, 1990), but was recently reported to also have impaired root hair formation (Gayomba and Muday, 2020). 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 (Chapman et al., 2019). H_2O_2 can then enter into the cell through aquaporins (Bienert et al., 2007), 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) (Poole and Schoneich, 2015).

There are 10 RBOH family members (*RBOHA*-*RBOHJ*) in *Arabidopsis* and each play distinct roles in organ development and stress response (Chapman et al., 2019). RBOHs can be regulated transcriptionally or enzymatically by a number of mechanisms, including calcium binding, phosphorylation, and phosphatidic acid binding (Kobayashi et al., 2007; Postiglione and Muday, 2020; Suzuki et al., 2011). 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 (Chapman et al., 2019; Kwak et al., 2003; Mittler et al., 2011; Postiglione and Muday, 2020). 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 (Kwak et al., 2003). Auxin induces root hair elongation through a localized increase in ROS (Mangano et al., 2017), suggesting that RBOH may drive hormone induced root hair elongation.

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 (Tanimoto et al., 1995). 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 (Binder, 2020; Bleecker, 1999). When ethylene is absent, the receptors are in the “on state” leading to activation of the CTR1 Raf-like kinase (Kieber et al., 1993); this turns the pathway off via phosphorylation and subsequent degradation of the EIN2 transmembrane protein (Alonso et al., 1999; Ju et al., 2012). 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 (Ju et al., 2012; Wen et al., 2012) 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 transcription factor (Feng et al., 2017) and that EIN3 also controls the formation of ectopic root hairs when ethylene reaches very high levels (Qiu et al., 2021). EIN3 physically interacts with RHD6, a known positive regulator of root hair development, to form a transcriptional complex that binds to and induces expression *RSL4*. This transcriptional regulation results in increased root hair length (Feng et al., 2017). However, the mechanistic events that drive ethylene-induced root hair initiation have not been fully described and the role of ROS as a downstream molecule in ethylene-induced root hair development has not been reported.

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

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 μ M ACC for 4 hours (Figure 1A). The root tip was divided into three 500 μ m zones starting from the root tip and the number of initiated root hairs (stage +2 and above as described by (Denninger et al., 2019) 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. It is also evident from Figure 1, that this treatment increases the length of root hairs in both zone 2 and 3, consistent with prior reports (Feng et al., 2017; Harkey et al., 2018). These data suggest that ACC-induced root hair initiation begins between 500 and 1000 μ m 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 (Harkey et al., 2018), therefore we asked whether the increase in root hairs seen with this brief 4 hour treatment with ACC 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 (designated zone 2A, 2B, and 2C in Figure S1). There was no difference in cell length in any of these 3 regions after 4 hours of ACC treatment compared to untreated controls. 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.

A ROS dependent transcriptional reporter 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 (Lim et al., 2019). This reporter construct contains the promoter of the ROS sensitive transcription factor, ZAT12, driving GFP and the constitutive ubiquitin10 promoter driving mCherry. We compared the fluorescent signal of ZAT12p-ROS in the presence and absence of ACC as visualized by laser scanning confocal microscopy (LSCM), with GFP reported as green and mCherry reported as magenta (Figure 1C). The ratio of signal of GFP (green) to mCherry was quantified across the entire root as a distance from the root tip. In ACC-treated roots, the GFP/mCherry ratio increased beginning at approximately 500 μ m from the root tip, which corresponded to zone 2, where root hair induction was maximal upon treatment with ACC (Figure 1D).

Ethylene increases the signal of the H₂O₂ selective dye, Peroxy Orange1, in trichoblast cells

We also used the hydrogen peroxide selective dye, Peroxy-Orange 1 (PO1), to ask whether there were cell type-specific increases in ROS in response to ACC or ethylene gas. PO1 is a permeable, boronate-based dye that is non-fluorescent in its reduced form, but becomes fluorescent when irreversibly oxidized by H₂O₂ (Dickinson et al., 2010). We treated Col-0 seedlings with either 1 μ M ACC or 0.05 ppm of ethylene gas for 4 hours followed by PO1 staining. In Col-0 roots, PO1 fluorescence was visualized using LSCM in zone 2 of roots treated with control, ACC, or ethylene gas for 4 hours (Figure 2). In Arabidopsis, root hairs form in alternating patterns, so that every root hair forming cell (trichoblast) is adjacent to a non-hair cell (atrachoblast). We quantified ROS accumulation by analyzing PO1 signal after confocal imaging, by drawing a line across the width of the root that spans 5 epidermal cell files so that the PO1 signal in 3 trichoblasts (numbered 1, 3, and 5) and 2 atrichoblasts (numbered 2 and 4) was quantified (Figure 2A). We found that PO1 accumulation in trichoblasts in zone 2 of untreated seedlings was slightly, but not significantly higher than atrichoblasts. In trichoblast cells in ACC treated seedlings, there was a significant increase in PO1 accumulation in ACC-treated trichoblasts as compared to trichoblasts of untreated seedlings and compared to atrichoblasts of both untreated and ACC treated seedlings. In contrast, there were no changes in PO1 in the atrichoblasts suggesting that ethylene and ACC treatment increased ROS levels in only the cells that formed root hairs, with PO1 fluorescence intensity values in trichoblasts after

both treatments showing the same magnitude increase as root treated with ACC (Figure 2B). These results are consistent with this short term and low dose treatment with ACC leading to efficient conversion to ethylene that in turn produces elevated ROS in trichoblast cells.

ROS accumulation increased prior to root hair emergence

To determine whether ethylene-induced ROS accumulation drives root hair emergence, we asked whether the ACC-induced ROS increases were detectable prior to the first ACC-induced root hair initiation. Wild type Col-0 seedlings were treated with ACC for either 2 or 4 hours and PO1 fluorescence was visualized in trichoblasts that did not have a root hair bulge (stages below +1) (Figure 3A). This PO1 signal is only reported in the 2-hour ACC treatment, as there were insufficient number of cells without root hair bulges to quantify in root treated for 4 hours. These trichoblasts were chosen for quantification to determine whether ACC-induced ROS increases were seen in hair forming cells prior 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 3B). Atrichoblast signal was not quantified as ROS levels did not change in those cells (Figure 2B). 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 3C). 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 (Harkey et al., 2018). *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 (Harkey et al., 2018; Hua and Meyerowitz, 1998). The double mutant *ein3eil1* has mutations in genes encoding EIN3 and EIL1 TFs (Chao et al., 1997; Solano et al., 1998). Both *etr1-3* and *ein3eil1* have reduced root hair initiation in response to ACC treatment (Harkey et al., 2018). 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 H₂O₂ in root hair cells and increased root hair initiation from these cells regardless of ACC treatment (Figure 4). 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 H₂O₂ 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.

The RBOHC knockout mutant, *rhb2-6*, 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 (Foreman et al., 2003). We have also shown that there is decreased ROS in root hairs of the *rhb2-6* mutant, which has an insertion mutation in the *RBOHC* gene (Gayomba and Muday, 2020). Therefore, we asked whether RBOHC is required for ACC-induced ROS increases and root hair initiation. We examined root hair numbers via light microscopy in ACC-treated *rhb2-6* (*rbohC*) seedlings (Figure 5). Root hair number in the ACC-treated *rhb2-6* mutant was significantly less than ACC-treated Col-0, suggesting that RBOHC contributes to ethylene-induced root hair initiation (Figure 5B). We also quantified the length of all root hairs in Zone 2 by measuring the length of all root hairs in zone 2 using the segmented line tool in ImageJ. A histogram showing the distribution of lengths of root hairs (reported as percentage in each length bin out of total number of roots) revealed that the length of root hairs increased after ACC treatment and that the *rhb2-6* mutant has reduced root hair elongation in the presence of ACC relative to Col-0.

We also visualized the PO1 fluorescence by LSCM in the *rhb2-6* mutant with and without ACC treatment (Figure 5D). We report the average of 2 trichoblast cells (1 and 5 from Figure 2) and two

atrachoblast cells (2 and 4) in Figure 5E. This method still reveals a significant increase in H₂O₂ fluorescence accumulation in Col-0 hair cells, while in *rhd2-6* there is no significant increase after ACC treatment (Figure 5E), suggesting that RBOHC contributes to ethylene-induced ROS accumulation. The data is reported for all 5 cell files in Figures S2. To determine if other RBOHs that are expressed in root tissue contributed to ethylene-induced ROS accumulation, PO1 accumulation patterns were examined in the *rbohdf* double mutant and compared to *rhd2-6*. No change was seen in ROS levels in hair cells of *rbohdf*, suggesting that these RBOHs do not contribute to ethylene-induced ROS synthesis in root hair cells (Figure S2).

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 (Harkey et al., 2018; Harkey et al., 2019) (Table S1). To determine if ethylene signaling regulated RBOH enzyme activity, a spectrophotometric 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 5F). The enzyme activity of roots of untreated *rbohcrhd2-6* 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 5F). These data are consistent with previous results indicating that the RBOHC enzyme constitutes the majority of the superoxide production in roots (Chapman et al., 2019; Gayomba and Muday, 2020).

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 (Harkey et al., 2018) 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 (Denninger et al., 2019), 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 (Mangano et al., 2017). 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 (Mangano et al., 2017), however, the role of ethylene signaling in regulating class III PRX expression to modulate root hair initiation or elongation has not been reported.

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 statistically significant >2-fold increase in GFP fluorescence, suggesting that the *PRX44* promoter is induced downstream of ethylene signaling (Figure 6A-B). We also asked whether these peroxidases participate in ACC-regulated root hair initiation. 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 6C-D). The root hairs in the ACC-treated mutant were also significantly shorter compared to Col-0, which is consistent with the phenotype that has been reported in response to auxin treatment (Figure 6E) (Mangano et al., 2017). 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 6F-G). 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 (Mangano et al., 2017) 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 S3) 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.

DISCUSSION

Ethylene is a key hormonal regulator of root hair initiation and elongation (Guzman and Ecker, 1990; Kieber et al., 1993). 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 (Foreman et al., 2003; Gayomba and Muday, 2020; Jones et al., 2007; Takeda et al., 2008). 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 (Foreman et al., 2003). The plant hormone auxin also increases ROS accumulation to drive root hair elongation (Mangano et al., 2017), 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 grown in the presence of low concentration of ethylene gas (0.5 ppm) and the ethylene precursor, ACC (1 μM), for 4 hours in both Col-0 and ethylene signaling mutant seedlings. There was an increase in the number of trichoblasts that formed root hairs in ethylene and 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 (Harkey et al., 2018) that 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 (Harkey et al., 2018; Lewis et al., 2011; Negi et al., 2010). These findings are also consistent with efficient conversion of ACC to ethylene and ACC effects root hair initiation due to ethylene signaling responses. A recent report described the ectopic

formation of root hairs when seedlings were treated with 10ppm ethylene for 2 days (Qiu et al., 2021). We do not see this response after our short 4-hour treatment with 20-fold less ethylene.

We asked whether ACC and ethylene 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 500-1000 μ m from the root tip (zone 2) in ACC treated roots compared to untreated controls. We also examined the effect of ethylene and ACC on hydrogen peroxide using a boronate based hydrogen peroxide selective sensor, PO1. Both ethylene and ACC lead to elevated H₂O₂ accumulation in the trichoblast cells in zone 2 after 4 hours of treatment, but PO1 signal was at constant low levels in atrichoblast cells. Consistent with this increasing ROS driving root hair formation, there was a significant increase in PO1 signal within 2 hours after ACC treatment, prior to the initiation of root hair bulges. These data are consistent with ROS acting 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 and with ACC acting through this canonical ethylene signaling pathway.

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 (Foreman et al., 2003; Gayomba and Muday, 2020), we asked whether this enzyme was contributing to ethylene-induced ROS accumulation and subsequent root hair initiation and/or elongation. ACC treated *rhb2-6*, 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 and to increase root hair elongation. The abundance of RBOHC transcripts showed small changes in response to ACC treatment (Harkey et al., 2018), 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 *rh2-6 (rboh)* 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 *rh2-6* mutant, these data suggest that initiation may require additional proteins.

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 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 and that this expression is localized to root hair cells. ACC treatment of the null mutant *prx73-4* and *prx44-2* led to decreased ROS accumulation in root hair forming trichoblasts in *prx44-2* and no effect in *prx73-4* as 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 and the length of these root hairs. Together these experiments implicate Class III peroxidase enzymes in ethylene-regulated 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 (Mangano et al., 2017). A recent report demonstrated that PRX enzymes function to modify the localization of extension proteins to control cell wall structure to allow increased elongation in response to auxin (Javier Martinez Pacheco, 2021). 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 (Shigeto and Tsutsumi, 2016). 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 (Liszak et al., 2003; Passardi et al., 2005). 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 (Mangano et al., 2017).

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 7. Low dose and short 4-hour treatments with ACC led to efficient conversion of ACC to ethylene by roots to initiate an ethylene signaling cascade. Ethylene acts through the ETR1 receptor and the canonical signaling pathway to increase the activity of EIN3/EIL1 TFs. EIN3/EIL1 binds to the root hair specific TF RHD6 to induce RSL4 expression and root hair elongation (Feng et al., 2017). Root hair growth is controlled by several proteins that eventually activate the RHD6 TF. Downstream of RHD6, the RSL4 TF is activated and it defines final root hair length based on its level of expression (Datta et al., 2015; Mangano et al., 2017). Previous work has shown that EIN3 physically interacts with RHD6 to form a transcriptional complex that coactivate RSL4 to promote root hair elongation (Feng et al., 2017), and that EIN3 and RSL4 both act to downregulate GL2 expression to drive ectopic root hair formation with long term treatments with high levels of ethylene (Qiu et al., 2021). However, in our microarray dataset (Harkey et al., 2018), *RSL4* transcript abundance only showed slight changes in response to ACC treatment (Table S1). It has also been shown that auxin treatment results in increased RSL4 transcript abundance, which binds to the promoters of *RBOH* and four class III peroxidase (*PRX*) genes, including *PRX44*, to drive ROS synthesis in root hair cells. RSL4 activation via auxin induces class III peroxidase and *RBOH* expression, which leads to ROS accumulation required to drive root hair elongation (Mangano et al., 2017). We find that ACC treatment increases activity of *RBOHC* enzymes leading to increased ROS and root hair elongation, without changing the abundance of *RBOHC* transcripts. Additionally, ACC treatment leads to the transcriptional regulation of class III peroxidases (Harkey et al., 2018), resulting in ROS production and root hair initiation. Further investigation regarding the mechanisms of ethylene-regulated ROS synthesis is required. For example, one important question to be answered is how ethylene signaling regulates *RBOH* activity. This could occur through a number of different

392 mechanisms, such as calcium binding or phosphorylation, which are known to regulate RBOH enzymes
393 (Postiglione and Muday, 2020). It is also unknown as to whether ethylene regulates class III peroxidase
394 expression via EIN3 or an alternate transcriptional mechanism, or if ethylene also regulates class III
395 peroxidase enzyme activity.

396

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 (Binder et al., 2007; Gayomba and Muday, 2020; Harkey et al., 2018; Hua and Meyerowitz, 1998). The ZAT12p-ROS construct was generously provided by Won-Gyu Choi (Lim et al., 2019). *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 (Marzol, 2021). 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.

Quantification of root hair number and root hair length

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 that were at stage +1 or +2 (or later stages) as defined by (Denninger et al., 2019) were quantified using Fiji/ImageJ software in three zones (0-500 µm, 500-1000 µm, 1000-1500 µm) starting from the root tip.

We also determined root hair length in the presence and absence of ACC treatment in Col-0 and mutants. All root hairs in zone 2 that had begun to elongate (were at stages above +2) were measured using Fiji/ImageJ software. The length of each root in microns was determined. To obtain the histogram of distribution, the roots were separated into bins of different lengths, and the % of root hairs in each length bin was determined by dividing by the total number of root hairs in Zone 2 in that root.

Confocal imaging of dyes and reporters of ROS levels

H₂O₂ was visualized with Peroxy-Orange 1 (PO1). PO1 was dissolved in DMSO to make a 500 µM stock and was further diluted in H₂O to make a 50 µM working solution. Seedlings were incubated in PO1 for 15 minutes in the dark and were then rinsed with H₂O and mounted in H₂O for imaging. Control, ACC-treated and ethylene-treated 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.

5-day old seedlings harboring the ZAT12p-ROS construct were mounted in H₂O 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 H₂O. 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 H₂O 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 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. 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 LiCl₂, 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 H₂O and stored at -20° C. Each PCR reaction contained 1X GoTaq Polymerase, 1 μM of each primer, 1 μL of DNA and 5 μL H₂O. To confirm that mutants were homozygous 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 (Harkey et al., 2018). 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 MgCl₂, 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 CaCl₂, 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⁻¹cm⁻¹ extinction coefficient. This assay was adapted in sweet peppers and has previously been described (Chu-Puga et al., 2019).

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COMPETING INTERESTS

The authors have no competing interests.

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DATA AVAILABILITY

All data in this manuscript are included in the text or in the supplementary file.

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Figure 1. ACC treatment induced root hair number and ROS dependent gene expression along the root 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 $\pm$ 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 $\pm$ SEM.

Figure 2. ACC and ethylene gas increase H_2O_2 accumulation in trichoblasts after 4 hours. (A)

Representative roots illustrating the alternating PO1 fluorescence between trichoblast and atrichoblast epidermal cell files of cells in Col-0 roots with and without ACC and ethylene gas 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). (B)

Quantification of PO1 fluorescence intensity in trichoblasts (orange bars: 1, 3 and 5) and atrichoblasts (black bars: 2, 4). Data are means $\pm$ SEM of 3 independent experiments (n=18-24 seedlings/experiment).

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.

Figure 3. 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 μ m. 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 μ m-1000 μ m from root tip) in untreated and 2 and 4h ACC-treated seedlings. Data are mean $\pm$ 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.

etr1-7: LOF ET receptor mutant that is constitutively ethylene signaling

etr1-3: GOF ET receptor mutant that is ethylene insensitive

Figure 4. 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-eil1-1* treated with and without ACC for 4 hours. 18-20 seedlings from each genotype and treatment were imaged. Scale bar is 100 μm.

Figure 5. 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 $\pm$ 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) Quantification of percent of root hairs within a certain micron length of Col-0 and *rhd2-6* treated with and without ACC. Columns with different letters indicate statistically significant differences determined by two-way ANOVA followed by Tukey's multiple comparisons test. (D) 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. (E) Quantification of PO1 fluorescence intensity in hair cells and non-hair cells. For each root PO1 fluorescence was quantified in two hair cells (cells 1 and 5 from Figure 2) and two nonhair cells (cells 2 and 4 from Figure 2) and the values pooled to obtain this average. Data are means $\pm$ 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. (F) Quantification of formazan concentration per

700 minute in roots of Col-0 and *rhd2-6* treated with and without ACC for 4 hours. Data are means $\pm$ SEM of 3
701 experiments. Columns with different letters indicate statistical significance as determined by two-way
702 ANOVA followed by Tukey's multiple comparisons test. Scale bars for all images are 100 μ m.

Figure 6. *pPRX44::GFP* expression increases in roots treated with ACC and PRX44 contributes to ethylene-induced root hair formation and H₂O₂ 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 $\pm$ 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 $\pm$ 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) Quantification of percent of root hairs within a certain micron length of *Col-0* and *prx44-2* treated with and without ACC. Columns with different letters indicate statistically significant differences determined by two-way ANOVA followed by Tukey's multiple comparisons test. (F) 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. (G) 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). For each root PO1 fluorescence was quantified in two hair cells (cells 1 and 5 from Figure 2) and two nonhair cells (cells 2 and 4 from Figure 2) and the values pooled to obtain this average. Data are means $\pm$ SEM of 3 experiments. Columns with different letters indicate

720 statistical significance compared to all other hair cells as determined by two-way ANOVA followed by
721 Tukey's multiple comparisons test. Scale bars for all images are 100 μ m.

Figure 7. 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 (Feng et al., 2017). 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 (Mangano et al., 2017). 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.