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Ethylene directs auxin to control root cell expansion

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SUMMARY

Root morphogenesis is controlled by the regulation of cell division and expansion. We isolated an allele of the *eto1* ethylene overproducer as a suppressor of the auxin-resistant mutant *ibr5*, prompting an examination of crosstalk between the phytohormones auxin and ethylene in control of root epidermal cell elongation and root hair elongation. We examined the interaction of *eto1* with mutants that have reduced auxin response or transport and found that ethylene overproduction partially restored auxin responsiveness to these mutants. In addition, we found that the effects of endogenous ethylene on root cell expansion in *eto1* seedlings were partially impeded by dampening auxin signaling, and were fully suppressed by blocking auxin influx. These data provide insight into the interaction between these two key plant hormones, and suggest that endogenous ethylene directs auxin to control root cell expansion.

Keywords

auxin; cell elongation; ethylene; hormone crosstalk; root hair

INTRODUCTION

Plants rely on differential cell expansion to shape root architecture. Root growth is indeterminate, proceeding by the continual succession of cell division, regulated cell expansion, and differentiation in the meristem and adjacent root regions. The outermost epidermal cell layer in radially symmetric *Arabidopsis thaliana* roots arises from the division of the meristematic epidermal/lateral root cap initials. Root epidermal cells have two fates, differentiating into files of either non-hair cells (atrichoblasts) or hair cells (trichoblasts), which bear root hairs that emerge as tube-shaped outgrowths of the root surface and function in water and nutrient uptake (reviewed in Grierson and Schiefelbein, 2002).

Epidermal cell lengthening and root hair tip growth are sensitive to a variety of environmental and developmental cues, including the plant hormones auxin and ethylene. Auxin inhibits root elongation (reviewed in Parry and Estelle, 2006) and promotes root hair lengthening (reviewed in Grierson and Schiefelbein, 2002). For example, mutants defective in AUXIN RESISTANT 1 (AUX1), which moves the auxin indole-3-acetic acid (IAA) into

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Markers used in MS34 mapping.

Table S2. PCR-based determination of mutant genotypes.

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cells (reviewed in Vieten *et al.*, 2007), are resistant to the inhibitory effects of exogenous and endogenous auxin on root elongation (Pickett *et al.*, 1990), and have short root hairs (Pitts *et al.*, 1998). Auxin response is regulated by a family of AUXIN RESPONSE FACTOR (ARF) transcription factors, which are repressed by interaction with Aux/IAA family members (reviewed in Parry and Estelle, 2006). This repression is relieved by the $SCF^{TIR1/AFB}$ family of ubiquitin-protein ligases, which promote the degradation of Aux/IAA proteins when bound to auxin (reviewed in Parry and Estelle, 2006). Several mutants that are defective in auxin response because of a failure to degrade Aux/IAA proteins, including *axr1-12* and axr2-1, display auxin-resistant roots with root hair elongation defects (Masucci and Schiefelbein, 1996; Cernac *et al.*, 1997). The dual-specificity protein phosphatase IBR5 also is required for full auxin responsiveness (Monroe-Augustus *et al.*, 2003; Lee *et al.*, 2009); however, IBR5 promotes auxin response without stimulating Aux/IAA protein degradation (Strader *et al.*, 2008a).

The gaseous hormone ethylene decreases root cell length (Le *et al.*, 2001) and increases root width (reviewed in Smalle and Van Der Straeten, 1997) and root hair length (Tanimoto *et al.*, 1995; Masucci and Schiefelbein, 1996). 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) enzymes catalyze the rate-limiting step in ethylene biosynthesis. Several ACS isozymes, including ACS5/ETHYLENE OVERPRODUCER 2 (ETO2), are targeted for degradation by the ETO1 ubiquitin-protein ligase (Chae *et al.*, 2003; Christians *et al.*, 2009). Slowing degradation of these ACS enzymes results in ethylene overproduction, which lengthens root hairs, shortens hypocotyls and roots of dark-grown seedlings, and shortens roots of light-grown seedlings (reviewed in Chae and Kieber, 2005). Ethylene is detected by transmembrane histidine kinase receptors that no longer activate the CTR1 Raf-like kinase upon ethylene binding: this relief from CTR1 repression allows ETHYLENE INSENSITIVE 2 (EIN2) to activate the EIN3 family of transcription factors to promote ethylene-responsive transcription (reviewed in Schaller and Kieber, 2002). Consequently, *ctr1* mutants have short roots with long root hairs (Kieber *et al.*, 1993; Cho and Cosgrove, 2002), whereas ethylene-resistant mutants, such as *ein2*, have long roots with short root hairs (Guzman and Ecker, 1990; Pitts *et al.*, 1998).

Multilevel crosstalk between ethylene and auxin affects the synthesis, signaling, and transport of these hormones. Auxin increases *ACS* transcription, thus stimulating ethylene synthesis (reviewed in Yang and Hoffman, 1984; Tsuchisaka and Theologis, 2004). Similarly, ethylene application promotes the expression of IAA biosynthetic genes (Stepanova *et al.*, 2005, 2008), increases IAA synthesis (Swarup *et al.*, 2007), and increases IAA levels (Růžička *et al.*, 2007) in root tips. Furthermore, root ethylene responses require basipetal (rootward) auxin transport (Růžička *et al.*, 2007), and blocking IAA influx or efflux results in ethylene resistance in the root (Pickett *et al.*, 1990; Luschnig *et al.*, 1998). Moreover, some aspects of auxin response require ethylene response, and some facets of ethylene response require auxin response,as evidenced by auxin resistance in many ethylene signaling mutants and ethylene resistance in many auxin signaling mutants (Stepanova *et al.*, 2007).

We have isolated and characterized modifier mutations that restore auxin responsiveness to *ibr5* (Strader *et al.*, 2008b). Here we report that the causative mutation in one of these suppressors is a new allele of the ethylene overproducer *eto1*. We examined the interaction of *eto1* mutants with other mutants that have reduced auxin responsiveness or transport, and found that the effects of endogenous ethylene on root cell expansion in untreated *eto1* seedlings were fully suppressed by blocking auxin influx. Our data suggest that endogenous ethylene directs auxin to control root cell expansion, and provide insight into the interaction between the plant hormones auxin and ethylene in shaping the root.

RESULTS

Ethylene overproduction suppresses the weak auxin-resistant mutant *ibr5-1*

The *ibr5* suppressor isolate MS34 displays restored responses to the naturally occurring auxins IAA and indole-3-butyric acid (IBA) (Strader *et al.*, 2008b). We mapped the *ibr5*-suppressing mutation to an interval containing *ETO1* (*At3g51770*; Figure 1a). Mutation of *ETO1* results in ACS protein hyperaccumulation and ethylene overproduction, resulting in short hypocotyls in dark-grown seedlings that can be rescued by the ACS enzyme inhibitor aminoethoxy-vinylglycine (AVG) (Guzman and Ecker, 1990). Like *eto1-1*, dark-grown MS34 seedlings had short hypocotyls that were rescued by AVG (Figure 1b). Sequencing *ETO1* in MS34 revealed a mutation causing an Asp-to-Asn missense mutation in a conserved amino acid (Figure 1a,c), disrupting a previously unannotated tetratricopeptide repeat (TPR) domain immediately N-terminal to the sixth previously annotated (Wang *et al.*, 2004) TPR domain (Figure 1c,d). We found that *eto1-1 ibr5* and *eto1-12 ibr5* recapitulated MS34 auxin-response phenotypes (Figure 1e), confirming that the identified lesion, which we named *eto1-34*, was the suppressing mutation in MS34. The *eto2-1* mutation, which overproduces ethylene by rendering the ACS5 (ETO2) enzyme resistant to ETO1-mediated degradation (Chae *et al.*, 2003; Christians *et al.*, 2009), also suppressed *ibr5* auxin resistance (Figure 1e), suggesting that the observed suppression was a general consequence of ethylene overproduction, and was not specific to *eto1*.

Because examining auxin-responsive root elongation in the *eto1 ibr5* double mutant is confounded by the short root of *eto1*, we examined the activity of the auxin-responsive transcriptional reporter DR5-GUS (Ulmasov *et al.*, 1997) in these lines. *ibr5* has reduced DR5-GUS activity both without treatment and in response to auxin (Figure 2; Monroe-Augustus *et al.*, 2003; Strader *et al.*, 2008a). We found that light- and dark-grown *eto1-34* seedlings displayed near wild-type DR5-GUS activity in whole seedling extracts (Figure 2b,c), and elevated activity in root tips (Figure 2a). Moreover, *eto1-34* fully restored DR5-GUS activity levels to both untreated and auxin-treated *ibr5* seedlings (Figure 2), demonstrating that ethylene overproduction restored auxin-responsive transcription to the *ibr5* auxin-response mutant.

Ethylene overproduction partially restores auxin responsiveness to auxin-resistant mutants

To determine the impact of ethylene overproduction on other mutants with deficient auxin responsiveness, we compared the *eto1-1 ibr5-1* phenotypes with *eto1-1* combined with the auxin receptor mutant *tir1-1*, the RUB-activating enzyme mutant *axr1-3*, the auxin influx mutant *aux1-7* or the ethylene response mutant *ein2-1* (Table 1). We found that *eto1-1* partially restored the responsiveness of *tir1, axr1* and *aux1* to IBA in root elongation assays (Figure 3a). Because interpreting auxin responses in *eto1* is complicated by its short root, we also examined IBA-induced lateral root formation. We found that *eto1* produced more emerged lateral roots per unit root length than wild type in response to auxin application, and fully restored auxin-responsive lateral root formation to the auxin-resistant mutants *ibr5, tir1* and *aux1* (Figure 3b). In contrast, *eto1* failed to restore auxin-responsive lateral root formation to the pleiotropic *axr1* mutant (Figure 3b).

Blocking auxin transport suppresses the short-root phenotype of ethylene overproduction

Loss of *ETO1* confers ethylene overproduction, resulting in short hypocotyls and roots in dark-grown seedlings, and short roots in light-grown seedlings (reviewed in Chae and Kieber, 2005). As previously reported (Roman *et al.*, 1995), *ein2* fully suppressed *eto1* root and hypocotyl elongation defects in dark- and light-grown seedlings (Figure 4). In contrast, hypocotyls of dark-grown *eto1 ibr5, eto1 tir1*, *eto1 axr1* and *eto1 aux1* seedlings resembled

hypocotyls of the *eto1-1* parent (Figure 4a), indicating that impeding auxin response or transport does not affect ethylene response in dark-grown hypocotyls, consistent with previous results of ACC application to auxin-response mutants (Stepanova *et al.*, 2007). However, *ibr5, tir1, axr1* and *aux1* each rescued *eto1* root elongation in both the dark (Figure 4a) and the light (Figure 4b) to a degree consistent with the relative auxin resistance of the parent (*ibr5-1* = *tir1-1* < *axr1-3* < *aux1-7*; Figure 3c). These data suggest that the short-root phenotype of *eto1* seedlings requires auxin signaling and transport. Indeed, blocking IAA influx with the *aux1* mutation restored *eto1* root elongation as efficiently as blocking ethylene signaling with the *ein2* mutation (Figure 4a,b).

Endogenous ethylene directs auxin to inhibit longitudinal root cell expansion

Because both cell division and cell elongation contribute to root length, we used confocal microscopy to examine fully differentiated (no longer expanding) root cells from light-grown wild-type, *eto1, aux1* and *eto1 aux1* seedlings. As expected, we found that epidermal and cortex cells (the two outermost root cell layers) of eto1 were shorter than those of the wild type (Figure 5a,b), suggesting that ethylene inhibits root elongation, at least in part, by inhibiting elongation of these cells. Moreover, *eto1* cortex cells were wider than those of the wild type (Figure 5a). In contrast, the epidermal and cortex cell lengths were longer in *aux1* than in the wild type (Figure 5a,b, Rahman *et al.*, 2002), although these cells were often longer than the objective allowed us to image. The length and width of eto1 *aux1* epidermal and cortex cells resembled those of *aux1* (Figure 5a,b), indicating that AUX1 is necessary for ethylene to affect root cell expansion.

We quantified the effects of auxin-response mutants on *eto1* cell length. *eto1* displayed shorter epidermal cells than the wild type (Figure 5c,d), whereas the auxin-resistant mutants *ibr5* (Figure 5d), *tir1* (Figure 5e), *axr1* (Figure 5f) and *aux1* (Figure 5c,g), and the ethylene-response mutant *ein2* (Figure 5h), all displayed longer epidermal cells than the wild type. As expected, *ein2* fully suppressed the short epidermal cell length of eto1: *eto1 ein2* epidermal cells were as long as *ein2* cells (Figure 5h). The mild auxin-resistant mutants *ibr5* (Figure 5d) and *tir1* (Figure 5e) partially suppressed *eto1* epidermal cell elongation defects, and *axr1* restored *eto1* cells to wild-type lengths (Figure 5f). Like *ein2*, *aux1* was fully epistatic to *eto1: eto1 aux1* epidermal cells were as long as *aux1* cells (Figure 5c,g). We concluded that auxin transport and signaling are required for epidermal cell elongation inhibition in response to endogenous ethylene.

Endogenous ethylene directs auxin to promote root hair expansion

In addition to inhibiting root epidermal cell elongation, both auxin and ethylene promote root hair elongation (reviewed in Grierson and Schiefelbein, 2002). As expected, *eto1* displayed longer root hairs than the wild type (Figure 5c,i), whereas the auxin-resistant mutants *ibr5* (Figure 5i), *tir1* (Figure 5j), *axr1* (Figure 5k) and *aux1* (Figure 5c,l), and the ethylene response mutant *ein2* (Figure 5m), all had shorter root hairs than the wild type. Also as expected, *ein2* fully suppressed the long root hairs of *eto1: eto1 ein2* root hairs were as short as *ein2* root hairs (Figure 5m). We found that the mild auxin-resistant mutants *ibr5* (Figure 5i) and *tir1* (Figure 5j) slightly reduced eto1 root hair length, whereas the more severe *axr1-3* mutant more fully suppressed *eto1* root hair length (Figure 5k), suggesting that auxin signaling is required for ethylene-promoted root hair lengthening. The auxin influx mutant *aux1* completely suppressed the long root hair phenotype of *eto1* (Figure 5c,l), indicating that AUX1-mediated IAA influx is required for root hair elongation in response to endogenous ethylene.

Blocking auxin transport does not disrupt ethylene signaling

Because auxin upregulates transcription of the *ACS* ethylene biosynthesis genes (reviewed in Yang and Hoffman, 1984; Tsuchisaka and Theologis, 2004), *aux1* might suppress *eto1* effects on root cell expansion by preventing ethylene overproduction, by blocking ethylene signal transduction, or by acting downstream of ethylene signaling. We therefore examined a late-acting component of ethylene signaling, the EIN3 transcription factor, which accumulates in response to ethylene application and is elevated in *eto1* (Guo and Ecker, 2003) (Figure 6). Because *ein2* blocks ethylene response upstream of EIN3, EIN3 levels were low in both *ein2* and *eto1 ein2* (Figure 6). Unlike *eto1 ein2*, however, we found that *eto1 aux1* accumulated EIN3 to *eto1*-like levels (Figure 6). Because *aux1* prevents phenotypes associated with ethylene overproduction in light-grown *eto1* seedlings (Figures 4 and 5) without reducing EIN3 levels (Figure 6), we concluded that AUX1 is needed downstream of ethylene signaling to control root hair expansion in response to endogenous ethylene. Because the only known role of AUX1 is to bring IAA into cells (reviewed in Vieten *et al.*, 2007), this epistatic relationship implies that auxin signaling acts downstream of ethylene signaling to promote root hair elongation (Figure 7).

DISCUSSION

Both ethylene and auxin alter root morphology by reducing longitudinal cell expansion and promoting root hair elongation. Ethylene decreases primary root length by reducing cell elongation (Le *et al.*, 2001; De Cnodder *et al.*, 2005; Růžička *et al.*, 2007; Swarup *et al.*, 2007). Mutants with decreased ethylene or auxin responsiveness display shorter root hairs (reviewed in Grierson and Schiefelbein, 2002); conversely, mutants with increased ethylene response or production (reviewed in Grierson and Schiefelbein, 2002), or decreased IAA (Santelia *et al.*, 2005; Cho *et al.*, 2007) or IBA (Strader and Bartel, 2009) efflux, display longer root hairs.

The similar influences of ethylene and auxin on root cell morphology suggest that the respective signaling or response pathways are dependent or interdependent. Indeed, many auxin-resistant mutants are also ethylene resistant, and many ethylene-resistant mutants also are auxin resistant, suggesting that some aspects of auxin and ethylene response require responsiveness to the other hormone (Stepanova *et al.*, 2007). In addition, ethylene responses in the root require basipetal (rootward) auxin transport machinery (Pickett *et al.*, 1990; Luschnig *et al.*, 1998; Růžička *et al.*, 2007), and ethylene enhances acropetal (shootward) auxin transport (Negi *et al.*, 2008). However, interpretation of these studies is complicated by the use of applied hormones or inhibitors. Applied hormones may or may not mimic endogenous hormone production, and recent studies reveal that the commonly used ethylene inhibitors AVG and $AgNO_3$ are not suited for studying auxin–ethylene interactions. AVG not only inhibits the pyridoxal phosphate-requiring ACS ethylene biosynthesis enzymes (Yang and Hoffman, 1984), but also inhibits members of the recently described (Stepanova *et al.*, 2008; Tao *et al.*, 2008) pyridoxal phosphate-requiring TAA1 family of auxin biosynthesis enzymes (Soeno *et al.*, 2010). Thus AVG application can directly reduce both ethylene and IAA biosynthesis, and is not informative for dissecting auxin–ethylene interactions. $AgNO_3$ inhibits ethylene perception, but is also unsuitable for dissection of auxin–ethylene interactions because it promotes auxin efflux independently of its effects on ethylene responsiveness (Strader *et al.*, 2009).

We became interested in the interaction between auxin and ethylene response when we identified an *eto1* allele that suppressed certain phenotypes of the weak auxin-resistant mutant *ibr5*, including auxin-responsive root elongation inhibition (Figures 1e and 3a), lateral root promotion (Figure 3b) and gene expression (Figure 2). IBR5 is a dual-specificity protein phosphatase (Lee *et al.*, 2009) that promotes both auxin (Monroe-Augustus *et al.*,

2003) and ethylene (Strader *et al.*, 2008b) responsiveness. Intriguingly, IBR5 promotes auxin responsiveness without stimulating Aux/IAA protein degradation (Strader *et al.*, 2008a), unlike the well-characterized auxin-response components TIR1 and AXR1 (reviewed in Woodward and Bartel, 2005). Because IBR5 is unique among characterized factors that promote auxin-responsive transcription, we determined whether the *eto1* restoration of the *ibr5* auxin response was also unique.

We found that *eto1-1* partially restored the responsiveness of the auxin receptor mutant *tir1-1*, the RUB activating enzyme mutant *axr1-3* and the auxin influx mutant *aux1-7* to IBA in root elongation assays (Figure 3a). Moreover, *eto1-1* fully restored IBA-responsive lateral root formation in *ibr5, tir1*, and *aux1*, so we were surprised to find that *eto1-1* did not restore IBA-responsive lateral root formation to *axr1-3* (Figure 3b). AXR1 controls RELATED TO UBIQUITIN (RUB) modification of the CULLIN1 subunit of E3 ligase complexes to regulate E3 activity (reviewed in Schwechheimer and Caldero´n Villalobos, 2004). RUB-regulated E3 ligases include not only the TIR1 family of auxin receptors, but potentially hundreds of additional CULLIN-containing E3 ligases in Arabidopsis. As a result, disruption of RUB modification disrupts many plant processes, including responses to auxin (del Pozo *et al.*, 1998, 2002; Bostick *et al.*, 2004) and ethylene (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Stepanova *et al.*, 2007), as well as ethylene production (Bostick *et al.*, 2004; Woodward *et al.*, 2007). Because RUB modification of CULLIN affects diverse processes, it is difficult to pinpoint the cause of the *eto1* failure to restore auxin-responsive lateral root formation in *axr1*. In any case, our results clearly demonstrate that ethylene overproduction can compensate for attenuated auxin responsiveness caused not only by lesions in *IBR5*, but also by reduced *TIR1* or *AUX1* function, indicating that the observed suppression is not specific to the IBR5 pathway.

The *eto1* mutant was previously reported to produce fewer lateral roots than the wild type in the absence of treatment (Negi *et al.*, 2008). We also found that *eto1* exhibited fewer emerged lateral roots without treatment; however, it produced a greater lateral root density (emerged lateral roots per unit root length) in response to exogenous auxin than the wild type (Figure 3b). Treatment with low ACC levels promotes lateral root initiation and emergence in the wild type, but does not bypass the lateral root-deficient phenotype of mutants that lack lateral roots because of a strong block in auxin signaling, such as solitary *root-1* and *arf7 arf19* (Ivanchenko *et al.*, 2008), consistent with the possibility that ethylene promotion of lateral root initiation and emergence requires some residual auxin signaling. However, our demonstration that *eto1* restores auxin-responsive lateral root production to *ibr5, tir1* and *aux1* is not directly comparable with previous results showing that ACC treatment does not restore lateral root initiation and emergence to *slr-1* and *arf7 arf19* (Ivanchenko *et al.*, 2008), because Ivanchenko *et al.* used low levels of ACC that may not yield as much ethylene as is found in *eto1*. It will be interesting to learn whether *eto1* can restore auxin-responsive lateral root production to *slr-1* or *arf7 arf19*. Our demonstration that *eto1* restores auxin-responsive lateral root formation to several mutants with attenuated auxin responsiveness (Figure 3b) suggests that increased ethylene levels can prime the root for auxin-responsive lateral root production.

We used *eto1* to examine the effects of endogenous ethylene on root cell expansion in untreated auxin-response mutants and found that the effects of elevated ethylene on root cell expansion (inhibition of epidermal cell elongation and promotion of root hair elongation) were fully suppressed by blocking auxin influx (Figure 5). These data are consistent with previous reports that the application of 1-naphthaleneacetic acid (NAA) partially restores root hair elongation in the *ein2-1* mutant (Rahman *et al.*, 2002), and that the *aux1* mutant is resistant to the effects of the ethylene precursor ACC on the inhibition of root cell

elongation (Swarup *et al.*, 2007). Our epistasis data suggest that ethylene directs auxin to both promote root hair lengthening and inhibit root cell longitudinal expansion.

Animals require cell movement to determine the final form of organs. Because there is no morphogenic cell movement in plants, and because the cell wall is usually formed immediately after cell division, plant morphogenesis depends upon cell expansion. The plant hormones ethylene and auxin alter root morphology by decreasing root cell length and by increasing root hair length. We found that the ethylene effects on these processes are entirely dependent on auxin influx and responsiveness. Because ethylene is volatile, using the tightly regulated phytohormone auxin to influence root growth and morphology may provide additional control and specificity to ethylene effects. Our data suggest a model (Figure 7) in which ethylene promotes auxin biosynthesis (Růžička *et al.*, 2007; Swarup *et al.*, 2007) and/or auxin transport (Růžička *et al.*, 2007) to increase auxin levels in the epidermal cell layer, which inhibits longitudinal cell elongation and promotes root hair elongation. Future studies may reveal whether modulation of auxin biosynthesis or auxin transport by ethylene play a larger role in the control of root cell expansion.

EXPERIMENTAL PROCEDURES

eto1-34 identification

The previously described (Strader *et al.*, 2008b) *ibr5-1* suppressor MS34 (in the Col-0 background) was outcrossed to Ws-introgressed *ibr5-1* for mapping (Strader *et al.*, 2008b). The resulting F_2 seeds were plated on 10 lM IBA, and DNA from sensitive individuals was used for mapping using polymorphic markers (Table S1). A candidate gene (*ETO1/At3g51770*) within the mapping interval was sequenced from 51 bp upstream of the putative translation start site to 159 bp downstream of the stop codon to reveal the *eto1-34* missense mutation.

Plant materials, growth conditions and phenotypic assays

Mutants were in the Colombia (Col-0) accession of *A. thaliana*. PCR analysis of F_2 plants was used to identify double mutants (Table S2). Surface-sterilized seeds were plated on plant nutrient medium (PN; Haughn and Somerville, 1986) supplemented with 0.5% (w/v) sucrose (PNS) solidified with 0.6% (w/v) agar. Seedlings were grown at 22°C under continuous illumination unless indicated otherwise.

To examine hypocotyl elongation, seedlings were grown for 1 day in the light, followed by 4 days in darkness.

To examine auxin-responsive root elongation, seedlings were grown under yellow long-pass filters to slow indolic compound breakdown (Stasinopoulos and Hangarter, 1990) for 8 days on PNS with the indicated auxin.

To examine root hairs, 4-mm root sections adjacent to the root–shoot junction from 5-day-old vertically grown seedlings were imaged using a dissecting microscope, and root hair lengths were measured using the public domain NIH IMAGE program (developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image).

To examine epidermal cell lengths, 8-day-old seedlings were fixed in 3:1 ethanol:acetic acid, mounted in a chloral hydrate solution (80 g chloral hydrate, 20 ml glycerol and 10 ml water) and imaged using a Zeiss Axioplan 2 microscope (http://www.zeiss.com). Epidermal cell lengths (from both trichoblast and atrichoblast cell files) were measured using NIH IMAGE.

GUS assays

For histochemical GUS assays, 5-day-old dark-grown seedlings carrying DR5-GUS (Ulmasov *et al.*, 1997) were incubated in 90% acetone for 20 min at –20°C. Seedlings were rinsed twice in GUS buffer {0.1 M $NaPO_4$, pH 7.0, 0.5 mM $K_3[Fe(CN_6)]$, 0.5 mM $K_4[Fe(CN_6)]$, 10 mM EDTA, 0.01% Triton X-100}, and then incubated overnight at 37°C in GUS buffer supplemented with 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, fixed in 3:1 ethanol:acetic acid, mounted and imaged using a Zeiss Axioplan 2 microscope.

For 4-methylumbelliferyl-β-D-glucuronide hydrate (MUG) assays, 16 replicates of three 8-day-old seedlings carrying DR5-GUS (Ulmasov *et al.*, 1997) were treated for 2 h on a PNS plate supplemented with the indicated auxin. Extracts were prepared and GUS activity was monitored as previously described (Strader and Bartel, 2009).

Confocal fluorescence microscopy

Eight-day-old seedlings were stained for 10 min in an aqueous solution of 10 μg ml^{-1} propidium iodide (Sigma-Aldrich, http://www.sigmaaldrich.com), then mounted in water under a cover-slip. Samples were excited with an argon 488-nm laser line and imaged with a Zeiss LSM 510 Meta laser scanning confocal microscope through a 63x oil-immersion lens. Fluorescence was filtered through a 560-nm high-pass filter, and detected pixels were false-colored red. Images were converted using NIH IMAGE software.

Immunoblot analysis

To monitor EIN3 levels, protein from 12 5-day-old seedlings was analyzed by immunoblotting as previously described (Strader *et al.*, 2009), except that a 1:4000 dilution of anti-EIN3 antibody (Guo and Ecker, 2003) was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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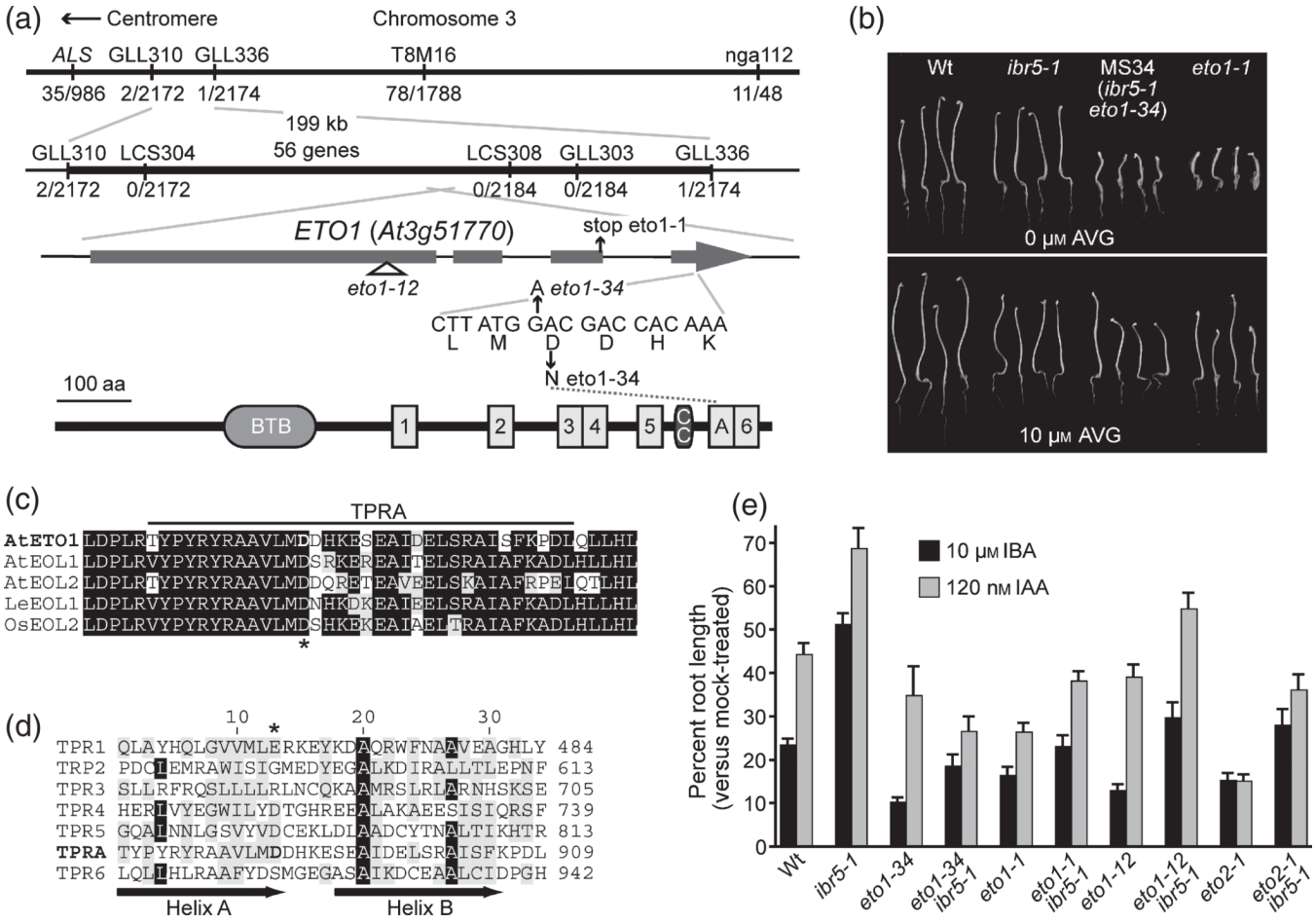

Figure 1. Ethylene overproduction suppresses the *ibr5* auxin-response mutant

(a) Recombination mapping with the indicated PCR-based markers (Table S1) localized the suppressing mutation in MS34 to a region containing 56 predicted genes between GLL310 and GLL336, with 2/2172 north and 1/2174 south recombinants. Examination of the *ETO1 (At3g51770)* gene in this region revealed a G→A mutation at position 3426, resulting in an Asp879 → Asn substitution. The ETO1 protein schematic, based on output from the domain-predicting programs SMART (Schultz *et al*, 1998) and PROSITE (de Castro *et al*, 2006), illustrates the ETO1 BTB/POZ domain (BTB), a coiled-coil region (CC) and seven TPR domains (labeled 1–6 and A).

(b) The MS34 *(ibr5-1 eto1-34)* short hypocotyl is rescued by AVG: photographs of 5-day-old dark-grown seedlings grown in the absence (top panel) or presence (bottom panel) of AVG.

(c) and (d) The *eto1-34* mutation alters a conserved Asp in TPR repeat A (asterisks).

(c) Alignment showing the newly recognized TPR domain *in Arabidopsis thaliana*ETO1 (At3g51770), Arabidopsis ETO1-LIKE1 (At4g02680), Arabidopsis ETO1-LIKE2 (At5g58550), LeEOL1 (DQ223268) and *Oryza sativa*ETO1-LIKE2 (AP003826).

(d) Alignment of TPR domains from Arabidopsis ETO1. Sequences were aligned using the MEGALIGN program (DNAStar, http://www.dnastar.com). Identical and chemically similar residues are boxed in black and gray, respectively.

(e) Normalized primary root lengths (mean + SE versus mock-treated control) of 8-day-old Col-0 (Wt), *ibr5-1, eto1-34, eto1-34 ibr5-1* (MS34), *eto1-1, eto1-1 ibr5, eto1-12, eto1-12 ibr5, eto2-1* and *eto2-1 ibr5-1* seedlings grown under yellow-filtered light at 22°C on

medium supplemented with the indicated auxin ($n \geq 12$). *eto1-34 ibr5-1, eto1-1 ibr5-1, eto1-12 ibr5-1* and *eto2-1 ibr5-1* normalized root lengths on indole-3-butyricacid (IBA) were significantly shorter than *ibr5-1* on 10 μM IBA ($P \leq 0.0001$) and 120 nM indole-3-acetic acid (IAA) ($P \leq 0.01$) in two-tailed Student's *t*-tests assuming unequal variance.

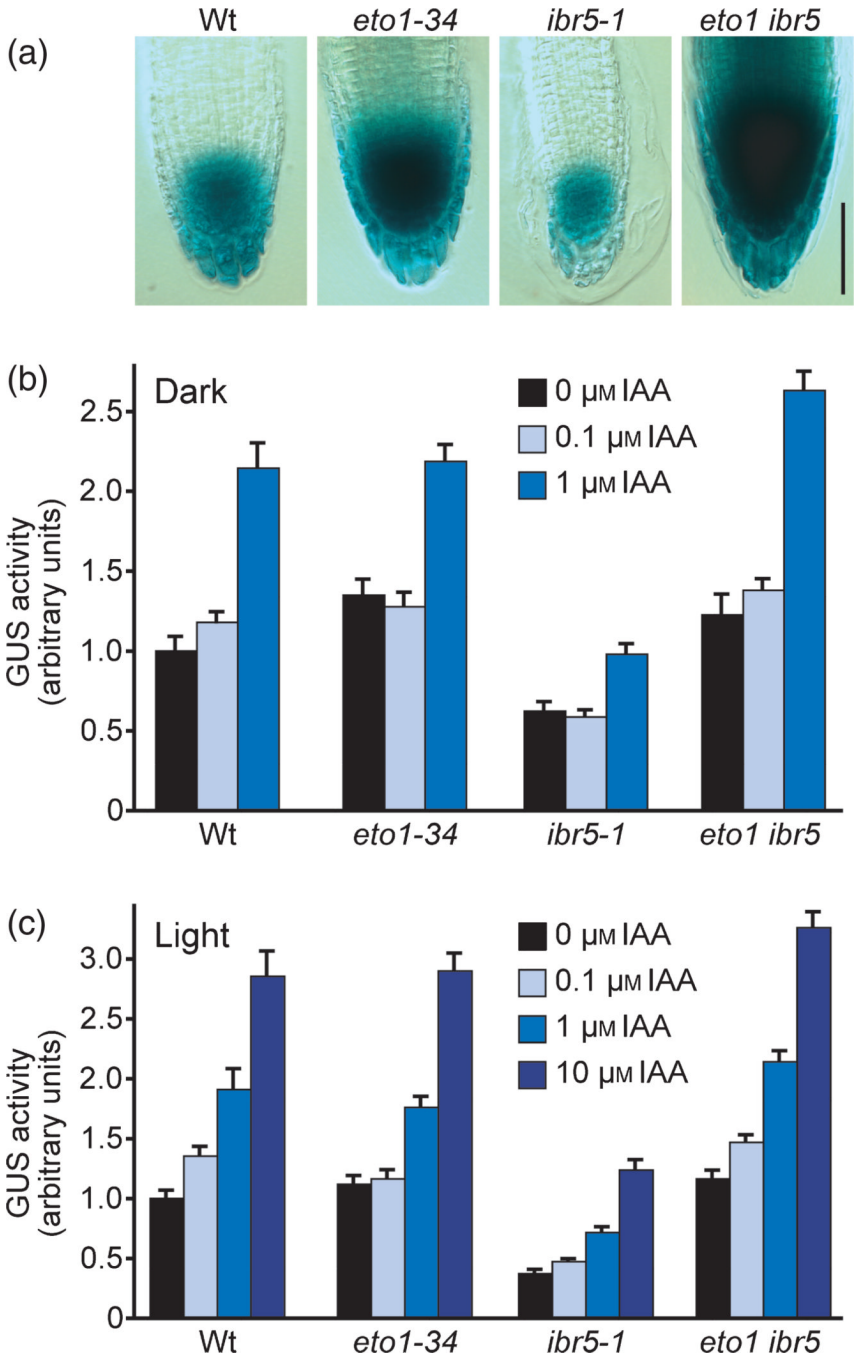

Figure 2. *eto1* restores auxin-responsive transcription to *ibr5*

(a) Primary root tips of untreated 8-day-old Col-0 (Wt), *eto1-1, ibr5-1* and *eto1-1 ibr5-1* seedlings carrying the DR5-GUS construct (Ulmasov *et al.*, 1997) stained to visualize GUS activity. Scale bar: 50 μm.

(b, c) Col-0 (Wt), *eto1-1, ibr5-1* and *eto1-1 ibr5-1* seedlings carrying the DR5-GUS construct were grown for 5 days in the dark (b) or 8 days in the light (c), and were then transferred to medium supplemented with various concentrations of indole-3-acetic acid (IAA) for 2 h. GUS activity in seedling extracts is presented as normalized fluorescence (mean + SE). GUS activity in *ibr5-1* was significantly lower than GUS activity in the wild type in both dark- and light-grown seedlings in both the absence and presence of IAA

treatment ($P \leq 0.001$ in two-tailed Student's *t*-tests assuming unequal variance). GUS activity in *eto1-34 ibr5-1* was significantly higher than GUS activity in *ibr5-1* in both dark- and light-grown seedlings in both the absence and presence of IAA treatment ($P \leq 0.0001$ in two-tailed Student's *t*-tests assuming unequal variance).

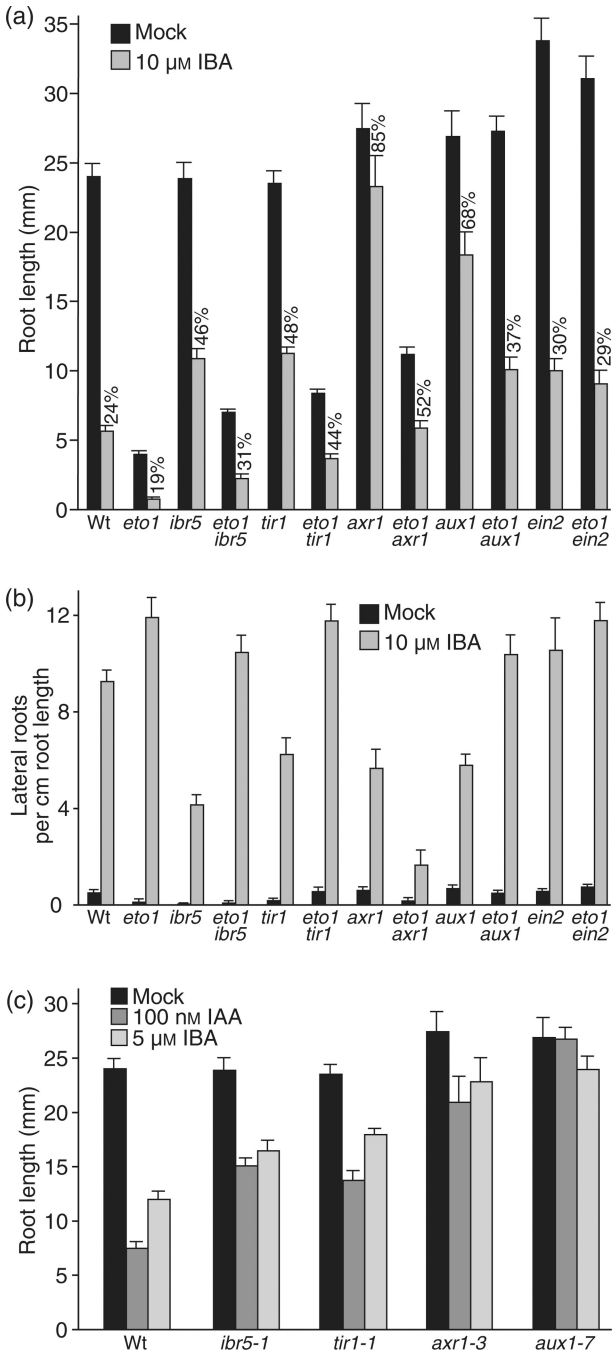

Figure 3. *eto1* partially restores auxin responsiveness to auxin-resistant mutants

(a) Mean primary root lengths of Col-0 (Wt), *eto1-1, ibr5-1, eto1-1 ibr5-1, tir1-1, eto1-1 tir1-1, axr1-3, eto1-1 axr1-3, aux1-7, eto1-1 aux1-7, ein2-1* and *eto1-1 ein2-1* seedlings grown under yellow-filtered light at 22°C on medium supplemented with ethanol (mock) or 10 μM indole-3-butyricacid (IBA; $n \geq 15$; error bars indicate SE). The percentage of root length of seedlings grown in the presence of 10 μM IBA compared with control seedlings is indicated above the bars. Root lengths of *eto1 ibr5, eto1 tir1, eto1 axr1* and *eto1 aux1* were significantly shorter than the root lengths of the respective auxin-resistant parent when grown in the presence of 10 μM IBA ($P \leq 0.0001$ in two-tailed Student's t-tests assuming unequal variance).

(b) Mean numbers of emerged lateral roots per centimeter root length of Col-0 (Wt), *eto1-1, ibr5-1, eto1-1 ibr5-1, tir1-1, eto1-1 tir1-1, axr1-3, eto1-1 axr1-3, aux1-7, eto1-1 aux1-7, ein2-1* and *eto1-1 ein2-1* seedlings 4 days after transfer of 4-day-old seedlings to medium supplemented with ethanol (mock) or 10 μM IBA ($n \geq 10$; error bars indicate SE). The number of emerged lateral roots per cm root length in *eto1* was significantly higher than the number in the wild type when grown in the presence of 10 μM IBA ($P \leq 0.01$ in two-tailed Student's *t*-tests assuming unequal variance). The number of emerged lateral roots per cm root length in *eto1 ibr5,eto1 tir1 and eto1 aux1* was significantly greater than the number for the respective auxin-resistant parent when grown in the presence of 10 μM IBA ($P \leq 0.0001$ in two-tailed Student's *t*-tests assuming unequal variance).
(c) Mean primary root lengths of Col-0 (Wt), *ibr5-1, tir1-1, axr1-3 and aux1-7* seedlings grown under yellow-filtered light at 22°C on medium supplemented with ethanol (mock) or the indicated auxins ($n \geq 15$; error bars indicate SE).

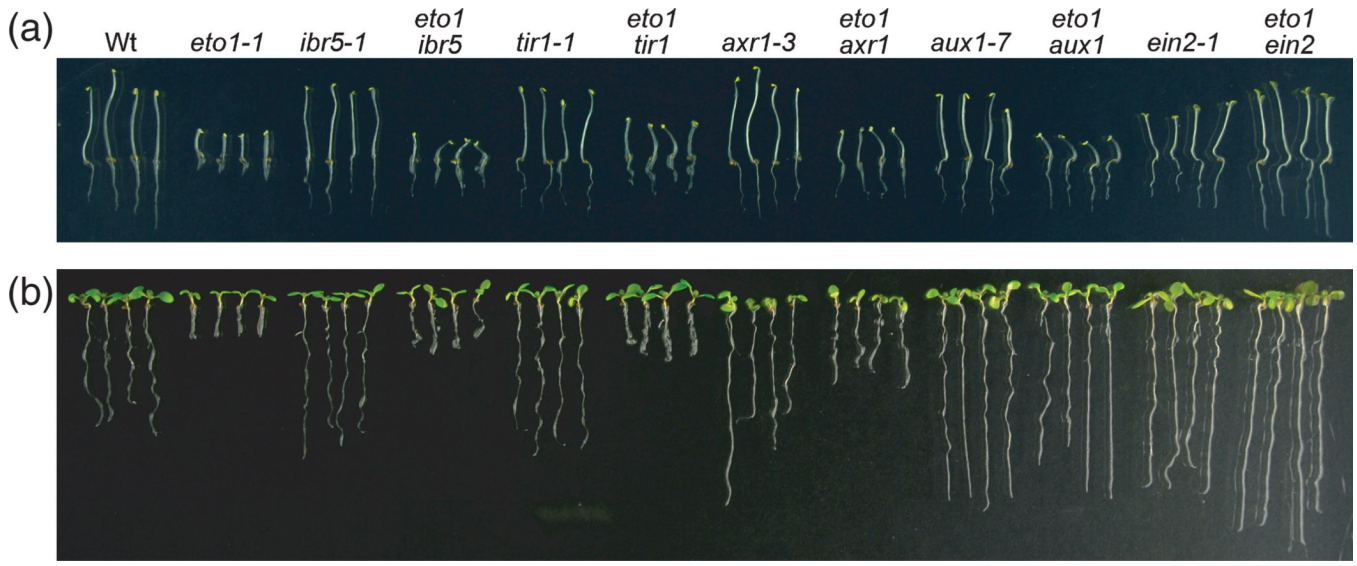

Figure 4. Blocking auxin response or influx restores *eto1* root phenotypes

Five-day-old dark-grown (a) and 8-day-old light-grown (b) Col-0 (Wt), *eto1-1, ibr5-1, eto1-1 ibr5-1, tir1-1, eto1-1 tir1-1, axr1-3, eto1-1 axr1-3, aux1-7, eto1-1 aux1-7, ein2-1 and eto1-1 ein2-1* seedlings representing the range of observed pheno-types.

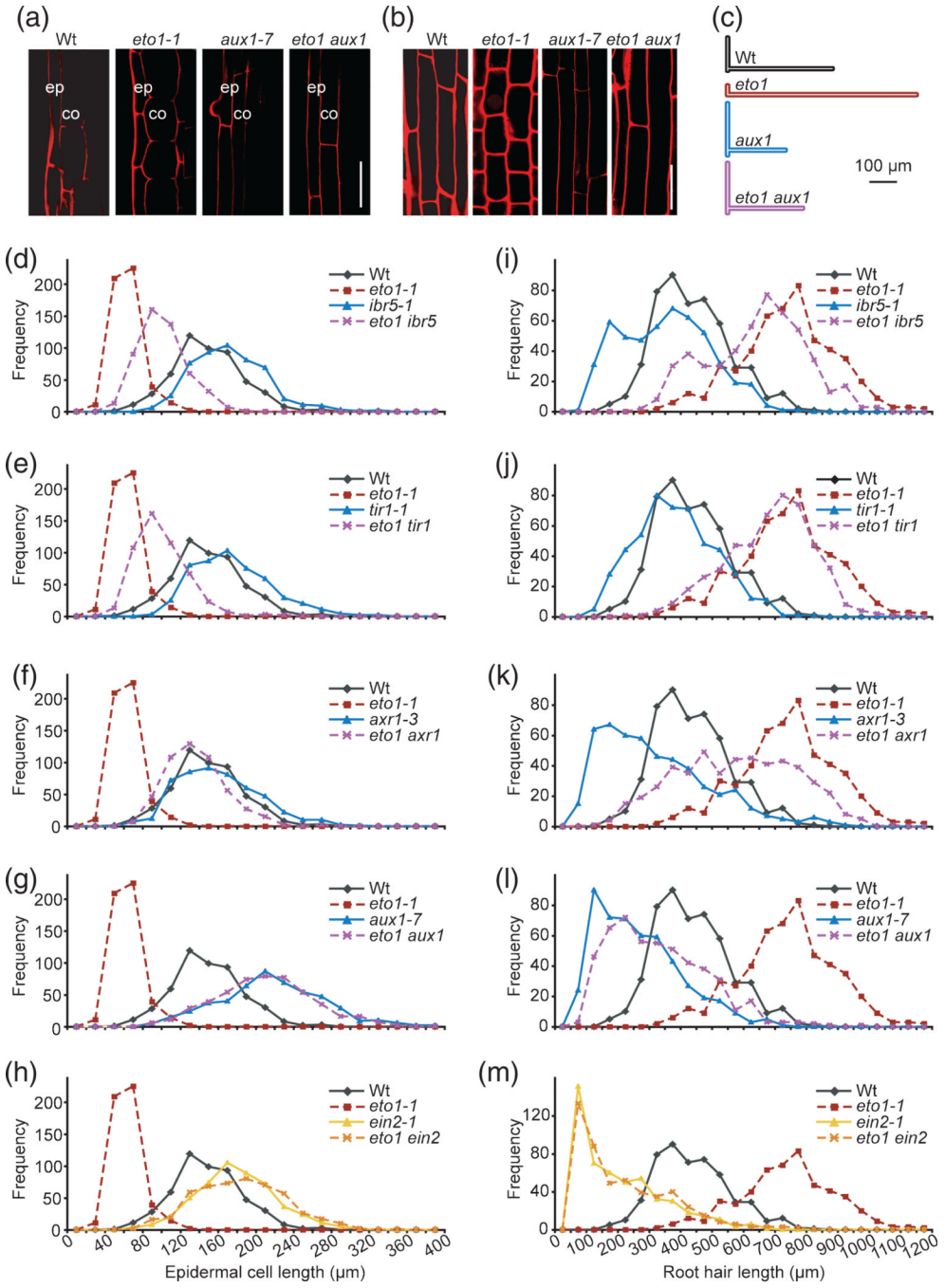

Figure 5. Blocking auxin response or influx restores *eto1* root cell elongation phenotypes

(a, b) Confocal images of differentiated root cells stained with propidium iodide from 8-day-old Col-0 (Wt), *eto1-1, aux1-7* and *eto1-1 aux1-7* seedlings. (a) Longitudinal section of the root showing epidermal (ep) and cortex (co) cells. Scale bar: 50 µm.

(b) Longitudinal section of epidermal cells. Scale bar: 50 µm.

(c) Schematic depicting mean dimensions of root hair cells from Col-0 (Wt), *eto1-1, aux1-7 and eto1-1 aux1-7* seedlings. Mean epidermal cell lengths of *eto1 were* significantly shorter, and cell lengths of *aux1* were significantly longer, than those of the wild type ($P \leq 0.0001$ in two-tailed Student's *t*-tests assuming unequal variance). Mean epidermal cell lengths of *eto1 aux1* were significantly longer than those of *eto1* ($P \leq 0.0001$ in two-tailed Student's *t*-tests

assuming unequal variance). Mean root hair lengths of *eto1* were significantly longer, and root hair lengths of *aux1* were significantly shorter, than those of the wild type ($P \leq 0.0001$ in two-tailed Student's *t*-tests assuming unequal variance). Mean root hair lengths of *eto1 aux1* were significantly shorter than mean root hair lengths of *eto1* ($P \leq 0.0001$ in two-tailed Student's *t*-tests assuming unequal variance).

(d–h) A decreased auxin response or influx suppresses *eto1* inhibition of root cell elongation. Histograms show epidermal cell lengths of 8-day-old Col-0 (Wt) seedlings compared with the indicated single and double mutants. For each genotype, 500 cells from ~12 seedlings were measured. The same wild-type and *eto1-1* data are depicted in each panel for ease of comparison.

(i–m) Decreased auxin response or influx suppresses *eto1* promotion of root hair elongation. Histograms show root hair lengths of 5-day-old Col-0 (Wt) seedlings compared with the indicated single and double mutants. For each genotype, 500 root hairs from ~12 seedlings were measured. The same wild-type and *eto1-1* data are depicted in each panel for ease of comparison.

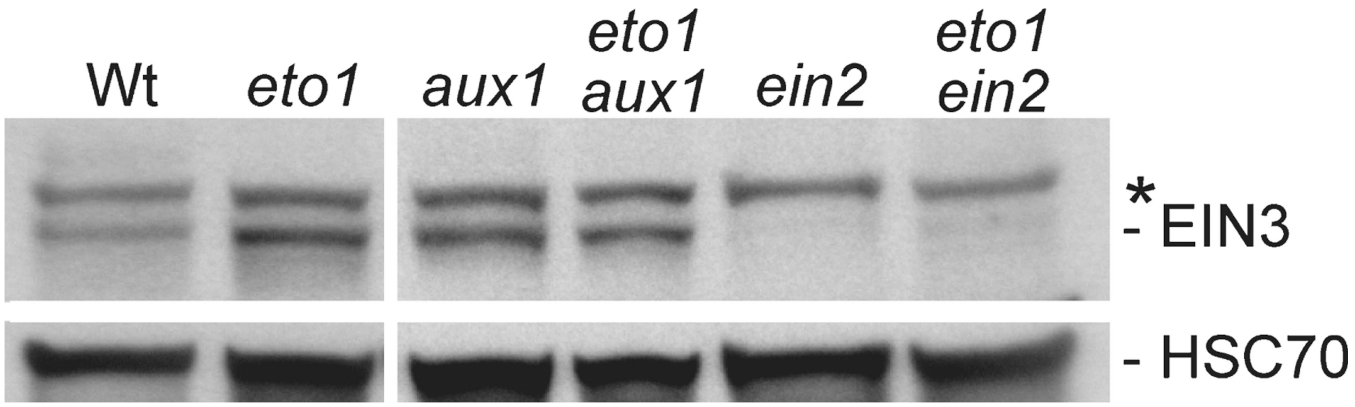

Figure 6. EIN3 protein accumulates in *eto1 aux1*

Anti-EIN3 (top panels) and anti-HSC70 (bottom panels) antibodies were used to probe immunoblots of protein prepared from 5-day-old light-grown Col-0 (Wt), *eto1-1, aux1-7, eto1-1 aux1-7, ein2-1* and *eto1-1 ein2-1* seedlings. The asterisk marks a protein that cross-reacts with the EIN3 antibody.

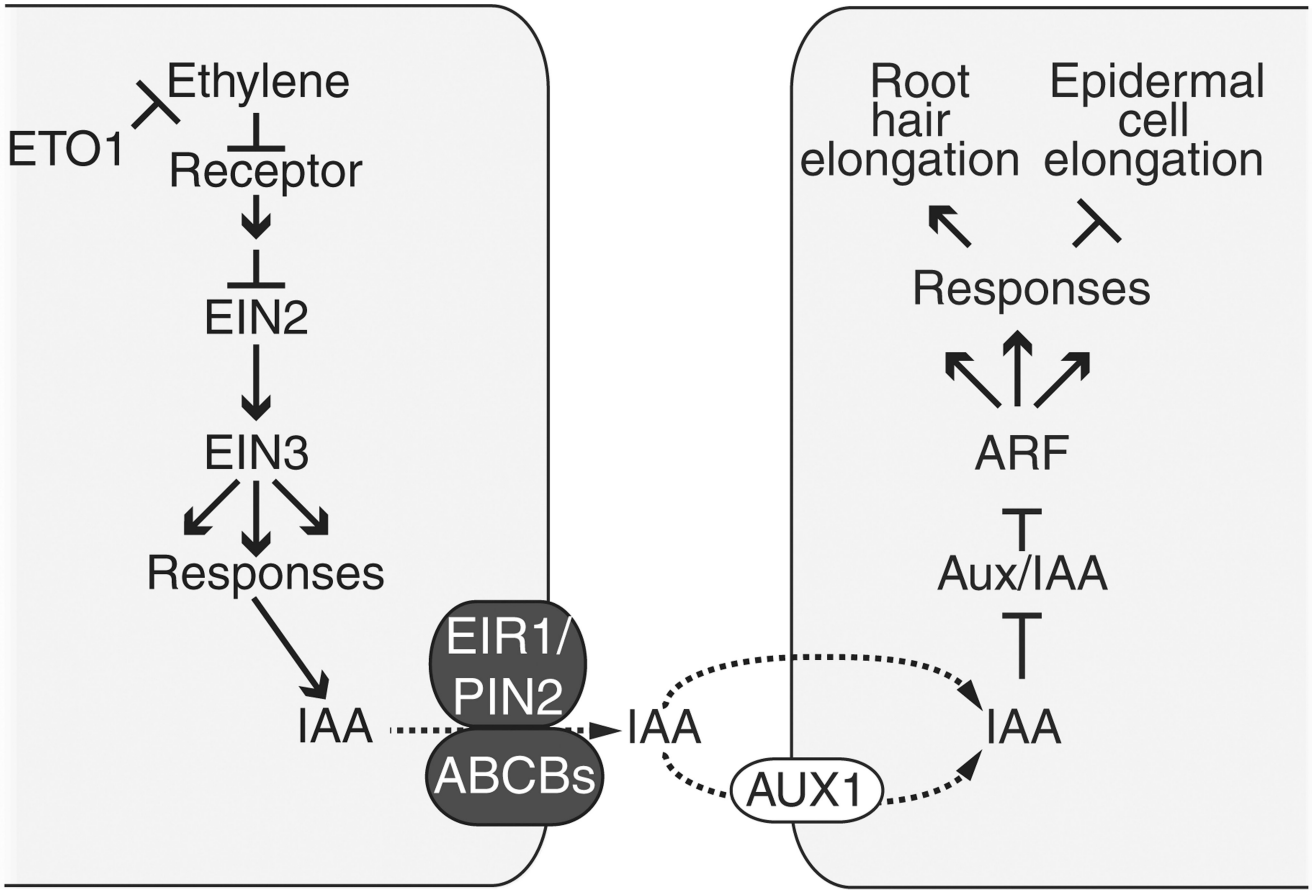

Figure 7. Model for ethylene–auxin interactions controlling root cell expansion

Ethylene promotes auxin biosynthesis (Swarup *et al.*, 2007) and/or auxin transport (Růžička *et al.*, 2007) to direct auxin in the epidermal cell layer to inhibit root epidermal cell elongation and promote root hair elongation. This process is dependent on atrichoblast-localized AUX1 (Pitts *et al.*, 1998; Jones *et al.*, 2009), suggesting that this increased auxin biosynthesis or transport may occur in cells other than the trichoblast.

Table 1

Mutant alleles used in this study

Allele	Gene	Gene product	Original isolation	References
eto1-1	*At3g51770*	BTB protein	Ethylene overproducing	Guzman and Ecker (1990); Wang *et al.* (2004)
eto1-12 (SALK_061581)	*At3g51770*	BTB protein	Reverse genetics	Gingerich *et al.* (2005)
eto1-34	*At3g51770*	BTB protein	Suppressor of *ibr5* IBA resistance	Strader *et al.* (2008b)
eto2-1	*At5g65800*	ACC synthase	Ethylene overproducing	Kieber *et al.* (1993); Vogel *et al.* (1998)
ibr5-1	*At2g04550*	MAP kinase phosphatase	IBA resistance	Zolman *et al.* (2000); Monroe-Augustus *et al.* (2003)
tir1-1	*At3g62980*	F-box protein auxin receptor	Auxin transport inhibitor resistance	Ruegger *et al.* (1998)
axr1-3	*At1g05180*	Subunit of RUB-activating enzyme	2,4-D resistance	Estelle and Somerville (1987); Leyser *et al.* (1993)
aux1-7	*At2g38120*	Auxin influx carrier	2,4-D resistance	Maher and Martindale (1980); Bennett *et al.* (1996)
ein2-1	*At5g03280*	Transmembrane protein	Ethylene resistance	Roman *et al.* (1995); Alonso *et al.* (1999)