Ethylene inhibits lateral root development, increases IAA transport and expression of PIN3 and PIN7 auxin efflux carriers

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SUMMARY

We used genetic and molecular approaches to identify mechanisms by which the gaseous plant hormone ethylene reduces lateral root formation and enhances polar transport of the hormone auxin. *Arabidopsis thaliana* mutants, *aux1*, *lax3*, *pin3* and *pin7*, which are defective in auxin influx and efflux proteins, were less sensitive to the inhibition of lateral root formation and stimulation of auxin transport following treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). By contrast, *pin2* and *abcb19* mutants exhibited wild-type ACC responses. ACC and indole-3-acetic acid (IAA) increased the abundance of transcripts encoding auxin transport proteins in an ETR1 and EIN2 (ethylene signaling)-dependent and TIR1 (auxin receptor)-dependent fashion, respectively. The effects of ACC on these transcripts and on lateral root development were still present in the *tir1* mutant, suggesting independent signaling networks. ACC increased auxin-induced gene expression in the root apex, but decreased expression in regions where lateral roots form and reduced free IAA in whole roots. The ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG) had opposite effects on auxin-dependent gene expression. These results suggest that ACC affects root development by altering auxin distribution. PIN3- and PIN7-GFP fluorescence was increased or decreased after ACC or AVG treatment, respectively, consistent with the role of PIN3 and PIN7 in ACC-elevated transport. ACC treatment abolished a localized depletion of fluorescence of PIN3- and PIN7-GFP, normally found below the site of primordia formation. These results suggest that ACC treatment increased PIN3 and PIN7 expression, resulting in elevated auxin transport, which prevented the localized accumulation of auxin needed to drive lateral root formation.

KEY WORDS: PIN3, PIN7, Auxin, Auxin transport, Ethylene, Lateral roots

INTRODUCTION

The process of lateral root formation begins when quiescent pericycle cells of primary roots are activated and undergo a precise series of divisions leading to formation of a new lateral root meristem. Lateral roots might undergo additional branching to form higher order roots, which ultimately defines root system architecture and overall plant fitness (Lynch, 1995). The best characterized signal modulating this process is auxin, of which indole-3-acetic acid (IAA) is the most abundant molecular species. Auxin stimulates lateral root initiation by activating quiescent pericycle cells to initiate division and then expansion to facilitate lateral root emergence (Fukaki and Tasaka, 2009). Appropriate synthesis, signaling and transport of auxin are all required for root formation (Peret et al., 2009).

Auxin is synthesized at the shoot and root apices and transported with a single basipetal, or rootward, polarity in the shoot (Baskin et al., 2010; Zazimalova et al., 2010). In roots, there are two auxin transport polarities localized to distinct tissue layers that are linked to distinct developmental and physiological processes. Shootward (or basipetal) transport through the epidermal cell file is tied to

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gravitropism (Chen et al., 1998; Lewis et al., 2007; Rashotte et al., 2000) and root elongation, whereas rootward (acropetal) transport through the center cylinder of the root provides the auxin essential for lateral root emergence and the subsequent elongation or growth of these roots (Bhalerao et al., 2002; Reed et al., 1998). Inhibition of auxin transport by mutations or chemical inhibitors blocks both initiation (Casimiro et al., 2001) and elongation of lateral roots (Reed et al., 1998).

IAA transport is mediated by influx proteins such as AUXIN RESISTANT 1 (AUX1) and Like AUX (LAX1, 2 and 3) (Bennett et al., 1996; Marchant et al., 2002; Marchant et al., 1999; Swarup et al., 2008), and by efflux proteins such as PIN FORMED 1 (PIN1) and ATP BINDING CASSETTE B 19/P-GLYCOPROTEIN 19/MULTIDRUG RESISTANT 1 (ABCB19/PGP19/MDR1) (Galweiler et al., 1998; Noh et al., 2001; Teale et al., 2006). Defects in AUX1, LAX3, PIN1 and ABCB19 negatively impact initiation and/or elongation of lateral roots due to reduced movement of auxin (Benkova et al., 2003; Marchant et al., 2002; Swarup et al., 2008; Wu et al., 2007). Additionally, changes in the abundance and localization of auxin transport proteins may define the position of lateral root formation (Zazimalova et al., 2010). The PIN auxin efflux proteins have distinct expression patterns in lateral root primordia (LRP), which change during lateral root development (Benkova et al., 2003; Sauer et al., 2006). PIN3- and PIN7-GFP fluorescence was reduced below the site of lateral root initiation, whereas AUX1-YFP was increased at the point of root formation (Laskowski et al., 2008). These results suggest that regulation of proteins that mediate auxin transport creates local auxin maxima, which specify the location of lateral root development. Consistent with this model, regions of expression of an auxin responsive

luciferase construct (DR5:*LUC*) mark the position of future lateral roots at a pre-branch site (Moreno-Risueno et al., 2010). In addition, localized IAA accumulation by sporadic expression of the *iaaM* gene under the control of a *cre-lox* system specified the site of pericycle cell differentiation into lateral root founder cells (Dubrovsky et al., 2008).

Recent genetic studies in *Arabidopsis* and tomato have shown that the gaseous plant hormone ethylene inhibits lateral root formation (Ivanchenko et al., 2008; Negi et al., 2008; Negi et al., 2010). Mutations in *CTR1*, which confer constitutive ethylene signaling (Huang et al., 2003; Kieber et al., 1993), and the ethylene overproducing *eto1* mutant (Guzman and Ecker, 1990), both negatively affect root branching (Negi et al., 2008; Strader et al., 2010). Additionally, treatment with ethylene or the ethylene precursor, 1-aminocyclopropane carboxylic acid (ACC) reduces lateral root formation in both species (Negi et al., 2008; Negi et al., 2010). By contrast, elevated numbers of lateral roots are produced by *ethylene resistant 1* (*etr1*; also known as *ein1*) and *Never-ripe* (*Nr*) (Negi et al., 2008; Negi et al., 2010), which are dominant-negative ethylene receptor mutations in *Arabidopsis* and tomato, respectively (Hua et al., 1998; Wilkinson et al., 1995; Yen et al., 1995), and *ethylene insensitive 2* (*ein2*), which causes a defect in an ethylene signaling protein (Alonso et al., 1999). XBAT32, an E3 ubiquitin ligase that functions in proteolysis, increases lateral root number by reducing the abundance of two ACC synthase isoenzymes that function in ethylene biosynthesis (Prasad et al., 2010). Ethylene affects root branching at the earliest stages of lateral root initiation (Ivanchenko et al., 2008) and alters auxin transport, suggesting that cross talk with auxin might regulate ACC repression of root branching (Negi et al., 2008; Negi et al., 2010).

Auxin-ethylene crosstalk regulates root elongation (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007). The effects of the interactions of these hormones on elongation of the primary root are fundamentally different from those of lateral root formation, as they act synergistically to reduce primary root elongation, but antagonistically in lateral root formation. Screens for mutants with reduced ethylene-inhibited primary root elongation identified auxin transport mutants, such as *aux1* and *eir1* (a *pin2* allele) (Luschnig et al., 1998; Roman et al., 1995) and auxin synthesis mutants, including *weak ethylene insensitive* (*wei2, 7* and *8*) (Stepanova et al., 2005; Stepanova et al., 2007). Treatments with ACC and ethylene increased free IAA and IAA synthesis at the root tip (Ruzicka et al., 2007; Swarup et al., 2007), with a requirement for functional ethylene signaling proteins, EIN2 and ETR1, and auxin signaling proteins, ARF19/NPH4 and AXR1, AXR2, AXR3 and AXR4 (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007). Ethylene treatment elevated fluorescence of AUX1 and PIN2 fluorescent protein fusions at the root tip. This suggests increased auxin synthesis and reduced auxin transport contribute to the elevation of auxin signaling that mediates the reduction in growth after ACC treatment (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007). Ethylene also inhibits other auxin-dependent process including hypocotyl and root gravity response (Buer et al., 2006; Li, 2008; Muday et al., 2006), root waving (Buer et al., 2003) and hypocotyl hook opening (Vandenbussche et al., 2010; Zadnikova et al., 2010). By contrast, root hair development is stimulated by both auxin and ethylene (Rahman et al., 2002).

This study explores the mechanism of the antagonistic, rather than synergistic, cross-talk between ethylene and auxin in the control of lateral root development. We measured gene expression, auxin-transport protein localization, and free IAA levels to identify

ACC-mediated changes linked to repression of root branching. We identified auxin transport proteins whose regulation contributes to this process, finding important roles for PIN3 and PIN7. We also used auxin and ethylene signaling mutants to uncover distinct hormonal signaling pathways that control expression of these genes. These analyses suggest a model in which auxin transport is enhanced by ACC, which prevents the localized accumulation of IAA that is needed to drive lateral root formation.

MATERIALS AND METHODS
Plant material and growth conditions
Arabidopsis thaliana etr1-3, *ein2-5*, *aux1-7*, DR5rev:GFP and *eto1-1* mutants have been described previously (Buer et al., 2006; Negi et al., 2008; Sukumar et al., 2009). Seeds were provided by Malcolm Bennett, University of Nottingham, UK (*lax3-1*, *aux1-7/lax3-1* and AUX1-YFP) (Swarup et al., 2008), Marta Laskowski, Oberlin College, Oberlin, OH, USA (*pin3-1*, *pin7-1*, PIN3-GFP, PIN7-GFP and DR5:vYFP) (Laskowski et al., 2008) and Mark Estelle, University of California, San Diego, CA, USA (*tir1-1*). Wild-type seeds were of the Columbia (Col) ecotype. Surface-sterilized seeds were sown on control medium: 0.8% (w/v) agar, MS nutrients (macro and micro salts), vitamins (Murashige and Skoog, 1962), 1.5% (w/v) sucrose, 0.05% (w/v) MES, pH 5.6, and stratified for 2 days at 4° C. Seedlings were then grown on unsealed plates held vertically under constant fluorescent lighting at 100 μmol/m^2/second at 23°C. IAA and napthaleneacetic acid (NAA) treatments were performed under yellow filters to prevent auxin degradation (Stasinopoulos and Hangarter, 1989).

Lateral root quantification
Five-day-old seedlings were transferred to new media, containing either no additions or the indicated amounts of 1-naphthoxyacetic acid (NOA), ACC, NAA, naphthylphthalamic acid (NPA), aminoethoxyvinylglycine (AVG) or IAA. Concentrated stocks of 10 or 30 mM of these additives in ethanol (NAA and IAA), water (ACC and AVG) or DMSO (NOA and NPA) were added to agar medium cooled to 50°C. The number of emerged lateral roots was quantified under a dissecting microscope after an additional 5 days of growth.

Confocal microscopy
For all confocal microscopy, roots were stained with 25 μg/ml propidium iodide. Images were captured with a Zeiss 710 meta laser scanning confocal microscope (LSCM; Zeiss, Jena, Germany). Gain and pinhole settings were held constant for comparisons. For the analysis of auxin-dependent changes in gene expression in the whole root (DR5-GFP or DR5-vYFP shown in Figs 1 and 2), plants were grown on control medium for 5 days, then transferred to media containing the indicated concentrations of IAA, NAA, ACC, AVG or combinations thereof. After 5 days (Fig. 1A) or 3 days (Fig. 2A), tiled micrographs were collected.

For the micrographs in Fig. 1C, a 24-hour ACC or AVG treatment of DR5*rev*:GFP-expressing roots was followed by imaging at 20× in a z-stack configuration, with 8.5 μm optical sections through the entire root tip and a pinhole of 1 AU. The central slice and a maximum projection of the same root are shown in Fig. 1C. For the analysis of AUX1-, PIN3- and PIN7-fluorescent protein constructs, the pinhole diameter was set to 1.5 AU for Fig. 6A and 2.7 AU for Fig. 6B.

For the analysis of AUX1, PIN3 and PIN7 in bent roots, 24 hours after ACC treatment roots were bent at 90°, at 3 mm from the root tip. Eight hours after roots were bent, transport protein reporter constructs were examined as described above. In all situations where fluorescence was quantified, a region of interest was drawn over an equivalently sized area located a set distance from a landmark (root apex, LRP or bend apex); *n*>12.

Auxin transport assays
Seedlings were germinated on control medium and transplanted to control or treatment plates on the fifth day after sowing. After 24 hours of treatment, an agar droplet containing 100 nM [³H]IAA (Amersham, Buckinghamshire, UK) was applied below the aligned root-shoot junctions. After 18 hours, the amount of radioactivity was quantified in a 5 mm apical

segment using previously described methods (Lewis and Muday, 2009). Because the *eto1* roots were substantially shorter, we also quantified radioactivity in the whole root and middle segments. All segments had similar elevations in IAA accumulation (data not shown).

RNA isolation for qRT-PCR comparison of transcript abundance
RNA was isolated from seedlings grown on a nylon screen (03-100/32 Sefar Filtration, Depew, NY) as described previously (Levesque et al., 2006). On the sixth day after sowing, the nylon filter and attached plants were transferred to a control plate or to media supplemented with 1 µM ACC or IAA for 6 hours. The roots were excised, excluding the root-shoot junction. Samples containing 900 ng RNA were used for cDNA synthesis with a 1:1 mixture of oligo(dT) and random hexamer primers. Quantitative real-time PCR analysis of RNAse-digested cDNA was performed on an Applied Biosystems 7600-fast thermal cycler using SYBR Green detection.

Primers were designed with primer3 primer design software (Rozen and Skaletsky, 2000). A standard curve verified that all primer sets were linear over a 1000-fold concentration range. The efficiency coefficient E (Pfaffl, 2001) was calculated for all primer sets by plotting the relationship between the C_t value and log[cDNA]. E-values were as follows: *ACT2*=1.925, *PIN3*=2.022, *PIN7*=2.094, *AUX1*=1.908. The transcript levels of the reference gene for actin, did not vary by more than 1 C_t between treatments. All qRT-PCR values are from three biological replicates with three technical replicates each. Efficiency-corrected relative expression measured in fold increase over untreated control was calculated using an efficiency-corrected $\Delta\Delta C_t$ formula (Pfaffl, 2001). The sequence of the primers were: actin gene, forward (F) 5′-TGAGAGATTCAGATGC-CCAGAA-3′, actin gene, reverse (R) 5′-GCAGCTTCCATTCCCACAA-3′; *AUX1* F 5′-CAGCCGCCGCACATG-3′, *AUX1* R 5′-ACCCTGACTC-GATCTCTCAAAGA-3′; *PIN3* F 5′-CCCAGATCAATCTCACAACG-3′, *PIN3* R 5′-TTCTCCTCCGAAATCTCCAC-3′; *PIN7* F 5′-CATTGCCAT-ACCAAGACCAG-3′, *PIN7* R 5′-GCTCTTGTTGCTTTCAGGTG-3′.

Free IAA quantification by gas chromatography-mass spectroscopy
For quantification of free IAA levels, 24 hours after control or ACC treatment 5-day-old roots were excised and frozen in liquid nitrogen. 50-80 mg of frozen root tissue was homogenized with 4 ng of [$^{13}C_6$]IAA as an internal standard (Barkawi et al., 2008). The homogenates were incubated for 1 hour, centrifuged, purified over two successive columns using an automated robotic system, methylated, dried and re-dissolved in ethyl acetate (Barkawi et al., 2008). The samples were then analyzed using gas chromatography-selected ion monitoring-mass spectroscopy (GC-SIM-MS). The free IAA was quantified by isotope dilution analysis using the [$^{13}C_6$]IAA internal standard. Values are reported relative to fresh weight, in ng/gFW.

RESULTS
ACC reduces lateral root formation and auxin-dependent gene expression
We examined the effect of changing ethylene levels on auxin-induced gene expression along an entire *Arabidopsis* root. The fluorescence of the DR5rev:GFP reporter in the presence of a propidium iodide counter stain was examined 3 days after transfer to media with and without 1 µM ACC or AVG, in order to raise or lower ethylene levels, respectively. Images were captured using a LSCM set on tile mode to collect multiple images along the root, observed at confocal resolution (Fig. 1A). The position of the root apex at the time of transfer to new medium is indicated by an arrowhead. These images illustrate the complete inhibition of lateral root formation in the region of the root that formed after ACC treatment (below the arrowhead), the decrease in DR5 activity along the entire root, as well as the reduced elongation caused by elevated ethylene levels. By contrast, AVG treatment lead to slightly higher fluorescence in the region of lateral root formation and increased LRP formation.
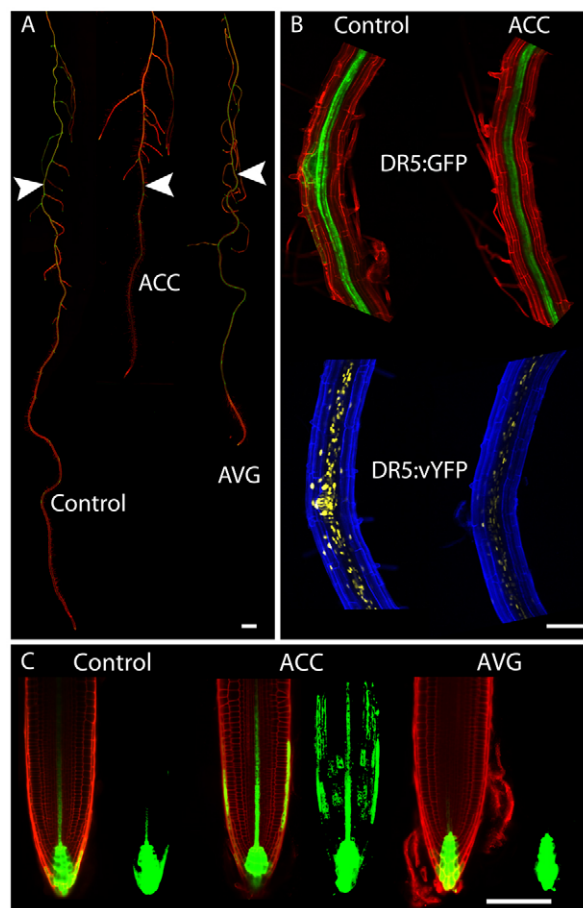
Fig. 1. ACC reduces DR5rev:GFP expression and lateral root formation. (A) DR5rev:GFP roots imaged 5 days after transfer to control medium or media containing 1 µM AVG or ACC, with roots aligned at the root shoot junction. Propidium iodide (PI) counterstain is shown in red. Arrowheads indicate the position of the root apex at the time of transfer to new medium. Scale bar: 1 mm. (B) After 5 days of growth, DR5:revGFP and DR5:vYFP seedlings were transferred to control or ACC plates. After 24 hours of treatment, roots were bent 90° 3 mm from the root tip and micrographs were taken of the bent region after 8 hours. PI is shown in red for DR5rev:GFP and blue for DR5:vYFP. Representative images from at least six individuals per marker and per treatment are shown. Scale bar: 100 µm. (C) Representative micrograph of DR5rev:GFP-expressing seedlings 24 hours after transfer to control, AVG- or ACC-containing media. Cross sections and a z-stack of the same roots are shown with and without PI. Scale bar: 100 µm.

We examined the effect of ACC on auxin-induced gene expression at higher magnification in regions of the root forming lateral roots. Lateral roots form when primary roots are bent, either as a result of asymmetric growth or manual bending (Ditengou et al., 2008; Laskowski et al., 2008; Richter et al., 2009). The auxin responsive promoter DR5:vYFP is expressed at the earliest stages of lateral root development at the bend (Laskowski et al., 2008). Using both this reporter and DR5:revGFP, we saw strong expression in LRP at all stages, on the convex side of all bent roots (Fig. 1B). ACC treatment blocked the initiation of lateral roots at the bend and prevented formation of the auxin maxima that precede lateral root initiation, suggesting ethylene acts downstream of pre-branch site specification, as negative regulation of root initiation still

occurs in regions of the root that were allowed to form prebranch sites under normal conditions. By contrast, ACC treatment increased auxin-induced gene expression in the root apex (Fig. 1C), consistent with previous reports (Negi et al., 2008; Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007), whereas AVG reduced apical DR5:revGFP fluorescence. These results indicate that in lateral-root-forming regions of the primary root, elevated ethylene levels reduce auxin-induced gene expression and root formation, whereas reduced ethylene levels result in the opposite effects.
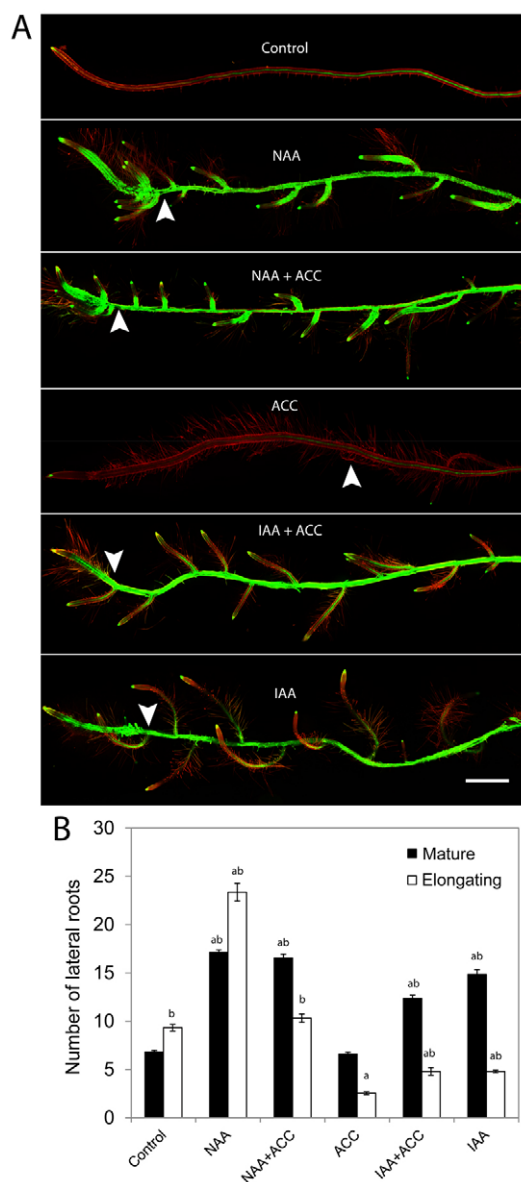
NAA, but not IAA, reverses the inhibition of root formation by ACC

Lateral roots were examined in the presence and absence of auxin to determine whether auxin rescued the ACC repression of root branching. We used two auxins with different specificity for auxin transport proteins. IAA is a substrate for both influx and efflux proteins, whereas the synthetic auxin, NAA, is specific for efflux proteins, as shown by its ability to move into plant cells in influx-carrier-defective mutants, reversing the mutant phenotypes (Parry et al., 2001; Yamamoto and Yamamoto, 1998). Both auxins enhanced DR5rev:GFP fluorescence all along the primary root including the apices of both primary and lateral roots (Fig. 2A). By contrast, at the base of the lateral roots, NAA increased DR5rev:GFP fluorescence more than IAA.

Both auxins increased the number of lateral roots and reduced root elongation (Fig. 2A,B). Lateral roots proliferated in the region formed shortly after transfer to NAA, but not IAA. NAA increased lateral root formation in both the mature and elongating regions (above and below the arrowhead), whereas IAA stimulated root formation only in the mature region. In fact, IAA led to a reduction in root formation in the region formed after transfer, consistent with another recent report (Ivanchenko et al., 2010). Treatment with ACC in addition to either NAA or IAA had no effect in the mature region relative to the auxin only treatment. In the elongating region, NAA and ACC co-treatment had an intermediate effect, whereas IAA and ACC together produced the same number of roots as IAA alone. The ability of NAA, but not IAA, to partially reverse the effect of ACC in the elongating region suggests that limited synthesis of influx carrier proteins in this region after ACC treatment might restrict auxin uptake needed to stimulate lateral root formation.

Free IAA levels in whole roots are reduced when ethylene levels are elevated in *eto1*

We quantified free IAA in whole wild-type roots treated with 1 μM ACC for 24 hours, and in the roots of the ethylene-overproducing mutant, *eto1-1*, and ethylene signaling mutants, *ein2-5* and *etr1-3* (Fig. 3A). There was a reduction in free IAA content in the whole root when ethylene levels were elevated genetically or pharmacologically. This decrease was significant in the *eto1-1* mutant, but not after exogenous ACC treatment. In addition, we found that mutations in *ETR1* and *EIN2* lead to slight, but not significant increases in free IAA content in whole roots. These results differ from those of previous studies that observed an increase in free IAA levels in root tip after ethylene treatment, and tip-localized ACC-mediated increases in auxin signaling (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007). Localized increases in free IAA at the root tip might have occurred, but could have been masked by reduced IAA levels in the more mature regions of the root. We also quantified the free IAA levels in the *aux1* mutant, which has fewer lateral roots than wild type

Fig. 2. NAA, but not IAA, reverses the inhibition of root formation by ACC. (**A**) DR5rev:GFP roots imaged 3 days after transfer to control medium, or media containing 1 μM of ACC, IAA or NAA, or a combination. The position of the root apex at the time of transfer is indicated with an arrowhead. Scale bar: 1 mm. (**B**) Col seedlings were grown on control medium and transferred to media containing IAA, NAA, ACC or a combination for 5 days. After an additional 5 days the number of lateral roots was quantified in the region of the root formed before transfer (mature region), and in the region of the root formed after transfer (elongating region). Values are mean±s.e.m., n=20. [a]Significant difference between control and treatment as judged by a Student's t-test (P<0.05); [b]significant difference relative to ACC-only treatment.

(Marchant et al., 2002) and is less responsive to the inhibition of lateral root formation by ACC treatment (Negi et al., 2008). Although there was less free IAA in the *aux1* mutant, there was no significant change in free IAA content with ACC treatment in *aux1* roots (Fig. 3B). The data shown in Fig. 1C are consistent with elevated IAA signaling and synthesis at the root tip after ethylene treatment. However, the results presented in Fig. 3 suggest that neither free IAA, nor auxin signaling is elevated in the entire root
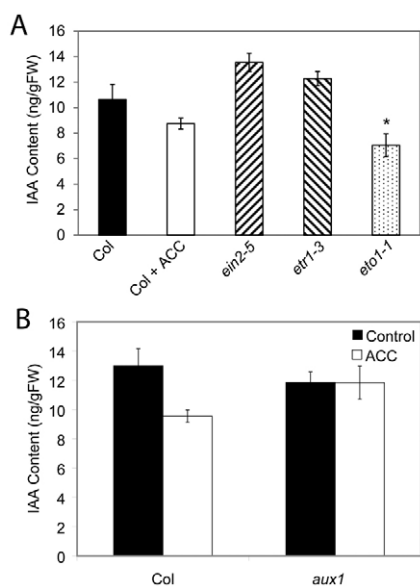
Fig. 3. Free IAA is reduced in whole *Arabidopsis* roots by ACC treatment in an AUX1-dependent manner. (**A**) Free IAA was quantified in 5-day-old Col seedlings with and without the addition of ACC for 24 hours, and in untreated *ein2*, *etr1* and *eto1-1* mutants. *Significant difference relative to untreated Col seedlings as judged by a Student's *t*-test (*P*<0.05). (**B**) Free IAA was quantified in 5-day-old Col and *aux1* seedlings with and without ACC treatment. Values are mean±s.e.m., *n*≥3.

upon ACC treatment. Therefore, the effect of ACC on root branching might result from altered auxin distribution, mediated by regulation of IAA transport proteins.

Auxin influx inhibitors abolish the effects of ethylene on lateral root development and auxin transport

We utilized three strategies to separate the roles of auxin influx and efflux activity in ACC suppression of LRP development: auxins that bypass auxin influx carriers, inhibitors that are selective for influx and efflux carriers, and a diversity of auxin transport mutants. We examined the effect of ACC treatment on lateral root formation and auxin transport in the presence and absence of NOA and NPA, compounds that specifically block IAA influx and IAA efflux, respectively (see Fig. S1A in the supplementary material). Both inhibitors reduced the number of lateral roots, but produced different responses to combined treatment with ACC. ACC treatment further reduced the inhibition in root formation by NPA. By contrast, NOA treatment combined with ACC treatment, or the ethylene overproducing mutant, *eto1-1* (Bleecker et al., 1998; Guzman and Ecker, 1990), yielded identical inhibition of lateral root formation to NOA added alone. The ethylene-insensitive mutants *etr1* and *ein2* show reduced responses to NOA treatment. In addition, the elevation of auxin transport by ACC was substantially reduced upon treatment with NOA (see Fig. S1B in the supplementary material), whereas NPA did not prevent the enhancement of auxin transport by ACC (see Fig. S1C in the supplementary material). These results provide strong evidence for a role of auxin influx in the response to ACC, but a more complex role of auxin efflux in this process, with NPA-sensitive and insensitive pathways differentially contributing to the effect of ACC on root

branching and auxin transport. These observations led directly to the analysis of specific auxin transport mutants, as detailed in the following section.

AUX1, LAX3, PIN3 and PIN7 proteins play roles in ACC-inhibited root formation and ACC-enhanced auxin transport

We examined the effect of ACC on root branching in auxin influx and efflux carrier mutants to identify the proteins required for the ACC effect on root architecture and auxin transport. We observed substantial variation between genotypes in the number, position and length of lateral roots, as well as the growth orientation and rates of elongation of these seedlings under control conditions (Fig. 4A). The number of lateral roots formed by each genotype in the absence and presence of ACC was quantified (Fig. 4B; see Fig. S2 in the supplementary material) and analyzed by a two-way between groups ANOVA. The differences between genotype (F=148.72, P<10^{-3}) and by ACC treatment (F=267.45, P<10^{-3}) and the genotype by environment (treatment; F=72.01, P<10^{-3}) were all significant. We also performed post-hoc comparisons using the least squares means and found that ACC treatment causes a reduction in root formation, especially in the younger part of the root, which was significant only for Col (wild type), *abcb19* and *pin2* (Fig. 4A,B). The magnitude of the inhibition of root formation by ACC is shown in the inset of Fig. 4B. These data indicate roles for AUX1, LAX3, PIN3 and PIN7 in lateral root formation and for reduction in lateral root formation by ACC. This study examined the number of lateral roots and not lateral root density, which was used in a previous study examining some of the same genotypes (Laskowski et al., 2008). Both studies show reductions in numbers and density of lateral roots in *aux1* and *pin3* mutants; however, our analyses differ in that we observed a stronger reduction in the number of roots in the *pin7* single mutant and *pin3 pin7* double mutant.

Acropetal (rootward) IAA transport was measured in the presence and absence of ACC in wild type and auxin transport mutants (Fig. 4C) and the results were analyzed by a two-way between groups ANOVA. The difference between genotype (F=87.01, P<10^{-3}), by ACC treatment (F=656.41, P<10^{-3}), as well as the genotype by environment (treatment; F=68.44, P<10^{-3}) were all significant. Specific comparisons for genotype or ACC results were all judged significant by post-hoc comparison using the least squares means (P<10^{-3}). *abcb19*, *pin3 pin7* and *aux1 lax3* were the only untreated genotypes, grown on agar media without sealing the plates, with significantly reduced acropetal IAA transport. This differs from a previous report in which *aux1* and wild type had significant differences in auxin transport when ethylene was elevated in Parafilm-sealed plates, which enhanced transport in wild type, but not *aux1* (Negi et al., 2008) genotypes. All genotypes except *pin3 pin7* showed significant increases in transport after ACC treatment relative to untreated seedlings within the same genotype. The magnitude of the response to ACC was attenuated in all mutants to ~200%, except *abcb19* and *pin2*, which, like wild type, showed a 500% increase after ACC treatment (Fig. 4C, inset). These results indicate important roles of specific auxin transport proteins in the ACC stimulated IAA transport.

IAA and ACC enhance *AUX1*, *PIN3* and *PIN7* gene expression through distinct receptors

Our results strongly suggest that ACC-enhanced IAA transport requires the presence of the IAA influx and efflux carriers, AUX1, PIN3 and PIN7. We therefore examined the accumulation of transcripts encoding these proteins after treatment with 1 μM ACC
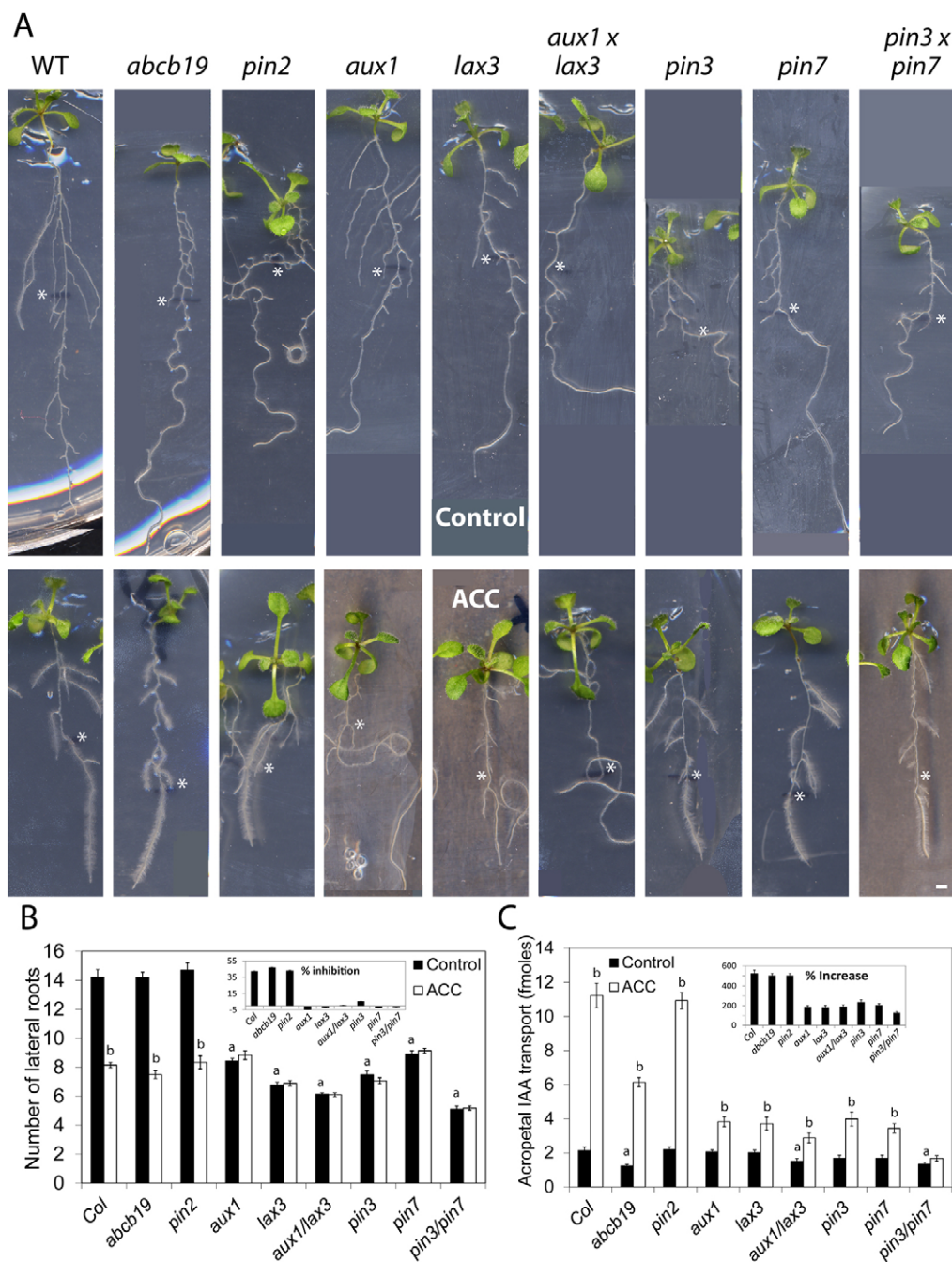
Fig. 4. Auxin transport mutants exhibit altered ACC-mediated lateral root inhibition and enhancement of acropetal auxin transport. (**A**) Representative images of plants 5 days after transfer to control or ACC-containing media are shown. Asterisk indicates root apex position at time of transfer, 5 days after plating. Scale bar: 1 mm. (**B**) Quantification of lateral root number in auxin transport mutants after 5 days of treatment with ACC, with the percentage inhibition by ACC treatment shown in the inset for each genotype. Values are mean±s.e.m., $n=27$. (**C**) Acropetal IAA transport in the indicated genotypes 24 hours after transfer to control plates or plates containing 1 µM ACC. Inset depicts percentage increase after ACC treatment. Values are mean±s.e.m., $n=21$. [a]Significant difference between untreated wild type and mutant ($P<0.05$); [b]significant difference between control and treatment within genotype ($P<0.05$).

or IAA for 6 hours, using qRT-PCR to determine whether transcriptional changes are linked to the elevated transport. The message levels of *AUX1*, *PIN3* and *PIN7* were elevated by two- to fourfold in the wild type after ACC treatment (Fig. 5A-C). *AUX1*, *PIN3* and *PIN7* transcript abundance was significantly reduced in the ethylene signaling mutants *etr1-3* and *ein2-5* in the presence of ACC. Although the transcripts were also reduced in these mutants in the absence of ACC, the effect was not significant These results are consistent with the absence of elevated transport when these mutants are treated with ACC, suggesting that ACC enhances *AUX1*, *PIN3* and *PIN7* gene expression in an ETR1- and EIN2-dependent manner.

IAA treatment also increased the abundance of *AUX1*, *PIN3* and *PIN7* transcripts with a four- to sevenfold enhancement (Fig. 5A-C). We examined the abundance of these three transcripts in the

tir1 mutant and found that all three showed reduced accumulation relative to wild type. Additionally, the enhanced accumulation of transcripts of *AUX1*, *PIN3* and *PIN7* after IAA treatment required TIR1, but not ETR1 or EIN2. Mutations in *TIR1* did not affect the ACC-responsiveness of the three transcripts. These results are consistent with independent auxin and ethylene signaling pathways controlling transcript levels.
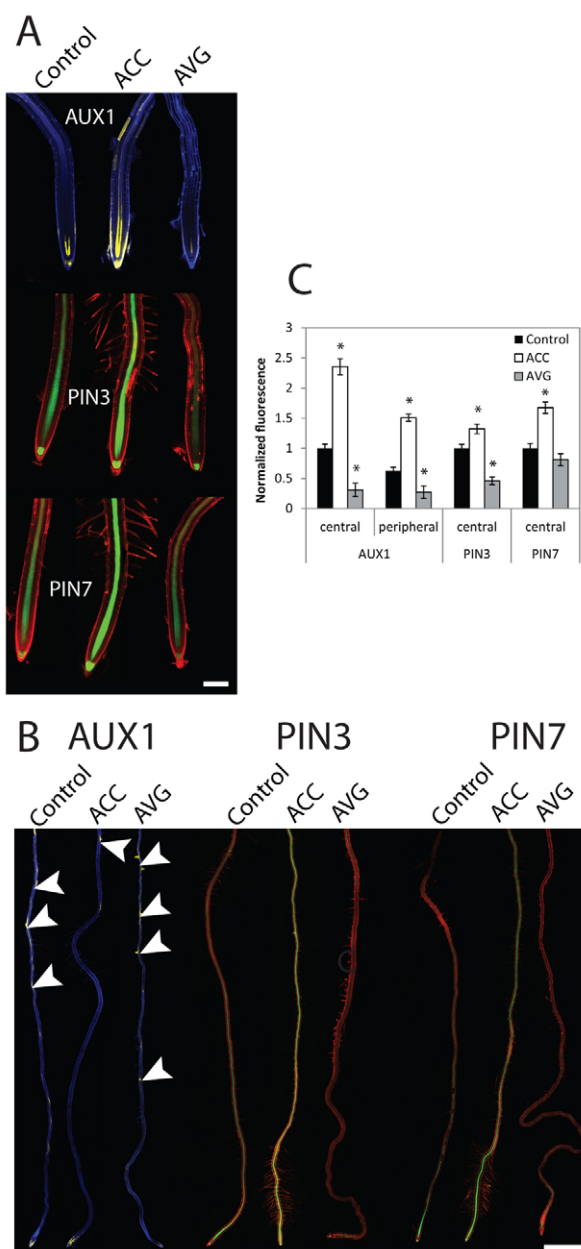
To further demonstrate that ACC inhibits lateral root formation independently of ethylene effects on auxin signaling, we examined the effect of ACC on lateral root formation in the *tir1* mutant. In the absence of ACC, *tir1* plants formed half as many lateral roots as wild-type plants (Fig. 5D). Wild-type and *tir1* plants exhibited similar decreases in root formation after ACC treatment, consistent with ACC inhibition of root formation that occurs independently of TIR1-mediated auxin signaling.
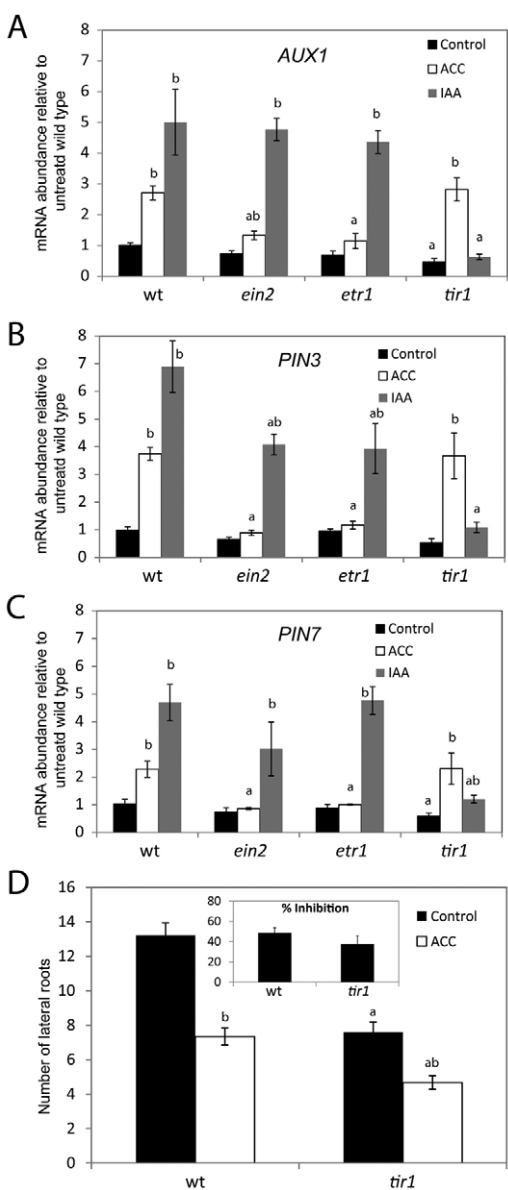
Fig. 5. Independent ethylene and auxin signaling pathways mediate increases in *AUX1*, *PIN3* and *PIN7* mRNA in response to IAA and ACC. Fold increase in (**A**) *AUX1*, (**B**) *PIN3* and (**C**) *PIN7* transcript abundance relative to actin 6 hours after mock, IAA or ACC treatment of 5-day-old seedling roots of the indicated genotypes. Values are mean±s.e.m., with *n*=3 biological replicates each containing three technical replicates. (**D**) Quantification of lateral roots in wild-type and *tir1* plants 5 days after transfer to control or ACC-containing media. The inset shows the percentage inhibition after ACC treatment. Values are mean±s.e.m., *n*=32. [a]Significant difference between wild type and mutant within treatment (*P*<0.05); [b]significant difference between control and IAA or ACC treatment within genotype (*P*<0.05).

Fig. 6. Ethylene modulates the localization and abundance of auxin influx and efflux proteins in many axial zones of the root. Native promoter-driven AUX1-YFP, PIN3-GFP and PIN7-GFP seedlings were grown on control medium and after 5 days transferred to media containing 1 µM ACC or AVG, or control medium. After 8 hours, confocal micrographs were taken with a propidium iodide counterstain, shown in red for GFP fusions and blue for the YFP fusion. (**A**) AUX1, PIN3 and PIN7 fluorescent protein fusions were used to visualize changes in the abundance of these proteins in root tip after treatment with ACC. Representative images from more than ten individuals. Scale bar: 100 µm. (**B**) Tile scan micrographs of the whole root showing a global view of changes in AUX1, PIN3 and PIN7 protein localization and abundance in central and peripheral tissues in response to ACC and AVG. Arrowheads indicate the position of LRP. Scale bar: 1 mm. (**C**) Quantification of increases in reporter fluorescence in the apical regions of the root. Values are mean±s.e.m.; *n*=10. Fluorescence was quantified 100-200 mm from the root apex and is reported relative to the signal of an untreated root central cylinder. *Significant difference between control and treatment as judged by a Student's *t*-test (*P*<0.05).

ACC enhances PIN3 and PIN7 abundance throughout the root, but only enhances AUX1 at the root tip

Because ACC increases *AUX1*, *PIN3* and *PIN7* transcript abundance, we examined whether treatment with 1 µM ACC or the ethylene synthesis inhibitor AVG, also changed the fluorescence of GFP and YFP reporters for these transport proteins. AUX1-YFP,

PIN3-GFP and PIN7-GFP exhibited enhanced expression at the root tip after ACC treatment and reduced expression after AVG treatment (Fig. 6A,C). When the fluorescence intensity was quantified in the region 100 and 200 μm from the root tip, we found significant increases in fluorescence after ACC treatment for all three reporters. AVG treatment reduced AUX1-YFP, PIN3-GFP and PIN7-GFP fluorescence. The analysis also revealed that the root-tip domain of epidermal AUX1-YFP signal was expanded basally after ACC treatment, while the expression domains of the other reporters were largely unchanged. These results are in concert with the transcript abundance changes in these genes described above.

Although these changes in AUX1, PIN3 and PIN7 reporters in the root tip are positioned appropriately to control elongation of primary roots, we also examined expression of these three reporter constructs in the region where lateral roots form (Fig. 6B). ACC and AVG treatment had opposite effects on the three proteins. The striking increase of PIN3-GFP and PIN7-GFP fluorescence after ACC treatment and decrease after AVG treatment extended into the mature regions of the root. AUX1-YFP fluorescence increased in the root apex and elongation zone after AVG treatment, but was undetectable in the mature regions of the root after ACC. In untreated seedlings, AUX1-YFP expression was detected in the pericycle cells and in LRP of all developmental stages, with LRP evident as small yellow regions (indicated by arrowheads in Fig. 6B). These results suggest that the abundance of PIN3 and PIN7 protein positively correlated with ethylene levels.

ACC abolishes lateral root formation in bent roots and alters local auxin-transport protein expression

We manually bent roots at a 90° angle 3 mm from the root tip in a region that had not yet formed LRP and examined localized induction of AUX1-YFP 8 hours after bending and 24 hours after ACC or control treatment. In the absence of ACC, AUX1-YFP was visible in the LRP (Fig. 7A,B). We observed root formation at this position in all untreated roots tested (n>40). By contrast, when roots were treated with ACC before bending, they all fail to initiate LRP (n>40) and the localized AUX1-YFP expression that occurs during lateral root formation was blocked (Fig. 7A). This expression pattern is consistent with a local role of AUX1 in auxin influx into developing roots, but with a more limited role in ACC enhancement of acropetal IAA transport.

By contrast, PIN3- and PIN7-GFP fluorescence decreased below the LRP forming at the bend in nearly all of >20 untreated roots (Fig. 7A,B). After ACC treatment, these localized decreases were never observed, but rather the central tissues of the root exhibited elevated PIN3-GFP and PIN7-GFP fluorescence (Fig. 7A,B) along with elevated acropetal auxin transport. Furthermore, the absence of localized depletion of PIN3 and PIN7 below the bent region of ACC-treated roots might prevent formation of auxin maxima that precede LRP development. This model is supported by the results in Fig. 1, in which localized DR5rev:GFP fluorescence at the point of lateral root formation is abolished after ACC treatment.

DISCUSSION

This study uncovers mechanisms by which ethylene enhances long-distance IAA transport, while reducing lateral root formation. The changing expression and localization of PIN3 and PIN7 with increasing or decreasing ethylene levels, as well as decreased ethylene responsiveness in *pin3* and *pin7* mutants, suggest that these proteins mediate both effects. This work builds on previous
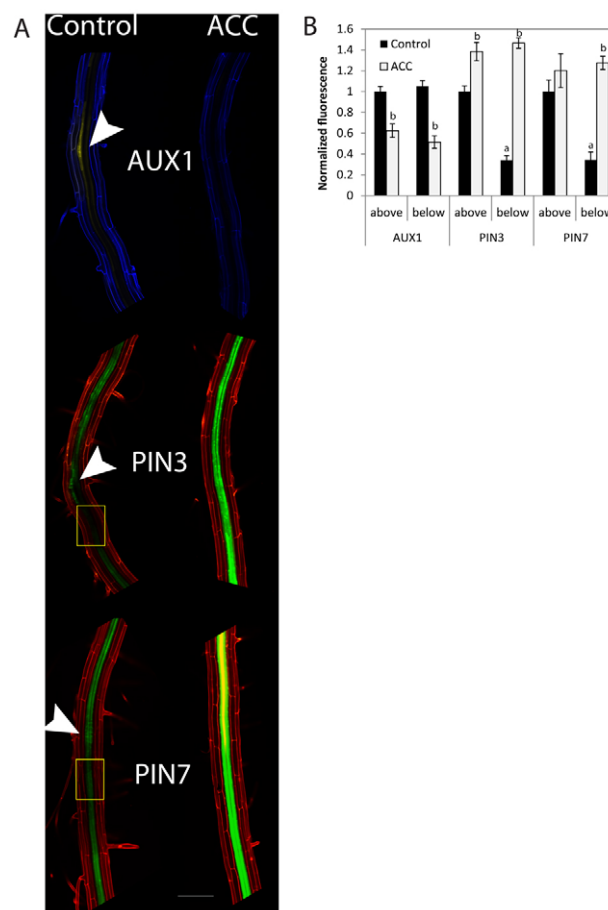
Fig. 7. ACC treatment blocks auxin-transport protein localization patterns necessary for lateral root formation. (**A**) AUX1-YFP, PIN3-GFP and PIN7-GFP fluorescence in the region of bending 8 hours after bending 5-day-old seedling roots. Representative images from three trials of at least six individuals are shown. Arrowheads indicate the position of LRP and yellow boxes indicate the area of depleted fluorescence below the LRP. Scale bar: 100 μm. (**B**) Quantification of reporter fluorescence above and below the site of LRP formation. Values are mean±s.e.m., n=10. [a]Significant difference in fluorescence between above and below the site of lateral root formation (P<0.05). [b]Significant difference between control and ACC treatment within location relative to the LRP (P<0.05).

observations that elevated levels of ethylene reduces lateral root formation, while enhancing auxin transport, with both events occurring through well-characterized ethylene signaling pathways (Ivanchenko et al., 2008; Negi et al., 2008; Negi et al., 2010). Yet, the mechanism by which ethylene positively regulates acropetal auxin transport and at the same time represses root branching has not been previously examined. The repression of LRP formation associated with elevated auxin transport is surprising, as studies have shown that auxin transport positively regulates LRP formation (Peret et al., 2009).

The mechanisms of synergistic inhibition of root elongation by ethylene and auxin have been described previously (Ruzicka et al., 2007; Stepanova et al., 2007; Strader et al., 2010; Swarup et al., 2007). This work explores the mechanisms of auxin and ethylene crosstalk, in a very different scenario in which auxin and ethylene act antagonistically, rather than synergistically. Expression of the DR5rev:GFP reporter increased in the root tip in response to ACC

treatment, consistent with previous reports and the synergistic inhibition of root elongation by these hormones (Swarup et al., 2007). By contrast, we observed depletion of DR5rev:GFP fluorescence after ACC treatment in the lateral root forming regions of roots, as well as at the site of lateral root formation in bent roots. Treatment with the ethylene synthesis inhibitor AVG caused opposite changes, reducing fluorescence in the root tip and increasing it in the mature root. We also examined free IAA levels in whole roots treated with ACC and in *eto1*, *ein2* and *etr1* mutants, and found that the level of free IAA is inversely correlated with ethylene signaling, mirroring the whole root DR5rev:GFP expression patterns, consistent with the opposite effect of ethylene and auxin on lateral root formation in this region of the root.

IAA, which enters cells through the action of influx carriers, and NAA, which bypasses these proteins, caused similar effects on DR5rev:GFP fluorescence but affected lateral root formation differently when combined with ACC. NAA enhanced lateral root formation in both mature and elongating regions, whereas IAA treatment increased LRP formation in the mature root but inhibited LRP formation in the region formed after transfer. This result is consistent with another recent report that described IAA repression of root branching (Ivanchenko et al., 2010). The ability of NAA, but not IAA, to strongly reverse the effect of ACC treatment in this apical region, suggests that influx carrier expression could be limited, consistent with the pattern of AUX1-YFP fluorescence in ACC-treated roots (Fig. 6).

We examined the effect of mutations in specific auxin influx and efflux transport proteins on ACC-regulation of long-distance polar IAA transport and root development. We demonstrated that auxin influx, mediated by AUX1 and LAX3, and auxin efflux, mediated by PIN3 and PIN7 are necessary for the full response to ACC, in which stimulation of transport and repression of branching were always linked. By contrast, neither PIN2 nor ABCB19 mediated the responses to ACC in either lateral root development or auxin transport.

We explored the mechanism of AUX1-, PIN3- and PIN7-mediated increases in auxin transport and associated reductions in lateral root number after ACC treatment by examination of their transcript abundance. *AUX1*, *PIN3* and *PIN7* transcript abundance increased after ACC treatment in an EIN2- and ETR1-dependent manner. *AUX1*, *PIN3* and *PIN7* transcripts were also elevated after IAA treatment in a TIR1-dependent manner, raising the question of whether the ethylene-dependent transcriptional increases are direct, or mediated by ethylene-dependent auxin signaling or synthesis changes. ACC-dependent increases in gene expression and decreases in lateral root formation were observed in the *tir1* mutant, suggesting the transcription of AUX1, PIN3 and PIN7 are independently regulated by auxin and ethylene.

The effects of ethylene on the spatial localization of auxin transport proteins were examined by observation of AUX1-YFP, PIN3-GFP and PIN7-GFP. At the root apex, the fluorescence of these three reporters increased with ACC treatment and decreased with AVG treatment. By contrast, in the mature region of the root, ACC decreased AUX1-YFP fluorescence and increased both PIN3-GFP and PIN7-GFP fluorescence. AVG treatment had opposite effects, increasing AUX1-YFP and the abundance of LRP, and decreasing PIN3- and PIN7-GFP. These results suggest clear roles for PIN3 and PIN7 in ethylene-dependent increases in acropetal IAA transport.

To observe the effects of ACC treatment on the early stages of lateral root initiation, we bent roots to predict the positions of LRP development and examined the expression of AUX1-YFP, PIN3-
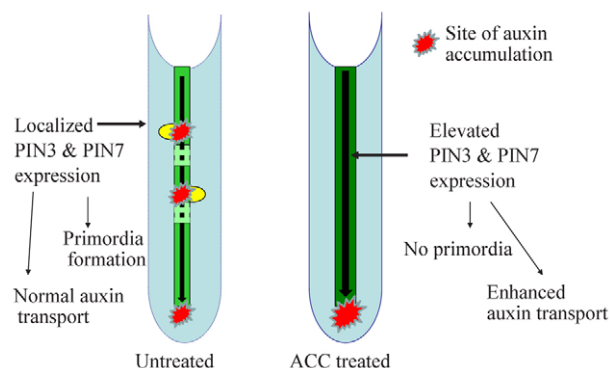
Fig. 8. Model of ACC-mediated inhibition of lateral root development. Diagrams of *Arabidopsis* roots in the absence (left) and presence (right) of ACC. Increases in long-distance transport after ACC treatment are correlated with increased synthesis and accumulation of PIN3 and PIN7. This enhanced acropetal auxin transport could contribute to the reduction in auxin signaling in the mature regions of the root and the increase in auxin signaling seen at the root apex. The enhanced PIN3 and PIN7 expression might thereby prevent the formation of local auxin maxima necessary for lateral root development.

GFP and PIN7-GFP. ACC treatment prevented the previously reported AUX1-YFP increase at the bend that preceded lateral root formation. ACC also enhanced the PIN3-GFP and PIN7-GFP fluorescence all along the root, preventing the previously described reduced accumulation immediately below the point of bending in untreated roots (Laskowski et al., 2008). Roots were bent 3 mm from the apex, at a position where the prepriming or prebranch sites have already been specified (De Smet et al., 2007; Moreno-Risueno et al., 2010). This suggests that bending induces predictable development of lateral roots from prebranch sites, which is supported by the observation that LRP emerge not at the geometric apex of the curve, but at variable positions on the convex side of bend. From these results, a model emerges in which ACC inhibits lateral root development by blocking changes in local auxin-transport protein abundance needed to form local auxin maxima that drive lateral root formation from prebranch sites.

Our results are integrated in a model shown in Fig. 8, in which the central role of PIN3 and PIN7 in the simultaneous positive effect of ethylene on auxin transport and negative effect on lateral root initiation is depicted. ACC treatment stimulates PIN3 and PIN7 transcription, leading to increased protein abundance and enhanced acropetal auxin transport. The consequence of this global increase in auxin transport is the loss of auxin accumulation sites in the mature region of the root that will develop into LRP, which is evident in the examination of DR5-promoter-driven constructs. The observations of local depletions in PIN3 and PIN7 reporter fusions, suggest a mechanism for creating these auxin accumulation sites, and mutant analyses indicate the function of these proteins in the ACC-dependent changes in auxin transport and formation of LRP. The result of ACC-mediated increases in PIN3- and PIN7-dependent transport is redistribution of auxin accumulation away from the mature region of the root, where it is needed to form LRP, and towards the apex, where it inhibits root elongation.

Although this model is derived from experimental results in which roots were treated with ACC to increase ethylene levels, we used a range of genetic and inhibitor approaches to show that endogenous ethylene also has a negative effect on root formation.

Mutants with defects in ethylene signaling or wild-type plants treated with ethylene signaling and synthesis inhibitors exhibited increased LRP development and reduced acropetal transport. The changes in transcript abundance in response to ACC were lost in the ethylene signaling mutants, and changes in free IAA after ACC treatment or in the ethylene overproducing mutant *eto1* are also lost in *etr1* and *ein2* mutants. Finally, ACC and AVG treatment oppositely effect auxin transport proteins as seen by GFP-protein fusions. Together these experiments show that increases in endogenous ethylene or response to this molecule enhance auxin transport and reduce lateral root formation in a PIN3- and PIN7-dependent fashion.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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