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Ethylene Regulates Root Growth through Effects on Auxin Biosynthesis and Transport-Dependent Auxin Distribution [W]

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In plants, each developmental process integrates a network of signaling events that are regulated by different phytohormones, and interactions among hormonal pathways are essential to modulate their effect. Continuous growth of roots results from the postembryonic activity of cells within the root meristem that is controlled by the coordinated action of several phytohormones, including auxin and ethylene. Although their interaction has been studied intensively, the molecular and cellular mechanisms underlying this interplay are unknown. We show that the effect of ethylene on root growth is largely mediated by the regulation of the auxin biosynthesis and transport-dependent local auxin distribution. Ethylene stimulates auxin biosynthesis and basipetal auxin transport toward the elongation zone, where it activates a local auxin response leading to inhibition of cell elongation. Consistently, in mutants affected in auxin perception or basipetal auxin transport, ethylene cannot activate the auxin response nor regulate the root growth. In addition, ethylene modulates the transcription of several components of the auxin transport machinery. Thus, ethylene achieves a local activation of the auxin signaling pathway and regulates root growth by both stimulating the auxin biosynthesis and by modulating the auxin transport machinery.

INTRODUCTION

Plant signaling substances, also referred to as phytohormones, such as auxin, ethylene, cytokinin, jasmonic acid, abscisic acid, or gibberellin, are central modulators of plant growth and development. Recently, predominantly molecular-genetic studies have unraveled key components of single hormonal pathways and have helped us understand main principles of their signal perception and transduction (Davies, 2004). However, as indicated by previous physiological studies, it is apparent that complex interactions among hormonal pathways are common and of great importance for the final developmental outcome.

Synergistic effects of auxin and ethylene have been well defined in the regulation of hypocotyl elongation (Smalle et al., 1997; Vandenbussche et al., 2003), root hair growth and differentiation (Pitts et al., 1998), apical hook formation (Lehman et al., 1996: Li et al., 2004), root gravitropism (Lee et al., 1990; Buer et al., 2006), and root growth (Pickett et al., 1990; Rahman et al., 2001), suggesting that these two signaling pathways also interact at the molecular level.

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Genetic and biochemical studies have led to a detailed molecular characterization of both ethylene and auxin signaling pathways. Ethylene is perceived by a family of ethylene receptors ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE INSENSITIVE4 (EIN4) that are homologous to bacterial two-component His kinases. Upon ethylene binding, receptors inhibit the function of a Raf-like kinase, CONSTITUTIVE TRIPLE RESPONSE1, and thus allow EIN2, a protein with unclear function, to act as a positive regulator of the ethylene pathway. EIN2 activates transcription factors of the EIN3 family located in the nucleus. EIN3 binds to promoters of *ETHYLENE RESPONSE FACTOR* (*ERF*) genes and stimulates their transcription in an ethylene-dependent manner. These ERF transcription factors regulate the expression of many downstream genes (reviewed in Chen et al., 2005).

Recently, the molecular mechanisms underlying auxin perception and signal transduction have also been elucidated. Auxin signaling is primarily mediated by three protein families: the AUXIN RESPONSE FACTOR (ARF) family of transcription factors that is responsible for the regulation of auxin-inducible gene expression, the auxin/indole-3-acetic acid (AUX/IAA) transcriptional inhibitors that interact with the ARFs and prevent their action, and F-box proteins that are part of the ubiquitin protein ligase SCF^{TIR1} complex and control the rapid ubiquitin-mediated degradation of the AUX/IAA in response to auxin (reviewed in Leyser, 2006). TIR1 and related F-box proteins act as auxin receptors; binding of auxin strongly enhances their interaction

with AUX/IAA and ultimately leads to degradation of AUX/IAA inhibitors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

Although ethylene and auxin signaling pathways are relatively well characterized, the mechanisms of their interaction are still poorly understood. One interaction mode occurs at the hormone biosynthesis level: Auxin induces ethylene biosynthesis by upregulation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, the key enzyme in ethylene production (Abel et al., 1995). By contrast, ethylene might influence auxin levels because ethylene has been shown to regulate the expression of two *WEAK ETHYLENE INSENSITIVE* (*WEI2* and *WEI7*) genes that encode subunits of anthranilate synthase, a rate-limiting enzyme in Trp biosynthesis (Stepanova et al., 2005), from which pathway auxin is at least partially derived (Woodward and Bartel, 2005).

Apical hook formation implies a different mechanism of crosstalk, in which auxin and ethylene seem to interact at the level of hormonal responses. Formation of the apical hook structure is accompanied by differential auxin responses in hook tissue underlying the asymmetric cell elongation of the hypocotyl (Friml et al., 2002b). Ethylene enhances apical hook bending by activating *HOOKLESS1* (*HLS1*) transcription. One of the roles of *HLS1* is to inhibit the function of the auxin response factor *ARF2* that is a negative regulator of the differential auxin response in the apical hook. Thus, apical hook formation is an example of hormonal interaction at the response level because the auxin response is modulated by the upstream ethylene pathway (Lehman et al., 1996; Li et al., 2004).

Another process regulated by both hormones is gravitropic bending of the root. Asymmetric auxin redistribution in basipetal direction mediated by the auxin influx (AUX1) and the efflux carriers (PIN-FORMED3 [PIN3] and PIN2) is crucial for root gravitropism (Luschnig et al., 1998; Marchant et al., 1999; Friml et al., 2002b). Exogenous application of ethylene reduces (Buer et al., 2006) or delays (Lee et al., 1990) the root gravitropic response. Although ethylene has been shown to downregulate lateral auxin movement in maize (*Zea mays*) root tips (Lee et al., 1990) and auxin transport in pea (*Pisum sativum*) epicotyls (Suttle, 1988), the mode of this auxin–ethylene interaction is not known. Recent work of Buer et al. (2006) suggests a novel mechanism, in which ethylene inhibits the gravity response by altering the synthesis of flavonoids that have been considered prime candidates for in vivo regulators of auxin transport.

Besides apical hook formation and root gravitropism, root growth has been found to be influenced by both ethylene and auxin, which exhibit inhibitory effects on root growth (Swarup et al., 2002). Typically, in mutants impaired in ethylene signaling, root growth is insensitive to ethylene (Roman et al., 1995). Interestingly, ethylene-based mutant screens have identified components of auxin perception (*tir1*; Ruegger et al., 1998; Alonso et al., 2003) and auxin transport (*pin2*, also designated *ethylene insensitive root1* [*eir1*] [Luschnig et al., 1998]; and *aux1* [Pickett et al., 1990]). Nevertheless, the ethylene resistance of *pin2/eir1* and *aux1* is restricted exclusively to root growth (Roman et al., 1995; Stepanova et al., 2007), in contrast with the ethylene perception mutants that exhibit complex ethylene insensitivity in a number of developmental processes. Thus, both physiological and genetic studies point to an extensive crosstalk between auxin and ethylene in regulating root growth.

Here, we provide a mechanistic basis for the ethylene effect on root growth. By parallel stimulation of auxin biosynthesis and modulation of the auxin transport machinery, ethylene triggers auxin signaling locally in the elongation zone and thus regulates root growth.

RESULTS

Ethylene Inhibits Root Growth Primarily by Affecting Cell Elongation but Not Root Meristem Activity

Root growth depends on two basal developmental processes: cell division in the root apical meristem and elongation of cells that leave the root meristem (reviewed in Scheres et al., 2002). Ethylene has been shown to strongly inhibit the elongation of these cells as manifested by their short length (Le et al., 2001) (Figures 1B and 1D). Reduced meristematic activity or premature differentiation of cells within the root meristem also leads to root growth inhibition, in extreme cases resulting in complete collapse of the root meristem (Blilou et al., 2005). Therefore, we tested whether ethylene affects the activity of the root meristem and the differentiation of root meristem cells. We used the mitotic reporter *proCYCB1;1-GUS*$_{DB}$ (degradation domain fused to β-glucuronidase [GUS]) to monitor cell cycle activity within root meristem after exposure to ACC, which is a direct precursor of the ethylene biosynthetic pathway (Yang and Hoffman, 1984). CYCB1 belongs to the cyclin protein family that regulates cell cycle progression. Cyclin protein levels fluctuate during the cell cycle, determining the timing of the activation of the cyclin-dependent kinases. Therefore, cyclins can be used as markers of cell cycle progression (Doerner et al., 1996). ACC treatment had no dramatic effect on the intensity or extent of the *proCYCB1;1-GUS*$_{DB}$ expression in the root meristem when compared with control roots (Figure 1C).

To test whether ethylene affects the differentiation of root meristem cells, root meristem length was measured (Figure 1D) because premature differentiation of cells within the root meristem is evidenced by shortening of the root meristematic zone (Blilou et al., 2005). At 1 μM ACC, when the lengths of root and the epidermal cells are already strongly reduced (35% of mock-treated roots), no effects on the root meristem length were observed (Figure 1D). Furthermore, Lugol staining that visualizes differentiated root columella cells did not reveal any abnormalities in differentiation of columella initial cells (Figure 1E).

To confirm the requirement of the ethylene signaling pathway in these processes, we examined the lengths of the root meristem, the epidermal cells, and the root either in seedlings impaired in ethylene perception or with reduced endogenous ethylene levels. Ethylene insensitivity of both *etr1-3* and *ein2* mutants was demonstrated by ACC-resistant root growth and cell elongation (Figure 1D). Inhibition of ethylene perception by silver ions (Beyer, 1976; Rodriguez et al., 1999) completely suppressed the inhibitory effect of ACC on root growth and root cell elongation in wild-type seedlings (Figure 1F; data not shown). Consistently, reduction of endogenous ethylene levels by 2-aminoethoxyvinylglycin (AVG), which inhibits the conversion of *S*-adenosyl Met to ACC (Yang and Hoffman, 1984) or insensitivity to endogenous ethylene (typically in the *ein2*

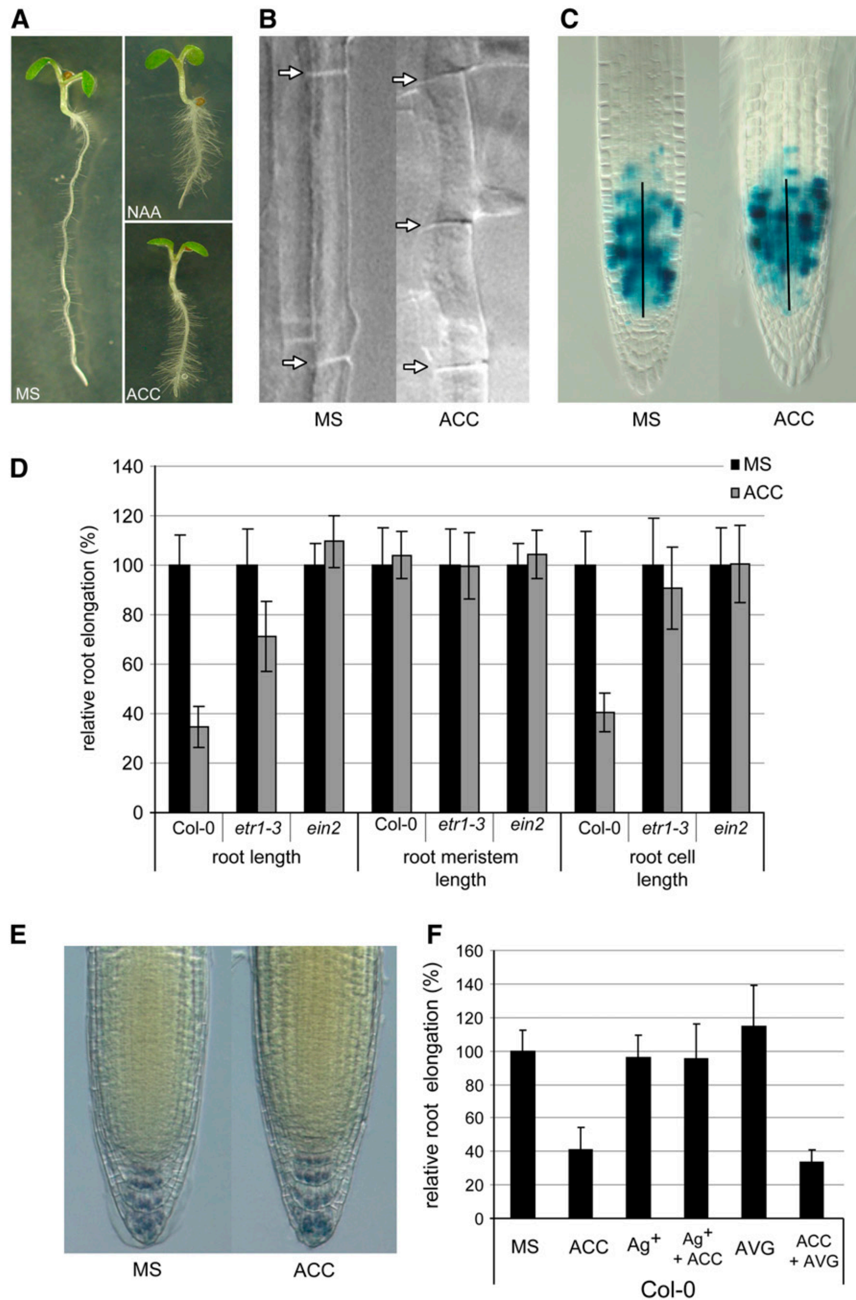

Figure 1. Ethylene Inhibits Root Growth through Regulation of Cell Elongation.

(A) Seedlings germinated on ethylene precursor ACC or synthetic auxin NAA exhibit comparably reduced root growth. NAA, 1-naphthylacetic acid.

(B) ACC strongly inhibits elongation of wild-type epidermal cells, as quantified in **(D)**.

(C) ACC does not affect cell cycle activity of the root meristem, as monitored by the *proCYCB;1-GUS*$_{DB}$ reporter.

(D) ACC inhibits total root length and epidermal cell length of control seedlings (ACC treated versus untreated wild-type; $P < 0.05$, analysis of variance [ANOVA]). Root and cell lengths of *etr1-3* and *ein2-1* are ethylene resistant ($P > 0.05$, ANOVA). Root meristem length is not affected by ACC in the wild type nor in the mutants ($P > 0.05$, ANOVA). 100% corresponds to 14.2 ± 1.76 mm of the wild type, 13.7 ± 2.03 mm of *etr1-3*, and 16.8 ± 1.52 mm of *ein2* root length.

(E) Columella differentiation in wild-type roots is not affected by ACC treatment. Starch granules are visualized by Lugol staining.

(F) ACC inhibits root elongation. The inhibitor of the ethylene perception (Ag^+) ($P > 0.05$, ANOVA) but not that of the ethylene biosynthesis (AVG) ($P < 0.05$, ANOVA) interferes with simultaneously applied ACC in root elongation. Reduction of the endogenous ethylene level by AVG treatment results in significantly longer roots than those of the control ($P < 0.05$, ANOVA).

Five-day-old seedlings were grown in the presence of 200 nM NAA, 1 μM ACC, 0.2 μM AVG, and 20 μM $AgNO_3$. Data are means ± SD.

mutant), resulted in longer roots than those of the wild type but not in aberrant meristem sizes (Figures 1D and 1F; data not shown).

Based on these results and taking into account that ethylene inhibits root cell elongation and overall root growth to a comparable extent, we conclude that ethylene affects root growth primarily by regulating the elongation of cells that leave the root meristem but without impact on the root meristem activity.

Ethylene Requires Basipetal Auxin Transport to Affect Root Growth

In addition to ethylene, auxin also inhibits root elongation (List, 1969) (Figure 1A). Moreover, genetic screens that were instrumental for the molecular characterization of the ethylene signal transduction pathway identified components of auxin perception (*tir1*) (Ruegger et al., 1998; Alonso et al., 2003) and auxin transport (*pin2*/*eir1* [Roman et al., 1995; Luschnig et al., 1998] and *aux1* [Pickett et al., 1990; Bennett et al., 1996]). To gain insight into the relation between the ethylene sensitivity of roots and the auxin transport, we inspected root growth of seedlings impaired in auxin transport, either after chemical inhibition or in other auxin transport mutants, such as *pin1* (Okada et al., 1991), *pin4* (Friml et al., 2002a), *pin7* (Friml et al., 2003a), or the *PIN1*-overexpressing line *35S-PIN1* (Benkova et al., 2003). Besides *aux1* (which was the most strongly affected) and *eir1*, *35S-PIN1* seedlings also exhibited ACC-resistant root and epidermal cell elongation (Figures 2A and 2B). By contrast, ethylene sensitivity of *pin1*, *pin4*, and *pin7* roots did not differ from that of control seedlings (Figure 2C), suggesting a specific requirement of a subset of auxin transport processes for ethylene effects on root growth. This finding was corroborated with general inhibitors of polar auxin transport, such as 1-*N*-naphthylphthalamic acid (NPA), 2,3,5-triiodobenzoic acid, 9-hydroxyfluorene-9-carboxylic acid, or brefeldin A (Morris, 2000; Rashotte et al., 2000). Chemical inhibition of auxin transport did not impair the ethylene effect on root growth and cell elongation (Figure 2D; see Supplemental Figure 1 online) in comparison to that of *eir1*, *aux1*, or *35S-PIN1*.

Auxin transport in roots occurs in two distinct directions, acropetally and basipetally, spatially separated in two different tissues (Figure 8; Tanaka et al., 2006). Our analyses showed that the overall reduction of polar auxin transport, as achieved by chemical inhibition, or of stele- and root tip–based transport after the functional loss of PIN1, PIN4, or PIN7, were not sufficient for ethylene-insensitive root growth. By contrast, loss of AUX1 and PIN2, both of which are required specifically for the basipetal transport of auxin through the outer root cell layers (Marchant et al., 1999; Rashotte et al., 2000), led to such ethylene insensitivity. *35S-PIN1*, which is also ethylene insensitive in roots, is possibly defective in the basipetal auxin transport as well because the ectopically expressed *PIN1* localizes to the side of epidermis cells opposite from that of PIN2, thus counteracting its action with root agravitropic phenotypes, similar to those of *aux1* and *eir1* (see Supplemental Figure 2 online; data not shown). We conclude that the ethylene sensitivity of root growth requires functional basipetal auxin transport.

Ethylene Mediates Basipetal Translocation of Auxin into the Root Elongation Zone

Previously, auxin transport has been shown to regulate plant development, including root growth, by controlling local asymmetric auxin distribution within plant tissues (reviewed in Tanaka et al., 2006). How does the lack of basipetal transport relate to ethylene-sensitive root growth? To address this question, we examined whether and how ethylene influenced auxin distribution in roots. For indirect monitoring of auxin distribution, we used well-established tools: the auxin-sensitive reporters *DR5-GUS* (Ulmasov et al., 1997; Sabatini et al., 1999), *DR5rev-GFP* (Benkova et al., 2003), and *proIAA2-GUS* (Luschnig et al., 1998). In control roots, *DR5* expression was restricted to the cells of the columella and the quiescent center and that of the *proIAA2* reporter in cells of the columella and the provascular tissue, as reported previously (Luschnig et al., 1998; Sabatini et al., 1999). The ACC-based increase of ethylene levels ectopically stimulated the expression of *DR5* and *proIAA2* in the root meristem and the elongation zone (Figures 3A to 3C) (see also Luschnig et al., 1998; Stepanova et al., 2005). Confocal sections revealed that the ectopic signal in the root meristem was mainly restricted to the outer cell layers, the lateral root cap, and the epidermis (Figure 3F). When ethylene perception was inhibited either chemically by silver ions or by mutation in the *etr1-3* and *ein2* mutants, ACC failed to induce this ectopic *DR5* expression (Figures 3A to 3D; *etr1-3* and *ein2* untreated control, see Figure 5C), confirming the requirement of the ethylene signaling. Consistently, Swarup et al. (2007) demonstrated that the *proIAA2-GUS* expression was ethylene insensitive in the *ein2* background.

The ethylene-dependent activation of the auxin response in the lateral root cap and the epidermis of root meristems and in the elongation zone might result from increased basipetal auxin delivery to these parts of the root. To test this hypothesis, we examined the auxin response in seedlings with auxin transport defects. Mutants defective in *PIN1* and *PIN7*, the two main regulators of acropetal auxin transport (Blilou et al., 2005), showed ectopic upregulation of *DR5* after ACC treatment comparable to that of control roots (Figure 3F). By contrast, mutants with defective basipetal auxin transport (*eir1*, *aux1*, and *35S-PIN1*) exhibited a very different *DR5-GUS* expression pattern in response to ethylene: treatment with 0.1 and 1 μM ACC failed to induce an ectopic *DR5* signal in the outer layers of the root meristem and the elongation zone of these mutants (Figure 3E; data not shown). A moderate ectopic *DR5* induction was observed only when high ACC concentrations (5 μM) were applied (Figure 3E). When the overall auxin transport was inhibited with NPA used simultaneously with ACC, the ectopic accumulation of the *DR5* signal in the roots similar to that of ACC treatment alone was observed (Figure 3G, top panel). Interestingly, the overall inhibition of auxin transport by NPA together with ACC treatment led to ectopic *DR5* expression in the roots of *eir1* as well (Figure 3G, bottom panel).

Thus, the ability of ethylene to activate *DR5* ectopically in the outer layers of the root meristem and the elongation zone in the wild type, *pin1*, *pin7* (cf. Figures 2C and 3F), the NPA-treated wild-type (cf. Figures 2D and 3G, top panel), or *eir1* (cf. Figures 2E and 3G, bottom panel) in all cases correlated with the

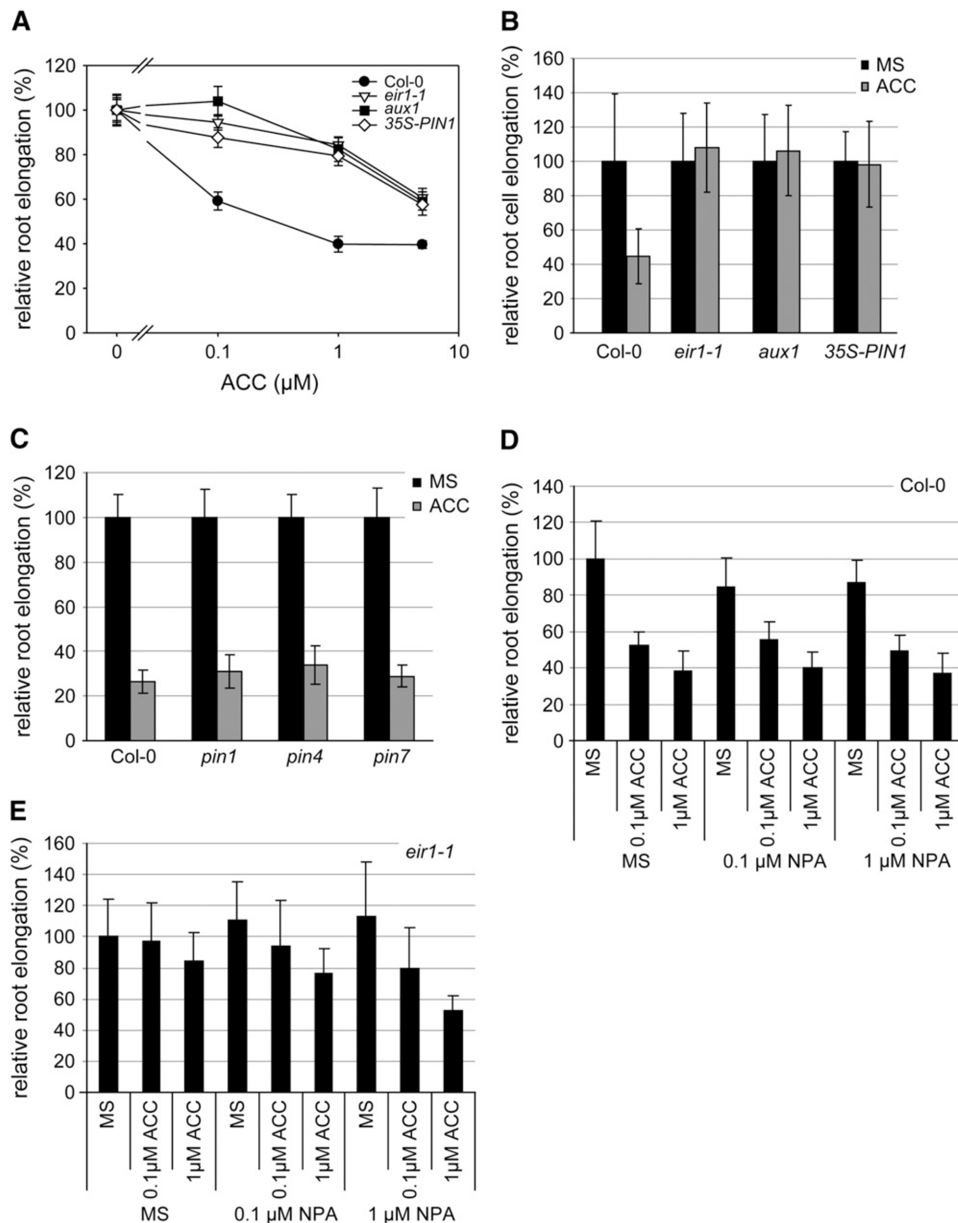

Figure 2. Ethylene Insensitivity in Mutants with Abolished Basipetal Auxin Transport.

(A) Lines affected in the root basipetal auxin transport (*eir1-1*, *aux1-T*, and *35S-PIN1*) exhibit ethylene-resistant root growth. The ethylene effects on the wild type compared with *eir1-1*, *aux1-T*, or *35S-PIN1* were significantly different ($P < 0.05$, ANOVA). 100% corresponds to 12.5 ± 1.95 mm of the wild type, 9.9 ± 2.33 mm of *eir1-1*, 9.1 ± 1.99 mm of *aux1*, and 13.0 ± 2.39 mm of *35S-PIN1* root length. For clarity, depicted data are mean ± SE.

(B) ACC does not inhibit the elongation of the epidermal cells in *eir1-1*, *aux1-T*, and *35S-PIN1* ($P < 0.05$, ANOVA).

(C) Auxin efflux carrier mutants *pin1-3*, *pin4-1*, and *pin7-2* (with normal root basipetal auxin transport) are ethylene sensitive. The ACC effect on *pin1*, *pin4*, and *pin7* roots was not significantly different from that on the wild type ($P > 0.05$, ANOVA). 100% corresponds to 24.4 ± 2.48 mm of the wild type, 23.3 ± 2.90 mm of *pin1*, 25.3 ± 2.35 mm of *pin4*, and 26.1 ± 2.71 mm *pin7* root length.

(D) Inhibitor of polar auxin transport (NPA) at any concentration does not interfere with ethylene sensitivity of the root growth. No significant difference was found between the root lengths of seedlings treated with ACC and simultaneously with NPA and ACC ($P > 0.05$, ANOVA).

(E) ACC sensitivity of *eir1-1* roots is increased by NPA treatment. The root length of *eir1-1* treated simultaneously with 1 µM NPA and 1 µM ACC was significantly shorter than that of seedlings germinated on 1 µM ACC ($P < 0.05$, ANOVA).

Seedlings were grown, if not specified differently, in the presence of 1 µM ACC. Data are mean ± SD (except in **[A]**).

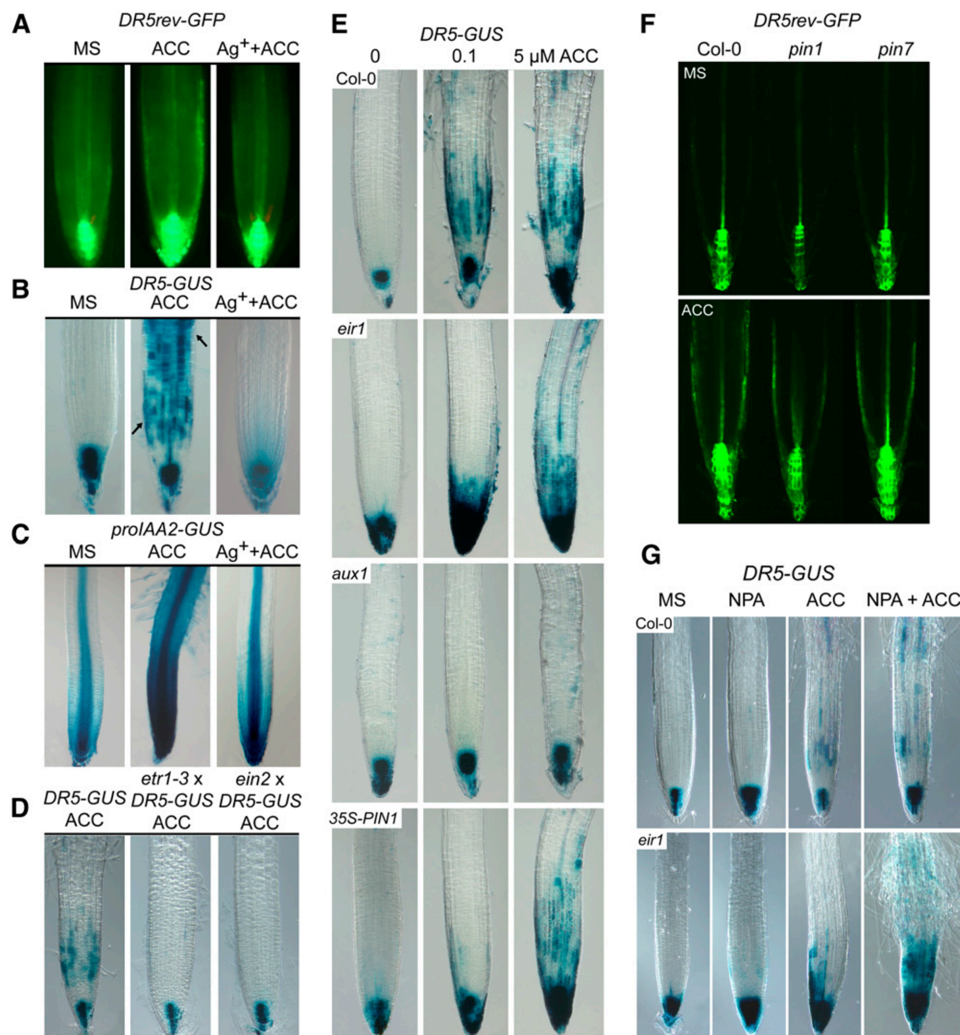

Figure 3. Induction of *DR5* Reporter Expression by Ethylene in the Root Meristem and the Root Elongation Zone.

(A) to **(C)** Auxin-sensitive reporters *DR5rev-GFP* **(A)**, *DR5-GUS* **(B)**, and *proIAA2-GUS* **(C)** are ectopically expressed after ACC treatment. Ag^{+} (ethylene perception inhibitor) blocks ACC-triggered ectopic expression. Prolonged GUS staining **(B)** reveals an increased auxin response in the outer layers of the root meristem (lateral root cap and epidermis) and in the elongating cells. The arrows point to the tissues with the ectopic *DR5* signal.

(D) ACC treatment induces ectopic expression of the *DR5-GUS* reporter in the wild type but not in the *etr1-3* and *ein2-1* mutant backgrounds.

(E) ACC treatment induces ectopic expression of the *DR5-GUS* reporter in the wild type but not in the *eir1-1*, *aux1-T*, and *35S-PIN1* backgrounds. Higher doses of ACC (5 μM) stimulate *DR5* expression in the epidermal cells of the meristem and partially also in the elongation zone of mutant roots.

(F) Confocal sections show that the ACC-induced ectopic expression of the *DR5rev-GFP* reporter is restricted to the lateral root cap and the epidermis of the root meristem in the wild type and a comparable expression pattern of the reporter in the *pin1-3* and *pin7-2* background.

(G) Inhibitor of the polar auxin transport (NPA) does not interfere with ACC-induced ectopic expression of *DR5-GUS* in the wild-type background. Simultaneous application of ACC and NPA induces the ectopic expression of *DR5-GUS* in the *eir1-1* mutant.

Five-day-old seedlings were grown, if not specified differently, in the presence of 1 μM ACC, 20 μM $AgNO_3$, and 1 μM NPA.

ethylene-dependent inhibition of root elongation. Consistently, ethylene-insensitive root growth was observed in all cases where ectopic activation of the auxin reporters failed in the outer cells of the root meristem and in the elongation zone, such as in the ethylene perception mutants *etr1-3* and *ein2* (cf. Figures 1D and 3D), wild-type seedlings treated with silver ions (cf. Figures 1F, 3A, and 3B), and the *eir1*, *aux1*, and *35S-PIN* lines impaired in basipetal auxin transport (cf. Figures 2A and 3E). These observations strongly suggest that a substantial part of the root growth regulation by ethylene is mediated through the basipetal delivery of auxin to cells of the elongation zone, where elongation is controlled.

An Auxin Response Is Required for Ethylene to Affect Root Growth

Our analysis of auxin reporters suggested that ethylene induces ectopic accumulation of auxin in the outer layers of the root

meristem and in the elongation zone, where it activates a local auxin response. If this auxin response participated in ethylene-mediated inhibition of root elongation, then root growth of mutants defective in auxin responses should be resistant to both auxin and ethylene. Mutants defective at different levels of the auxin signaling pathway were selected to test the auxin and ethylene responses. Root growth of the *tir1* mutant, which is impaired for the auxin receptor (Ruegger et al., 1998; Dharmasiri et al., 2005; Kepinski and Leyser, 2005), was comparably resistant to both auxin and ethylene treatments (Figure 4C). As another manner to inhibit auxin signaling, we used stabilized AUX/IAA-negative regulators of the auxin response. When AUX/IAAs are stabilized by a mutation in the degradation domain, they exhibit dominant auxin-insensitive phenotypes (Ramos et al., 2001). Such stabilizing mutations in AXR2/IAA7 (Wilson et al., 1990) and AXR3/IAA17 (Leyser et al., 1996) result in auxin-resistant root growth. Both *axr2-1* (Wilson et al., 1990; see Supplemental Figure 3 online) and *axr3-1* (Swarup et al., 2007) roots show ethylene-resistant root growth. Furthermore, we used a line carrying a stabilized allele of *AXR3/IAA17* driven by a heat shock promoter (*HS-axr3*). Heat shock leads to accumulation of AXR3 in roots (Knox et al., 2003). This resulted in auxin- and ethylene-resistant root growth (Figure 4A). In addition, transactivation studies (Swarup et al., 2007) specified that inhibition of the auxin response by accumulation of AXR3 in the cortex and the epidermis is sufficient to determine the auxin and ethylene insensitivities of the root growth. In reciprocal experiments, we tested the ethylene signaling mutants *etr1-3* and *ein2* for their sensitivity to auxin and ethylene in roots. Although *etr1-3* was clearly resistant to ethylene, its auxin sensitivity did not differ from that of the wild type. *ein2* showed only a slight auxin resistance besides the expected strong ethylene resistance (Figures 5A and 5B). These results suggest that a substantial part of the ethylene regulation of the root growth is mediated through auxin signaling and not vice versa.

Next, we addressed the question whether the auxin response is required for ethylene-induced ectopic *DR5* expression in the outer layers of the root meristem and in the elongation zone. As expected, both ethylene and auxin failed to induce ectopic expression of the *DR5* reporter in the *HS-axr3* seedlings after heat shock (Figure 4B). Also, terfestatin A (Yamazoe et al., 2005) prevented both auxin- and ethylene-induced ectopic *DR5-GUS* expression (Figure 4D). On the other hand, only auxin, but not ethylene, induced ectopic *DR5* expression in the *etr1-3* and *ein2* mutants (Figure 5C). Our results demonstrate that the ethylene effect on ectopic expression of the *DR5* reporter depends on auxin signaling but not vice versa.

In summary, these findings indicate that ethylene regulates root growth through downstream local auxin signaling in the epidermis and elongation zone of the root. However, not all data are consistent with this simple model. We noticed that auxin and ethylene induced a qualitatively similar ectopic upregulation of the *DR5* reporter in control roots. Nevertheless, auxin stimulated the *DR5* reporter more efficiently than did ACC, although both had a comparable inhibitory effect on the root elongation (cf. Figures 5A and 5C). Furthermore, mutants bearing a stabilized *SHY2/IAA3* allele (Tian and Reed, 1999) or *SLR/IAA14* (Fukaki et al., 2002) were strongly resistant against auxin but not against ethylene (Figure 4E; see Supplemental Figure 3 online). Similarly, some mutants and mutant combinations in the auxin-dependent ARF transcription factors, such as *nph4-1/arf7* and *arf19* and the corresponding double mutants (Okushima et al., 2005), showed pronounced resistance to auxin but none to ethylene in the roots (Figure 4F). A similar analysis performed previously (Li et al., 2006) is seemingly contradictory because it claims an ethylene-resistant root growth in the etiolated *arf7* and *arf19* mutants. However, we should note that at lower auxin and ACC concentrations with comparable inhibitory effects on control seedlings, both single and double *arf* mutants exhibited a more pronounced resistance to auxin than to ethylene. These results indicate that although the ethylene effect on root growth is mostly performed through the auxin pathway, there is also an ethylene-specific, auxin response–independent component to this regulation.

Ethylene Stimulates Local Auxin Biosynthesis

The next question concerned the mechanism by which ethylene mediates the transport-dependent activation of auxin responses in the outer layers of the root meristem and elongation zone. Conceptually, this mechanism might happen through direct interaction with auxin signaling in the root meristem and elongation zone, regulation of auxin delivery to this region, or upregulation of auxin biosynthesis. Previous reports suggested that auxin induced ethylene biosynthesis (Abel et al., 1995). However, the opposite might also be true because recently it was shown that ethylene regulates the expression of *WEI2* and *WEI7*, two genes encoding subunits of the anthranilate synthase, a rate-limiting enzyme in Trp biosynthesis (Stepanova et al., 2005). However, no direct support for ethylene-dependent regulation of auxin biosynthesis has been provided.

First, we tested whether the spatial changes in the pattern of the *DR5* expression were accompanied with an overall increase in *DR5* activity. We quantitatively examined *DR5* expression by a fluorimetric assay and found that in roots of intact seedlings treated with ACC, it had increased 4.5-fold compared with untreated seedlings (Figure 6A). This overall increase in *DR5* activity implies increased auxin levels in root tips of ethylene-treated seedlings. To confirm the results of the fluorimetric analysis, direct auxin measurements were performed by gas chromatography–selected reaction monitoring mass spectrometry. Auxin content in the last 1 mm of the root (including the root meristem and the elongation zone; Figure 6B) demonstrated increased IAA levels after ACC treatment (Figure 6C), corroborating the *DR5* activity measurements. As expected, the increase in *DR5* activity and auxin content did not occur when the ethylene signaling was inhibited in the *etr1-3* mutant (Figures 6A and 6C). By contrast, in the *eir1* mutant, the ethylene-dependent increase of *DR5* activity still occurred (Figure 6A), indicating that the ethylene resistance of the *eir1* mutant is not caused by the inability to increase auxin levels in the root tip but by the failure to deliver it basipetally to the responsive tissues. In summary, these data show that ethylene signaling mediates increased auxin levels in the root tip.

To test whether this effect might occur through direct stimulation of auxin biosynthesis, its rate was measured by D_2O-feeding experiments (Ljung et al., 2005). These measurements

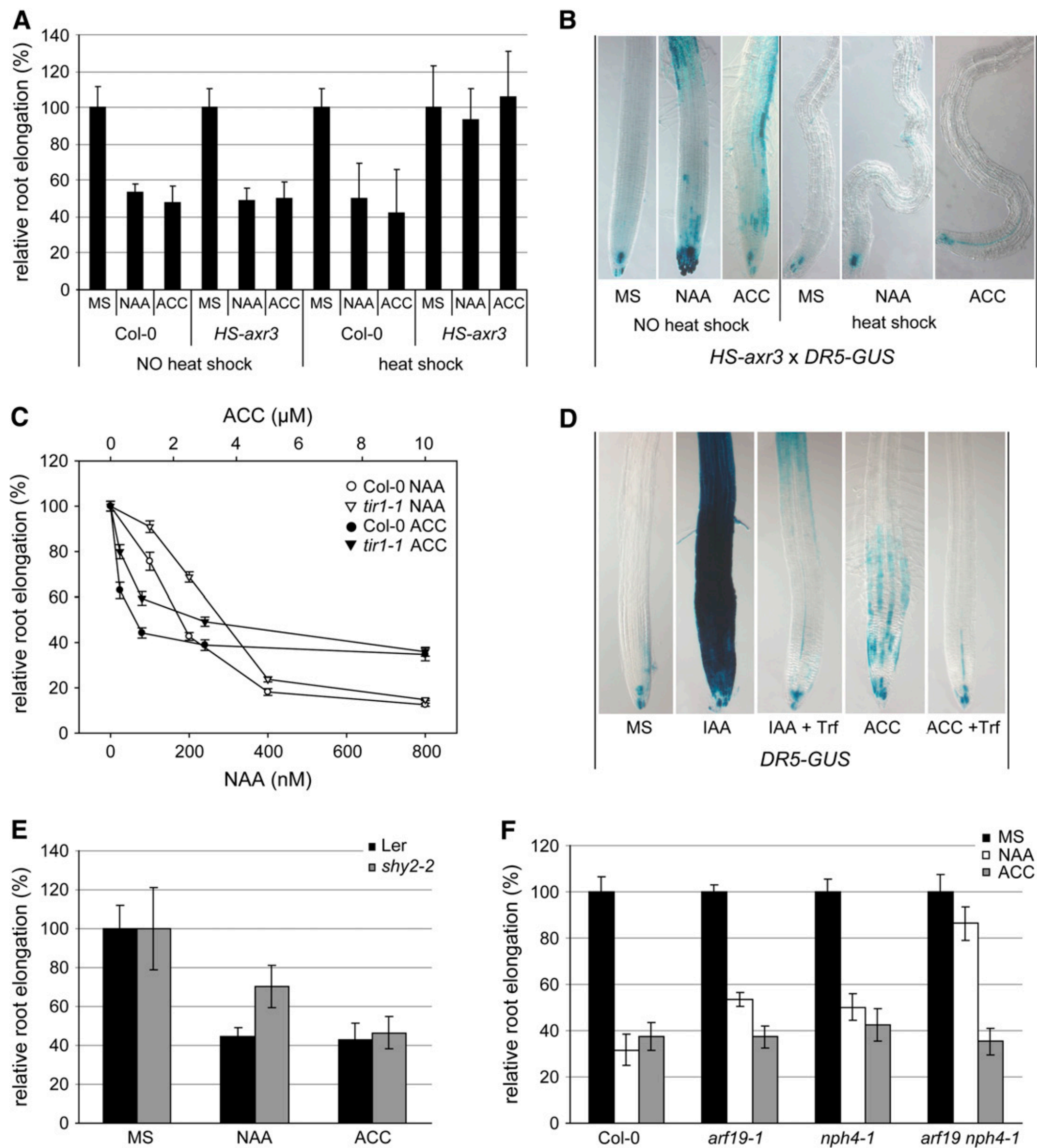

Figure 4. Auxin- and Ethylene-Resistant Root Growth of Auxin Signaling Mutants.

(A) Ectopic expression of *HS-axr3* induced by heat shock leads to auxin- and ethylene-insensitive root growth. Statistically significant difference was confirmed between nontreated and heat shock–treated seedlings germinated either on NAA or ACC ($P < 0.05$, ANOVA). 100% corresponds to 8.8 ± 0.99 mm and 10.4 ± 1.06 mm of non-heat-shocked or to 9.2 ± 0.92 mm and 3.6 ± 0.81 mm of heat-shocked wild type and *HS-axr3* root growth following heat shock, respectively.

(B) NAA- and ACC-dependent inductions of *DR5-GUS* in the epidermal cells of the root meristem and cells of the elongation zone do not occur in the *HS-axr3* line after the heat shock.

(C) The *tir1-1* mutant exhibits auxin- and ethylene-insensitive root growth ($P < 0.05$, ANOVA; validated for 50, 100, and 200 nM NAA and 0.3 and 1 μM ACC). 100% corresponds to 23.1 ± 2.37 mm of the wild type and 22.7 ± 2.28 mm of *tir1-1* root length. For clarity, depicted data are means ± SE.

(D) Terfestatin A (Trf), a chemical inhibitor of auxin response, interferes with auxin- and ethylene-mediated ectopic upregulation of the *DR5-GUS* reporter in roots. Seedlings were incubated for 24 h in liquid medium containing, as specified, 50 μM Trf, 5 μM IAA, or 1 μM ACC.

(E) *shy2-2* (carrying the *IAA3*-stabilized allele) exhibits strong auxin ($P < 0.05$, ANOVA) but not significant ethylene resistance ($P > 0.05$, ANOVA) compared with the control. 100% corresponds to 10.6 ± 1.29 mm of the wild type and 9.9 ± 2.09 mm of *shy2-2* root length.

(F) *arf19-1* and *nph4-1/arf7* single mutants and *arf19-1 nph4-1/arf7* double mutants exhibit auxin but not ethylene resistance. Roots of all mutants were significantly resistant to NAA ($P < 0.05$, ANOVA) but not to ACC ($P > 0.05$, ANOVA) in comparison to the control. 100% corresponds to 10.5 ± 1.24 mm of the wild type, 13.0 ± 1.28 mm of *arf19-1*, 11.5 ± 1.27 mm of *nph4-1/arf7*, and 11.0 ± 1.64 mm of *arf19-1 nph4-1/arf7* root length.

Seedlings were grown, if not specified differently, in the presence of 200 nM NAA and 1 μM ACC. Data are mean ± SD (except in **[C]**).

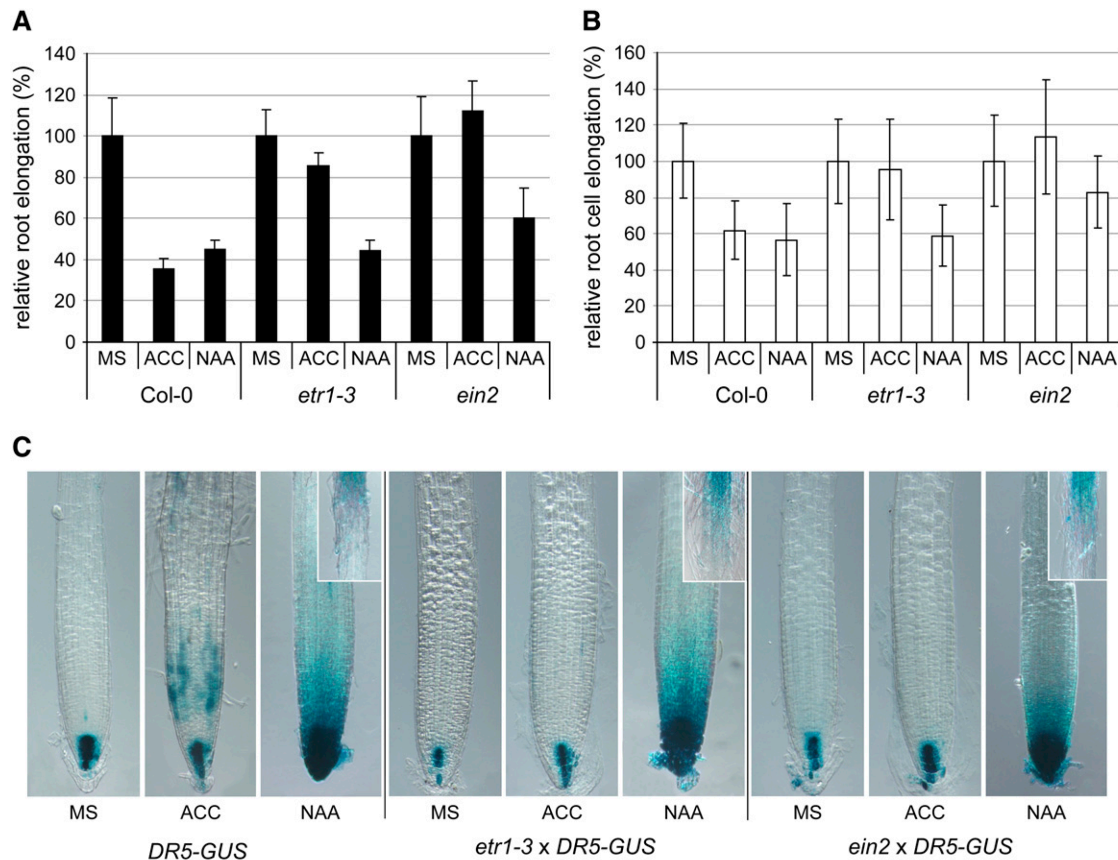

Figure 5. Root growth of ethylene-insensitive mutants on auxin.

(A) Root elongation of ethylene signaling mutants *etr1-3* and *ein2-1* is ACC resistant ($P < 0.05$, ANOVA). *etr1-3* is wild-type-like sensitive to auxin ($P > 0.05$, ANOVA), and *ein2-1* shows moderate, but statistically significant, auxin resistance ($P < 0.05$, ANOVA). 100% corresponds to 12.1 $\pm$ 2.53 mm of the wild type, 12.0 $\pm$ 2.75 mm of *etr1-3*, and 16.4 $\pm$ 0.89 mm of *ein2-1* root length.

(B) Root epidermal cell elongation of *etr1-3* and *ein2-1* is ACC resistant ($P < 0.05$, ANOVA). Root epidermal cells of *etr1-3* are wild-type-like sensitive to auxin ($P > 0.05$, ANOVA), and *ein2-1* shows moderate, but statistically significant, auxin resistance ($P < 0.05$, ANOVA).

(C) Ectopic *DR5-GUS* expression was induced by auxin but not by ACC in *etr1-3* and *ein2-1* mutants. Insets: strong *DR5-GUS* staining in the differentiation zone after auxin treatment.

Five-day-old seedlings were grown in the presence of 200 nM NAA and 1 μM ACC. Data are means $\pm$ SD.

revealed, similarly to those presented by Swarup et al. (2007), that ethylene regulates auxin biosynthesis in the root tips because AVG substantially reduced it (Figure 6D). However, no significant increase of auxin biosynthesis was observed (last 1 mm of the root tip) in response to the ACC treatment (Figure 6D), possibly reflecting an already saturated auxin biosynthesis rate in the roots. Thus, the exogenously applied ACC could not stimulate auxin biosynthesis additionally.

On the other hand, as shown by Swarup et al. (2007), when intact seedlings were treated with ethylene, a clear stimulatory effect on auxin biosynthesis was seen, suggesting an additional stimulation by ethylene of auxin biosynthesis in aerial tissues. To verify this possibility, we examined the ethylene response of roots detached from shoots by quantitative analysis of *DR5* activity. When compared with roots of intact, ethylene-treated seedlings, the increase of *DR5* activity in detached roots in response to ethylene was reduced. We observed a twofold higher *DR5* activity in dissected roots than that of the untreated roots, but a more than fourfold increased activity in roots of intact seedlings (Figure 6A). These findings suggest that ethylene stimulates auxin biosynthesis in different plant organs and that the additional auxin is delivered where it acts in the roots.

Ethylene Modulates Expression of Auxin Transport Components

Our results highlighted the important role of both auxin biosynthesis and auxin transport in ethylene-mediated regulation of root growth. To test whether ethylene, besides regulating auxin biosynthesis, might also modulate auxin transport, we investigated its effect on the expression and localization of major components of the auxin transport machinery. We used the *proPIN1,2,4,7-GUS* and *proAUX1-GUS* transgenic lines, previously established to be regulated similarly as the native genes

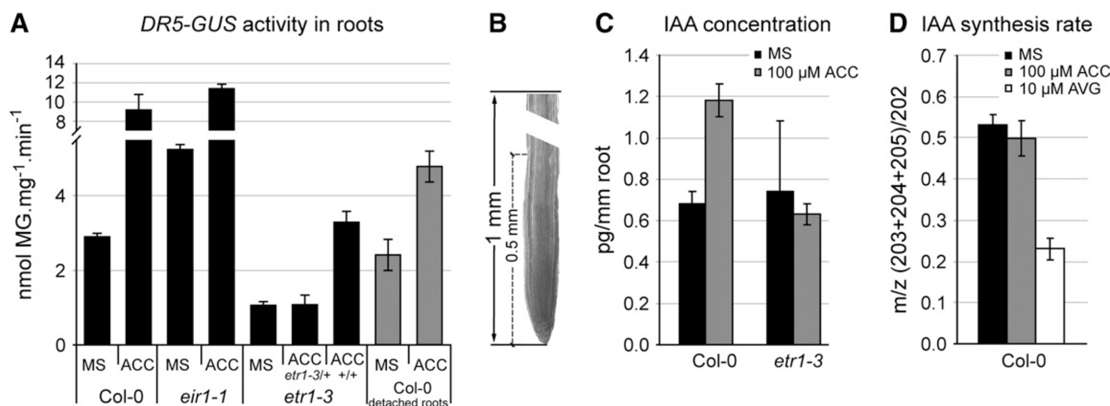

Figure 6. Local Stimulation of Auxin Biosynthesis by Ethylene.

(A) ACC induces *DR5* expression in roots of the wild type and *eir1-1* but not that of the *etr1-3* mutant (*etr1-3*/+ dominant phenotype, +/+ wild-type segregant). Intact seedlings (black bars) or detached roots (gray bars) were treated with or without 1 μM ACC. GUS activity in the roots was measured by a fluorimetric assay.

(B) The 1-mm root tip segment comprises the root meristem and elongation zone.

(C) ACC treatment increases auxin levels in the last 1 mm of the root tip of the wild type but not of the *etr1-3* mutant.

(D) AVG reduces the auxin biosynthesis rate in the root tip. By contrast, ACC treatment does not affect the auxin biosynthesis rate in the last 1 mm of the root tip.

Results of one of three independent experiments are presented. Data are means ± SD.

(Swarup et al., 2001; Vieten et al., 2005), and lines carrying functional *proPIN1,2-PIN1,2:GFP* and *proAUX1-AUX1:YFP* (Benkova et al., 2003; Blilou et al., 2005; Vieten et al., 2005; Abas et al., 2006). Whereas no ethylene-related effects on the subcellular localization of the auxin influx carrier AUX1 (Figure 7B) nor several auxin efflux carriers of the PIN family (Figure 7A; see Supplemental Figure 4A online; data not shown) were observed, these experiments revealed a complex regulation of multiple auxin transport components at the transcription level.

For example, expression of *proPIN2-GUS* and *proPIN2-PIN2:GFP* expression was upregulated after ACC treatment (Figures 7A and 7C). This upregulation was detectable after 5 to 10 h of incubation and was reduced by the inhibitor of ethylene perception Ag^+ (Figure 7A; data not shown). Also, the expression of other *PIN* genes, such as *PIN1* and *PIN4*, was regulated by ethylene as demonstrated by *proPIN1-GUS*, *proPIN1-PIN1:GFP*, and *proPIN4-GUS* reporters. A detectable increase in *PIN1* expression occurred within 10 h and was restricted to its endogenous expression domain of the root stele (see Supplemental Figure 4A online; data not shown). *PIN4* expression also was upregulated ectopically in the cortex and epidermal cells but reacted more slowly, first after ~24 h of ACC incubation (see Supplemental Figure 4B online; data not shown). By contrast, we did not see any clear effects of ethylene on the expression of *PIN7* (see Supplemental Figure 4B online).

In addition, the expression of the auxin influx component *AUX1* was influenced by ethylene, as demonstrated by the *proAUX1-GUS* and *proAUX1-AUX1:YFP* reporters. However, *AUX1* expression was upregulated more slowly; typically, 36 h were required to see the stimulatory effect (Figures 7B and 7C; data not shown). Similarly to *PIN2*, inhibition of ethylene perception by Ag^+ reduced the ethylene stimulatory effect on *AUX1* expression (Figure 7B).

The stimulatory effect of ethylene on the expression of the auxin transport components *PIN2* and *AUX1* was not confirmed by monitoring their overall transcript levels using quantitative RT-PCR (qRT-PCR) (Figure 7D). Possibly, the sensitivity of the qRT-PCR approach performed in the 2 mm of the root tip was not sufficient to detect the relatively weak effects of ethylene on *PIN* and *AUX1* transcription, which were furthermore confined to few tissue types of the extreme root tip. Nonetheless, the *AUX1* transcript levels were significantly reduced in *etr1-3* mutant roots compared with the wild type (Figure 7E), consistent with the notion that ethylene signaling is required for *AUX1* expression. In summary, these results show that ethylene regulates the expression of several *PIN* auxin efflux components and the *AUX1* influx carrier by a direct or indirect mechanism. Thus, ethylene stimulates both auxin biosynthesis and the capacity of auxin transport.

DISCUSSION

Ethylene Influences Root Growth through Local Auxin Signaling

Biochemical and genetic data show that auxin and ethylene interact in the regulation of root growth (Swarup et al., 2002). However, the mechanism of their crosstalk during this process is largely unknown.

Root growth rate is determined by cell production in the meristem and the cell elongation rate of cells leaving the meristem. We demonstrated that ethylene does not affect cell cycle activity nor the size of the root meristems but strongly reduces the elongation of epidermal cells. Consistently, reduction of endogenous ethylene levels by AVG enhances root elongation. The inhibitory effect of ethylene on cell and root elongation is prevented by either chemical (Ag^+) or genetic inhibition of the

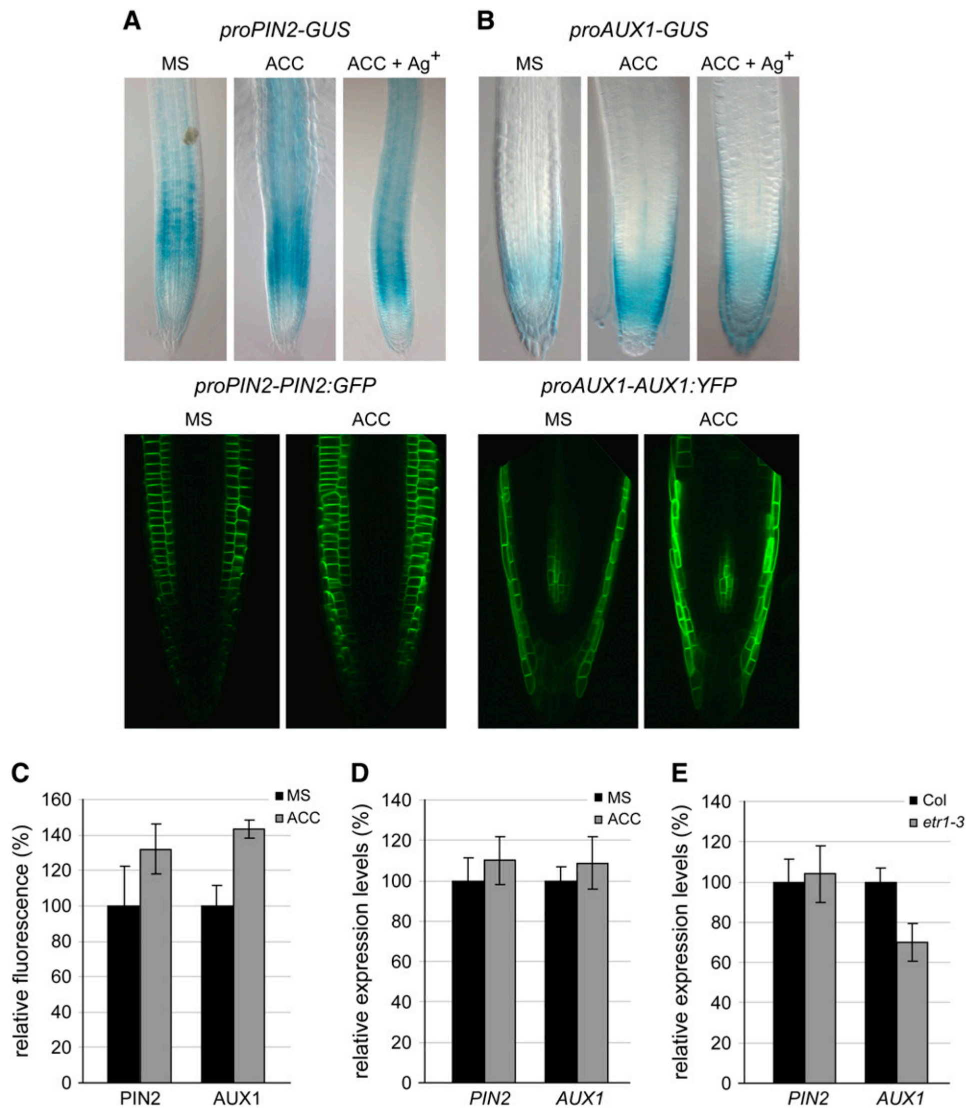

Figure 7. Ethylene-Modulated Expression of Auxin Transport Components.

(A) ACC upregulates the *PIN2* expression of *proPIN2-GUS* and *proPIN2-PIN2:GFP*. ACC effect is inhibited by silver ions.

(B) ACC upregulates the *AUX1* expression of *proAUX1-GUS* and *proAUX1-AUX1:YFP*. ACC effect is inhibited by silver ions.

(C) Quantification of membrane-located GFP/YFP fluorescence by image analysis of confocal sections; expression of *proPIN2-PIN2:GFP* and *proAUX1-AUX1:YFP* is significantly upregulated after ethylene treatment ($P < 0.05$, ANOVA).

(D) qRT-PCR analysis did not reveal ethylene stimulatory effect on *AUX1* and *PIN2* expression in the last 2 mm of the root tip. Results of one of three independent experiments are presented.

(E) qRT-PCR shows that *AUX1*, but not *PIN2* expression, is reduced in *etr1-3* in the last 2 mm of the root tip. *AUX1* RNA levels were significantly lower in the *etr1-3* mutant ($P < 0.05$, ANOVA). Results of one of three independent experiments are presented.

Five-day-old seedlings were grown in the presence of 1 μM ACC and 20 μM $AgNO_3$. Data are means ± SD.

ethylene signaling pathway (*etr1-3* and *ein2*). Our results suggest that the ethylene effect on root growth is primarily due to inhibition of cell elongation of cells leaving the root meristem.

Analysis of the role of ethylene signaling, auxin signaling, and auxin transport in root growth implies that an important part of the ethylene-driven inhibition of cell elongation is mediated by the downstream auxin signaling pathway. Indeed, auxin exhibits a negative effect on root growth, reminiscent of ethylene (Rahman et al., 2001; Swarup et al., 2002). Moreover, activation of the ethylene signaling pathway either by exogenously applied ethylene or in the *ctr1* mutant (Swarup et al., 2007) leads to a strong ectopic auxin response in the outer layers of the root meristem and cells of elongation zone. Furthermore, interference with the auxin response leads to both auxin- and ethylene-insensitive root growth, whereas impaired ethylene signaling leads to insensitivity to ethylene but not to auxin.

We propose that local activation of the auxin response in the epidermis of the meristem and of the elongation zone mediates a substantial part of the inhibitory ethylene effect on root growth. However, higher auxin than ethylene resistance in some auxin pathway-related mutants, along with less efficient induction of the *DR5* auxin reporter by ethylene than by auxin itself (at levels affecting root growth to the same extent), suggest an additional auxin-independent mechanism of the ethylene action. In agreement with this hypothesis, Stepanova et al. (2007) predicted at least three other types of interactions between auxin and ethylene, besides an auxin-mediated ethylene response, based on extensive gene expression analysis.

Ethylene Stimulates Auxin Biosynthesis

Mutual regulation of biosynthesis rates is a typical example of the interaction between plant hormonal pathways. A stabilizing effect of cytokinin (Cary et al., 1995; Chae et al., 2003; Wang et al., 2004) and auxin (Abel et al., 1995; Tsuchisaka and Theologis, 2004) on key enzymes in the ethylene biosynthesis of ACS5 and ACS4, respectively, has been reported; similarly, auxin and cytokinin have been shown to regulate each other's abundance (Eklof et al., 2000).

Increased auxin responses and direct auxin measurements suggest that auxin accumulates in the root tip in response to ethylene, indicating a stimulatory effect of ethylene on auxin biosynthesis. This process depends on the ethylene signaling pathway because impaired ethylene perception prevents accumulation of auxin in the root tip. Measurements of the biosynthetic rates revealed that ethylene is indeed involved in the regulation of auxin biosynthesis in roots, and, as elegantly demonstrated by Swarup et al. (2007), most of this biosynthesis is confined to the last 2 mm of the root tip. Although exogenously applied ethylene did not additionally upregulate auxin biosynthesis in the extreme root tip, probably because of an already saturated biosynthetic rate, reduction of endogenous ethylene levels decreased the auxin biosynthetic rate. Similar measurements in whole seedlings revealed that the auxin biosynthesis had increased significantly (Swarup et al., 2007). Consistently, comparison of the ethylene-induced increase of the auxin response in detached roots and roots of intact seedlings suggests that the auxin production in aerial tissues must be substantial and that at least part of the auxin detected in the root tip originates from the upper part of the plant. All these results emphasize that ethylene regulates the auxin biosynthesis rate in different plant organs.

The recent identification of *WEI2* and *WEI7* genes also provides an important molecular link between ethylene and auxin biosynthesis. These genes are expressed in roots, and their expression is stimulated by ethylene (Stepanova et al., 2005). It remains to be demonstrated whether this is the predominant molecular mechanism by which ethylene exerts its effect on auxin biosynthesis.

Ethylene Modulates the Capacity of Polar Auxin Transport

Our results suggest that auxin biosynthesis in response to ethylene is activated in various plant organs but that the ethylene-induced auxin response associated with the regulation of root growth occurs in specific regions of the root tip, namely, in the outer cell layers of the root meristem and in the elongation zone, implying specific delivery of auxin to these regions. To further support this scenario, mutants impaired in basipetal auxin transport, such as *pin2/eir1* and *aux1*, show ethylene-insensitive root growth (Roman et al., 1995; this article). A link between auxin transport and ethylene signaling is also supported by other mutants related to auxin transport, such as *root curl on NPA1*, with an altered response to NPA (Garbers et al., 1996), which is allelic to *enhanced ethylene response1* that exhibits ACC root resistance (Larsen and Chang, 2001; Larsen and Cancel, 2003).

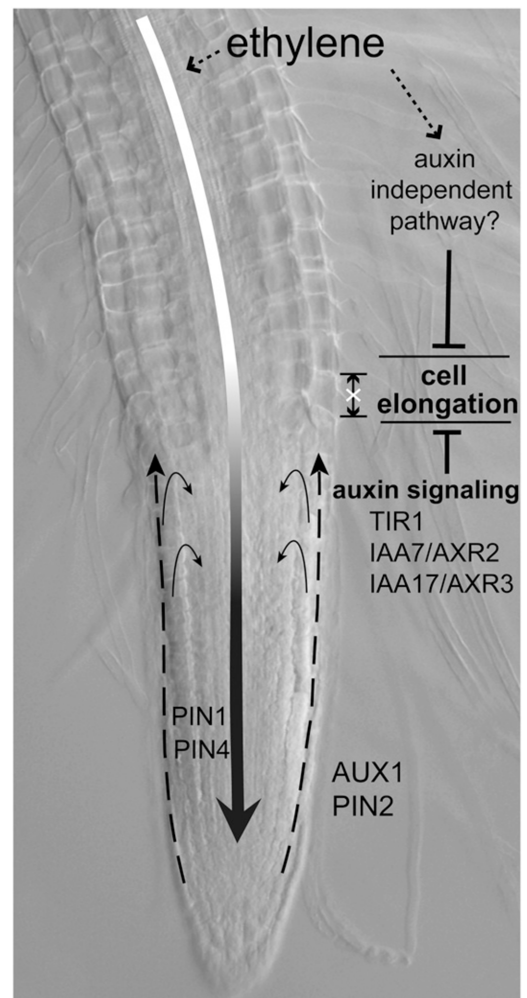

Figure 8. Model of Ethylene Action in the Root.

Ethylene stimulates the biosynthesis of auxin that is transported toward the root tip. Subsequently, basipetally transported auxin activates the local auxin response that is regulated by the auxin receptor (TIR1) and AUX/IAA (AXR2/IAA7 and AXR3/IAA17) and inhibits the cell elongation. Increased expression of auxin efflux carriers (PIN1, PIN4, and PIN2) and influx carrier (AUX1) enhances the capacity of acropetal and basipetal auxin transport. This mechanism can account for most but not all ethylene effects on the root growth.

Another example is *polar auxin transport inhibitor resistant2* that is allelic to *ein2* (Fujita and Syono, 1996). Together, these observations strongly indicate that auxin newly synthesized in response to ethylene is redistributed by the polar transport system.

Polar auxin transport regulates a variety of important developmental processes, such as embryogenesis, organogenesis, and tropisms (reviewed in Vieten et al., 2007). Polar auxin transport also plays an indispensable role in regulating root meristem actvity and root growth by maintaining auxin homeostasis and a stable asymmetric auxin distribution (Sabatini et al., 1999; Friml et al., 2002a; Blilou et al., 2005). Auxin has been shown to circulate in the root tip in an NPA-sensitive auxin reflux loop along both acropetal and basipetal streams (Blilou et al., 2005). Mutants impaired in basipetal transport were resistant to ethylene, whereas a reduction of the polar auxin transport in other than basipetal directions did not significantly affect root sensitivity to ethylene. Notably, inhibition of overall polar auxin transport by treatment with NPA did not prevent accumulation of auxin in the elongation zone nor the ethylene inhibitory effect on roots, despite the capability of NPA to inhibit root basipetal auxin transport (as shown by Rashotte et al., 2000). These results suggest that the basipetal auxin stream is the limiting factor for ethylene sensitivity of root growth and that the auxin levels in tissues supplied by this stream are critical. In agreement with our observations, Rahman et al. (2001) demonstrated that exogenously applied auxin can recover the ethylene response in *aux1* and *eir1* mutants. Similarly, we found that a further increase of auxin levels in responsive tissues by application of high ethylene concentrations (stronger stimulation of the auxin biosynthesis) elicits inhibition of the root growth in *eir1*. Furthermore, our data revealed that inhibition of polar auxin transport by NPA (increasing auxin levels in the root tip) (Sabatini et al., 1999; Friml et al., 2002a) sensitizes roots of *eir1* to ethylene. These observations collectively demonstrate that ethylene, besides stimulating auxin biosynthesis, also requires a functional auxin transport system to accumulate auxin in responsive tissues of the outer root meristem and in the elongation zone to achieve its specific physiological role of inhibiting root growth.

Here, we show that ethylene can also modulate the capacity of auxin transport. Several auxin efflux components, such as PIN1, PIN2, PIN4, and the influx carrier AUX1, are transcriptionally upregulated in response to ethylene. Upregulation of PIN1, PIN4, and AUX1 along the vasculature might increase the capacity of the main auxin transport pathway from the shoot to facilitate transport of newly synthesized auxin toward the root tip. PIN2 and AUX1 upregulation provides a means for basipetal auxin redirection because they are the pivotal transporters in this pathway.

Based on our results, we propose a model for ethylene-dependent root growth that accounts for a large portion of the ethylene effects on root growth. Ethylene stimulates auxin biosynthesis in different plant organs via its known signaling pathway. In addition, ethylene increases the capacity of auxin transport by regulating the transcription of auxin transport components. The additionally produced auxin is redistributed by polar auxin transport toward the root tip and, from there, through outer cell layers of the root meristem into the elongation zone. The major components of auxin transport in these tissues, AUX1 and PIN2, mediate auxin delivery into cells of the elongation zone, where auxin accumulates and induces local auxin responses that inhibit cell elongation and overall root growth (Figure 8).

METHODS

Plant Growth Conditions and Material

Seeds were sterilized with chloral gas, plated (0.5× Murashige and Skoog [MS] medium and 1% sucrose), stored for 2 d at 4°C in the dark, and then transferred to a plant growth room (20/18°C, 16-h photoperiod). Seedlings were harvested and processed 5 d after germination.

The transgenic *Arabidopsis thaliana* lines have been described elsewhere: *eir1-1* (Roman et al., 1995); *aux1-T* (R. Offringa, unpublished data); *DR5rev-GFP*, *35S-PIN1*, and *pin7-2* (Friml et al., 2003a); *pin1-3* (Okada et al., 1991); *pin4-1* (Friml et al., 2002a); *DR5-GUS* and *eir1-1* × *DR5-GUS* (Sabatini et al., 1999); *HS-axr3* (Knox et al., 2003); *axr2-1* (Wilson et al., 1990); *slr-1* (Fukaki et al., 2002); *tir1-1* (Ruegger et al., 1998); *etr1-3* and *ein2-1* (Guzman and Ecker, 1990); *proIAA2-GUS* (Luschnig et al., 1998); *proPIN2-GUS* (Friml et al., 2003b); *proPIN2-PIN2:GFP* (Xu and Scheres, 2005); *proAUX1-GUS* (Marchant et al., 1999); and *proAUX1-AUX1:YFP* (Swarup et al., 2004). *CYCB1-GUS*$_{DB}$ ensures rapid protein degradation (Ferreira et al., 1994).

A segregating F2 *etr1-3* × *DR5-GUS* line used for the fluorimetric assay was sorted according to root length and root hair phenotype when grown on ACC. ACC (Sigma-Aldrich) was prepared as 100 mM, AVG as 10 mM, Ag^+ ($AgNO_3$) as 20 mM stocks, dissolved with water, NAA (Duchefa) as 10 mM, NPA (Superco) as 20 mM, 2,3,5-triiodobenzoic acid (Sigma-Aldrich) as 50 mM, brefeldin A (Molecular Probes) as 50 mM, 9-hydroxyfluorene-9-carboxylic acid (Sigma-Aldrich) as 20 mM, dissolved with dimethylsulfoxide, and IAA (Duchefa) as 10 mM, dissolved with ethanol.

Phenotypical Analysis, Statistics, and Microscopy

Seedlings were photographed, and root lengths were measured (ImageJ; National Institutes of Health; http://rsb.info.nih.gov/ij). At least 20 seedlings were processed, and at least three independent experiments were performed, giving the same statistically significant results.

Root meristem lengths and epidermal cell lengths were measured on seedlings fixed in ethanol:acetic acid (3:1) and mounted in chloral hydrate. Microscopy was performed on a Zeiss Axiophot microscope equipped with an Axiocam HR CCD camera. Root meristem length was assessed as the distance between the quiescent center and the first elongating cell and measured with the ImageJ program. The same program was used also for measurements of the length of root epidermal cells. At least 20 seedlings were processed, and at least three independent experiments were done, giving the same statistically significant results.

Histochemical GUS staining analyses were done as described by Friml et al. (2003b). Immunodetection of PIN1 was performed as published by Sauer et al. (2006). For the confocal laser scanning microscopy, a Leica TCS SP2 AOBS was used. Images were processed in Adobe Photoshop. GFP fluorescence of membrane-localized proteins was quantified with the ImageJ program on confocal sections acquired with the same microscope settings. Approximately 10 seedlings/images were examined, and at least three independent experiments were done, giving the same statistically significant results.

To induce the heat shock in seedlings carrying the *HS-axr3* construct (Knox et al., 2003), they were incubated every 12 h for 60 min at 37°C. The first heat shock was applied 2 d after germination, the position of the root

tip was marked, and the difference between the points at days 2 and 5 was measured.

Data were statistically evaluated with NCSS 2007.

GUS Quantitative Assay

Seedlings or detached roots were incubated in control medium or in medium supplemented with ACC, respectively. Roots detached before or after incubation were collected in Eppendorf tubes (~50 roots per measurement) and homogenized with steel beads in GUS extraction buffer (50 mM potassium phosphate buffer, pH 7.2, 1 mM EDTA, 0.1% Triton X-100; all chemicals from Roth). The extract was centrifuged at 18,000*g* for 15 min at 4°C and the obtained supernatant used for measurements. In one experiment, all extractions were done in duplicates. Protein concentrations were normalized with the Bradford reagent (Bio-Rad). The fluorescence was measured in 96-well plates in total volumes of 250 μL with 455 μM 4-methylumbelliferyl-β-D-glucuronide hydrate (Sigma-Aldrich) on a Fluoroskan Ascent FL fluorometer (excitation of 365 nm and emission of 455 nm, 22°C). Each extraction was represented in doublets. Measurements were read each 15 min, and the standard curve was fitted. Enzyme activity was calibrated by concentration row of 4-methylumbelliferone (Sigma-Aldrich).

qRT-PCR

RNA was extracted with the RNeasy kit (Qiagen) from root samples (last 2 mm of the root tip). Poly(dT) cDNA was prepared from 1 μg of total RNA with Superscript III reverse transcriptase (Invitrogen) and quantified with an Icycler apparatus (Bio-Rad) with the qPCR core kit for SYBR green I (Eurogentec) according to the manufacturer's instructions. PCR was performed in 96-well optical reaction plates heated for 10 min to 95°C to activate hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 60 s at 95°C and annealing/extension for 60 s at 58°C. Targets were quantified with specific primer pairs designed with the Beacon Designer 4.0 (Premier Biosoft International). Expression levels were normalized to *ACTIN2* expression levels. All RT-PCR experiments were done in at least triplicates. The statistical significance was evaluated by the *t* test. The following primers were used: *ACTIN2* (5′-TTGACTACGAGCAGGAGATGG-3′ and 5′-ACAAACGAGGGCTGGAACAAG-3′), *PIN2* (5′-CCTCGCCGCACTCTTTCTTTGG-3′ and 5′-CCGTACATCGCCCTAAGCAATGG-3′), and *AUX1* (5′-TTGGTTCAGCTGCGCATCTA-3′ and 5′-GCAGTCCAGCTTCCTAGTAA-3′).

IAA Measurements

Seeds from *Arabidopsis* wild-type Columbia and the mutant line *etr1-3* were placed on agar plates (50 seeds in two rows/plate; 1% agar, 1% sucrose, and 1× MS medium, pH 5.7), stored for 3 d in 4°C, and grown for 5 d in vertical position at 23°C, with a 16-h photoperiod and light intensity of 150 μE.

For tissue-specific IAA biosynthesis measurements, seedlings were divided at the root-hypocotyl junction, and the root tissues were incubated for 24 h in liquid medium containing 30% D_2O, 1% sucrose, 1× MS, pH 5.7, ±100 μM ACC, or 10 μM AVG. The root tip (1 mm) was collected for analysis of the root-specific IAA biosynthesis. For each sample, 100 root tips were pooled and the samples were analyzed in triplicates after addition of 250 pg of $^{13}C_6$-IAA.

For the analysis of the IAA concentration in the root apex, intact seedlings were incubated for 24 h in liquid medium containing 1% sucrose, 1× MS, pH 5.7, and ±100 μM ACC. The root tip (1 mm) was collected and 50 root tips were pooled for each sample. Four replicates of the samples were purified after addition of 100 pg of $^{13}C_6$-IAA internal standard and analyzed by gas chromatography–selected reaction monitoring mass spectrometry as described (Ljung et al., 2005).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Influence of Polar Auxin Efflux Inhibitors on ACC-Mediated Root Growth Inhibition.

Supplemental Figure 2. Interference of PIN1 with PIN2 Function in *35S-PIN1*.

Supplemental Figure 3. Sensitivity of *axr2* and *slr* to Ethylene.

Supplemental Figure 4. Ethylene-Modulated Expression of Auxin Transport Components (*PIN1*, *PIN4*, and *PIN7*).

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