Trichoderma virens, a Plant Beneficial Fungus, Enhances Biomass Production and Promotes Lateral Root Growth through an Auxin-Dependent Mechanism in Arabidopsis[1][C][W][OA]

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Trichoderma species belong to a class of free-living fungi beneficial to plants that are common in the rhizosphere. We investigated the role of auxin in regulating the growth and development of Arabidopsis (Arabidopsis thaliana) seedlings in response to inoculation with Trichoderma virens and Trichoderma atroviride by developing a plant-fungus interaction system. Wild-type Arabidopsis seedlings inoculated with either T. virens or T. atroviride showed characteristic auxin-related phenotypes, including increased biomass production and stimulated lateral root development. Mutations in genes involved in auxin transport or signaling, AUX1, BIG, EIR1, and AXR1, were found to reduce the growth-promoting and root developmental effects of T. virens inoculation. When grown under axenic conditions, T. virens produced the auxin-related compounds indole-3-acetic acid, indole-3-acetaldehyde, and indole-3-ethanol. A comparative analysis of all three indolic compounds provided detailed information about the structure-activity relationship based on their efficacy at modulating root system architecture, activation of auxin-regulated gene expression, and rescue of the root hair-defective phenotype of the rhd6 auxin response Arabidopsis mutant. Our results highlight the important role of auxin signaling for plant growth promotion by T. virens.

Plant growth is affected by a plethora of environmental factors, including light, temperature, nutrients, and microorganisms. The region around the root, the rhizosphere, is relatively rich in nutrients, because as much as 40% of plant photosynthesis products can be lost from the roots (Bais et al., 2006). Consequently, the rhizosphere supports large microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth.

Trichoderma species are free-living fungi that are common in soil and root ecosystems. They have been widely studied for their capacity to produce antibiotics, parasitize other fungi, and compete with deleterious plant microorganisms (Harman et al., 2004a). Until recently, these traits were considered to be the basis for how Trichoderma exert beneficial effects on plant growth and development. However, it is becoming increasingly clear that certain strains also have substantial direct influence on plant development and crop productivity (Harman, 2006). Trichoderma enhancement of plant growth has been known for many years and can occur in axenic systems or in soils (Chang et al., 1986; Yedidia et al., 2001; Adams et al., 2007).

In maize (Zea mays) plants, Trichoderma inoculation affected root system architecture, which was related to increased yield of plants. Reported effects include enhanced root biomass production and increased root hair development (Bjorkman et al., 1998; Harman et al., 2004b). The root system is important for plant fitness because it provides anchorage, contributes to water use efficiency, and facilitates the acquisition of mineral nutrients from the soil (López-Bucio et al., 2005a). Many lines of evidence strongly support a role for auxin in the regulation of root system architecture. Application of natural and synthetic auxins increases lateral root and root hair development, whereas auxin transport inhibitors reduce root branching (Reed et al., 1998; Casimiro et al., 2001). The auxin-resistant mutants axr1 and axr2 produce fewer lateral roots than wild-type plants (Estelle and Somerville, 1987). Conversely, increased formation of lateral roots has been observed in Arabidopsis (Arabidopsis thaliana) mutants with elevated auxin content, including the rooty mu-

Figure 1. Effects of *T. virens* and *T. atroviride* inoculation on the growth of Arabidopsis seedlings. A, Photograph of 9-d-old Arabidopsis (Col-0) seedlings grown on the surface of agar plates containing 0.2× MS medium. Seedlings were treated with sterilized water at day 4 and photographed 5 d later. Bar = 1 cm. B, Representative photograph of Arabidopsis seedlings that were inoculated with *T. virens* at a distance of 5 cm from the root tip at 4 d after germination and grown for a further 5-d period. C, Photograph of Arabidopsis seedlings inoculated with *T. atroviride* at a distance of 5 cm from the root tip at 4 d after germination and grown for a further 5-d period. D, Effects of fungal inoculation on shoot biomass production. Photographs show representative individuals of four plates per treatment. Data from D show means ± SD from three groups of 10 seedlings that were recovered from the medium, excised at the root/shoot junction, and weighed on an analytical scale. Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results. [See online article for color version of this figure.]
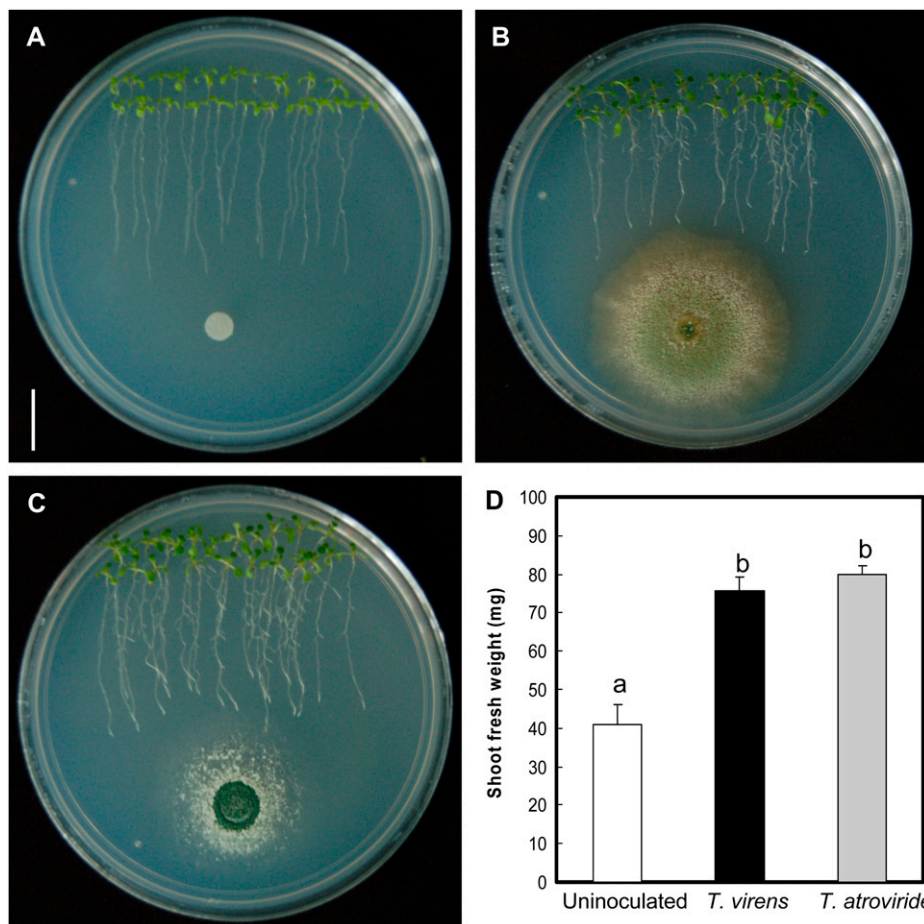
tant and its alleles *alf1* and *superroot1* (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995). Additional mutants with auxin-related phenotypes include *aux1*, *doc1*, and *eir1*. The *aux1-7* mutant is defective at the *AUX1* locus, which encodes an auxin influx facilitator participating in both acropetal and basipetal auxin transport at the root tip (Swarup et al., 2001). *doc1* is a mutant allele of *BIG*, which encodes a protein important for the correct location of certain auxin transport proteins (Gil et al., 2001), whereas *EIR1* encodes the auxin transporter *AtPIN2* (Luschnig et al., 1998). It has been determined that auxin deprivation keeps pericycle cells in G1 phase and readdition promotes the G1-S transition of the cell cycle, thus promoting lateral root initiation (Himanen et al., 2002). Despite auxin being a major player in root growth regulation, little is known about its role in plant growth promotion by fungi.

To elucidate the signaling mechanisms by which *Trichoderma* species promote plant growth and development, we evaluated the Arabidopsis response to inoculation with two *Trichoderma* species, *Trichoderma atroviride* (formerly known as *Trichoderma harzianum*) and *Trichoderma virens*. The two fungal species were found to promote Arabidopsis seedling growth under axenic conditions. Plant growth promotion elicited by these fungi correlated with prolific formation of lateral roots. A role for auxin signaling in mediating the observed developmental alterations by *T. virens* inoculation in plants was inferred from tests using the auxin-responsive marker constructs *DR5:uidA*, *BA3:uidA*, and *HS::AXR3NT-GUS* and the analysis of *aux1-7*, *doc1*, *eir1*, and *axr1* auxin-related mutants of Arabidopsis. We further show that *T. virens* is able to produce the indolic compounds indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), and indole-3-ethanol (IEt), which may play roles in mediating plant growth promotion by this fungus.

RESULTS

T. atroviride and *T. virens* Promote Growth and Development of Arabidopsis Seedlings

To study the plant growth-promoting activity of *T. atroviride* and *T. virens*, we used Arabidopsis as a model. Arabidopsis (ecotype Columbia [Col-0]) seedlings were germinated and grown for a 4-d period on petri plates containing agar-solidified 0.2× Murashige and Skoog (MS) medium. At day 4 after germination, the seedlings were treated with distilled sterilized water (control treatment) or with 10^6 spores of each
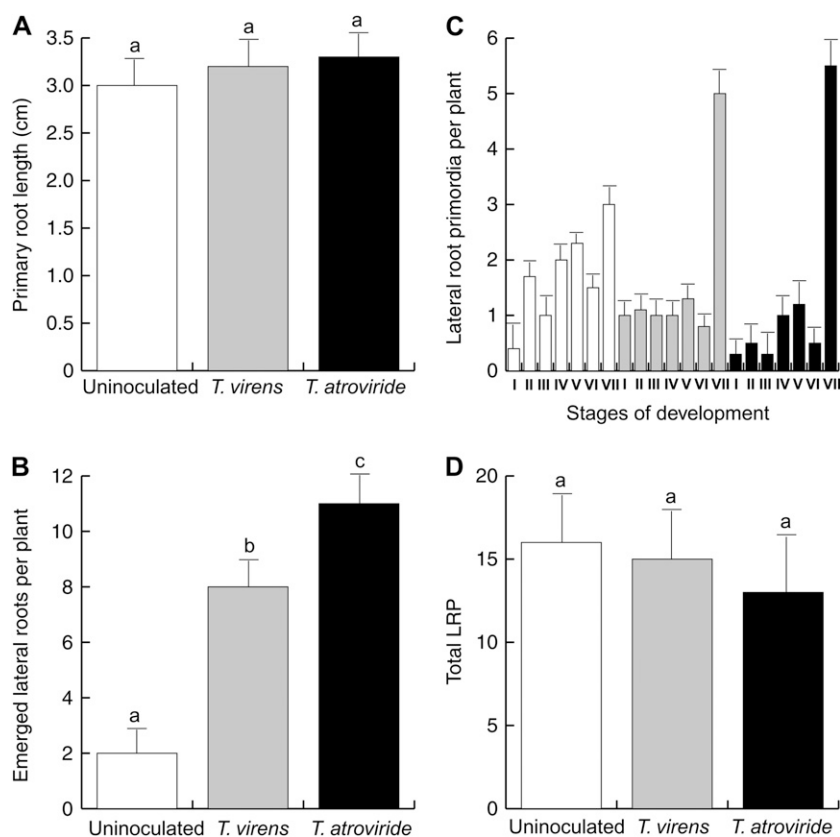
Figure 2. Effects of *Trichoderma* inoculation on Arabidopsis root system architecture. Arabidopsis Col-0 seedlings were germinated and grown for 4 d on the surface of agar plates containing 0.2× MS medium. Half of the plates were inoculated with *T. virens* or *T. atroviride* at a distance of 5 cm from the primary root tip and grown for an additional 5-d period. A, Primary root length. B, Lateral root number per plant. C, Stage number of lateral root primordia per plant. D, Total lateral root primordia per plant. Values shown are means ± SD (*n* = 30). Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

fungal species dissolved in water. Fungal spores were placed at a 5-cm distance from the primary root tip to test the possibility that diffusible fungal compounds could affect plant growth and development. After 5 d of growth in the presence of *T. atroviride* or *T. virens*, increases in shoot and root growth were observed (Fig. 1, A–C). Interestingly, fungal inoculation stimulated lateral root formation (Fig. 1, A–C) and increased shoot biomass production (Fig. 1D), indicating a beneficial effect of inoculation on plant growth and development.

T. atroviride and *T. virens* Alter Root System Architecture in Arabidopsis

To more closely analyze the effects of *Trichoderma* on plant development, primary root length and number of emerged lateral roots were determined in 9-d-old Arabidopsis seedlings grown on petri plates containing agar-solidified 0.2× MS medium after 5 d of fungal inoculation. No significant effects of inoculation with *T. atroviride* or *T. virens* were observed for primary root growth (Fig. 2A). However, a 4- to 6-fold increase in lateral root number was observed in seedlings inoculated with each fungus (Fig. 2B). The effect of *Trichoderma* at increasing the number of lateral roots could be due to the stimulation of lateral root growth or to the de novo formation of lateral root primordia (LRP) by activation of pericycle cells. To distinguish between

these two possibilities, LRP were quantified at day 5 after fungal inoculation. Seedling roots were first cleared to enable LRP at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997). We found that the stage distribution of LRP was affected by inoculation with *T. atroviride* or *T. virens*. In particular, stage VII LRP, which belongs to developing LRP with fully active meristems, was significantly increased in *T. atroviride*- and *T. virens*-inoculated seedlings (Fig. 2C). The total number of LRP per plant was similar between uninoculated and *Trichoderma*-inoculated seedlings (Fig. 2D). These data suggest that *Trichoderma* can promote root branching in Arabidopsis by inducing lateral root growth rather than by increasing de novo formation of LRP.

T. virens Alters Auxin-Inducible Gene Expression in Arabidopsis

The observed effect of *Trichoderma* in promoting lateral root development is similar to that described for auxins in plants (Casimiro et al., 2001). We next tested whether *T. virens* could alter auxin-regulated gene expression in Arabidopsis by inoculating *DR5:uidA* transgenic seedlings with this fungus. The *DR5:uidA* line has been used to study auxin-regulated gene expression in Arabidopsis (Ulmasov et al., 1997). *DR5:*
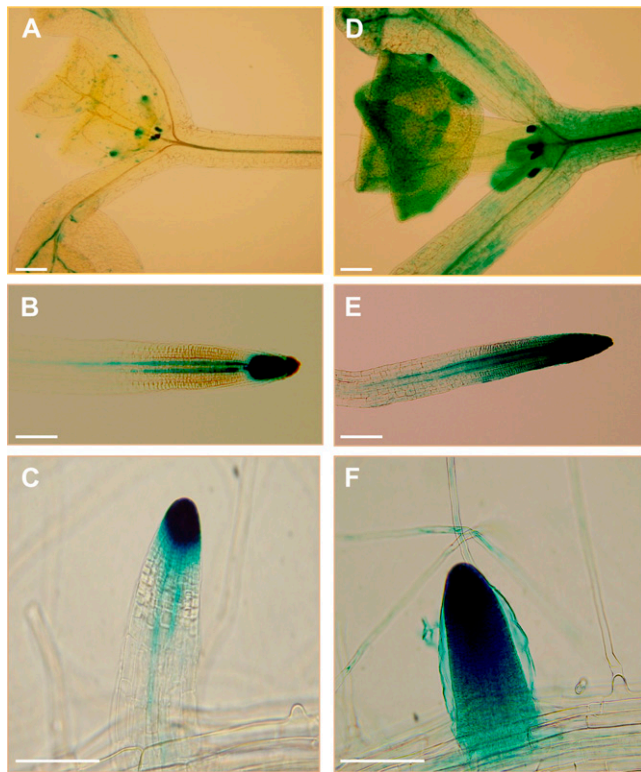
Figure 3. Effects of *T. virens* inoculation on auxin-regulated gene expression. Twelve-hour GUS staining of *DR5:uidA* primary roots of Arabidopsis seedlings grown for 4 d on agar-solidified 0.2× MS medium. A to C, Uninoculated seedlings. D to F, *T. virens*-inoculated seedlings. Photographs show representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. Bars = 100 μm. [See online article for color version of this figure.]

uidA seedlings were germinated and grown for 4 d on petri plates containing agar-solidified 0.2× MS medium and then inoculated with *T. virens* at 5 cm from the primary root tip. After an additional 5-d growth period, *DR5:uidA* seedlings were stained for GUS activity and further cleared to visualize changes in GUS expression. Although no significant effect of fungal inoculation was observed for primary root growth for wild-type (Fig. 2A) and *DR5:uidA* (data not shown) seedlings, an increase in GUS expression could be detected in shoots (Fig. 3, A and D), primary root tips (Fig. 3, B and E), and developing lateral roots (Fig. 3, C and F) from *T. virens*-inoculated seedlings when compared with uninoculated seedlings. These data suggest that *T. virens* inoculation increases auxin-regulated gene expression.

Effects of *T. virens* Inoculation on Growth and Lateral Root Development of Auxin-Related Arabidopsis Mutants

Next, we evaluated the effects of *T. virens* inoculation on growth of Arabidopsis wild-type seedlings and mutants defective in auxin transport (*aux1-7*, *doc1*, and *eir1*) or auxin response (*axr1-3*). Five days after plants were inoculated, *T. virens* increased by 62% shoot fresh weight in wild-type seedlings when compared with uninoculated seedlings. In contrast, all four mutant lines, *aux1-7*, *doc1*, *eir1*, and *axr1-3*, showed decreased or null responses in growth promotion by the fungus (Fig. 4A). We also quantified lateral root number in the wild type and all above mentioned mutants. It was found that *T. virens* inoculation induced up to a 4-fold increase in lateral root number when compared with uninoculated plants. Interestingly, a reduction in lateral root formation when compared with inoculated wild-type plants was observed for *aux1-7* and *axr1-3*
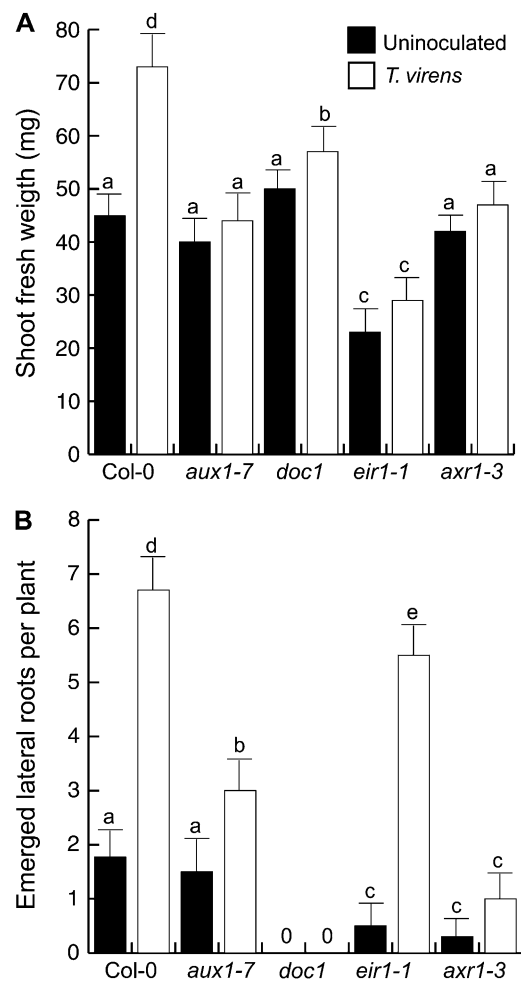

Figure 4. Effects of *T. virens* inoculation on biomass production and lateral root development in wild-type Arabidopsis (Col-0) and auxin-related mutants. Plant material was harvested 5 d after fungal inoculation. Shoots were excised at the root/shoot junction, and the fresh weight was determined on an analytical balance. A, Shoot fresh weight. B, Lateral root number per plant. Values shown represent means of four groups of 10 seedlings ± SD. Lateral roots were quantified for 30 seedlings. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated three times with similar results.
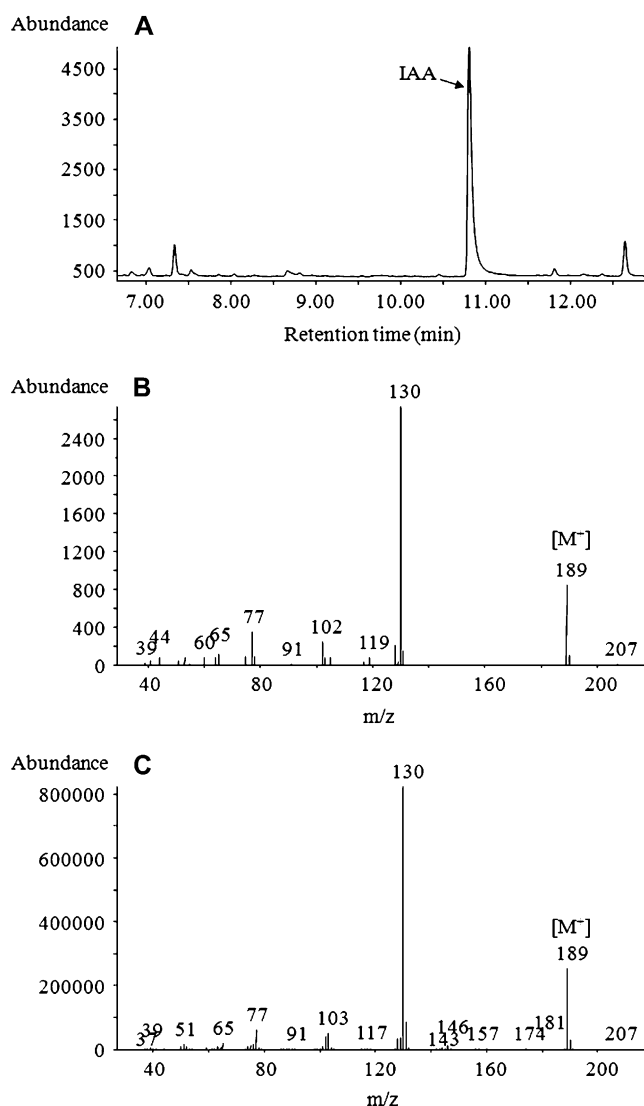
Figure 5. Determination of IAA from derivatized samples from *T. virens* growth medium by GC-MS. A, Total ion chromatogram of IAA from acidic ethyl acetate extract obtained from 1 L of culture medium of *T. virens*. B and C, The 70-eV electron-impact full-scan mass spectra from *m/z* 50 to 500 of IAA methyl ester identified in the extract (B) and the methylated IAA standard (C). Determinations were done from at least five independent samples.

inoculated seedlings, and no lateral root induction was registered for uninoculated or inoculated *doc1* seedlings (Fig. 4B). These results indicate that both normal auxin transport and response are important for promoting the effects of *T. virens* on plant growth and lateral root development.

T. virens Produces IAA, IAAld, and IEt

The induced expression of *DR5:uidA* by *T. virens* and the decreased response of auxin-related Arabidopsis mutants to fungal inoculation opens the possibility that the fungus could produce IAA or other auxin-like

compounds. We conducted experiments aimed at identifying IAA or IAA-related substances by growing *T. virens* on liquid cultures and determining indolic compounds from the supernatant by gas chromatography-mass spectrometry (GC-MS) analysis. We determined the actual (no Trp addition) and potential (100 mg L^{-1} Trp) production of indolic compounds produced by *T. virens* from either derivatized or underivatized samples from the growth medium. When derivatized samples were analyzed by GC-MS, we identified IAA (Fig. 5), which increases up to 17-fold in concentration in *T. virens* growth medium supplied with Trp (Table I). When underivatized samples from *T. virens* growth medium without Trp were analyzed for indolic compounds, the presence of IEt (retention time = 9.97 min) and IAAld (retention time = 8.83 min) was found (Fig. 6). The production of IEt was enhanced upon Trp addition, while a small yet significant increase in IAAld production was also detected in Trp-supplied cultures (Table I). IAA could not be further detected from underivatized samples.

IAAld Activates Auxin-Inducible Gene Expression

To determine if IAAld and IEt act in an auxin-related signaling pathway, we conducted analyses of the expression of the auxin-inducible *DR5:uidA* and *BA3:uidA* gene markers. Figure 7 shows histochemical staining for transgenic *DR5:uidA* and *BA3:uidA* seedlings that were grown for 6 d under IAA, IAAld, or IEt treatment. As reported previously (Ulmasov et al., 1997), in untreated control plants, *DR5:uidA* is absent from cotyledons and leaves and expressed primarily in the root tip region (Fig. 7, A and E). *DR5:uidA* seedlings grown at a concentration of 2 μM IAA showed GUS activity in the cotyledons and the primary root (Fig. 7, B and F). The pattern of GUS expression in *DR5:uidA* seedlings treated with 4 μM IAAld remained similar to that observed for IAA-treated plants (Fig. 7, C and G). In contrast, up to a 64 μM concentration of IEt showed a modest increase in expression of this marker (Fig. 7, D and H), indicating different auxin-related activity for the compounds. Untreated *BA3:uidA* plants did not show detectable levels of GUS activity (Fig. 7, I and M), whereas when treated with 2 μM IAA, they showed GUS expression mainly in petioles of cotyledons (Fig. 7J) and in the root elongation zone (Fig. 7N). GUS expression in plants treated with IAAld was clearly observed in the same regions as in IAA-treated seedlings (Fig. 7, K and O). IEt failed to activate *BA3:uidA* expression (Fig. 7, L and P). These results show that IAAld, IEt, and IAA treatments can differentially activate the expression of auxin-inducible gene markers.

IAAld Enhances Aux/IAA Protein Degradation

Auxin promotes the degradation of Aux/IAA repressor proteins via the ubiquitin-proteasome pathway and thereby induces primary auxin-responsive

Table I. *Quantification of auxin-like compounds from T. virens*

T. virens was inoculated in 1 L of nutrient solution with or without 100 mg of L-Trp, and determinations were performed by GC-MS after 3 d of growth. Data shown are means ± SE for samples from three independent cultures (n = 3). Different letters represent means statistically different at the 0.05 level.

| Compound	Retention Time	Concentration	
		(−) L-Trp	(+) L-Trp
	min	*μg L^{−1}*	
IAAld	8.83	59.4 ± 4.47[a]	70.15 ± 3.78[b]
IEt	9.97	72.33 ± 1.41[a]	141.88 ± 4.85[b]
IAA	10.81	13.48 ± 0.97[a]	233.64 ± 3.06[b]

gene expression (Gray et al., 2001). To address the effect of IAA, IAAld, and IEt on auxin-mediated degradation of Aux/IAA proteins, we examined the effects of these compounds on Aux/IAA stability using the Arabidopsis *HS::AXR3NT-GUS* line, in which a translational fusion between domains I and II of AXR3 and the GUS reporter protein is expressed under the control of a heat shock promoter (Gray et al., 2001). Seedlings expressing the *HS::AXR3NT-GUS* construct were heat shocked at 37°C for 2 h and further treated with 5 μM IAA, IAAld, or IEt for 5, 10, 20, and 60 min. Treatment with IAA or IAAld showed enhanced degradation of the fusion protein in a similar way, but IEt failed to induce degradation of the fusion protein even after 60 min of treatment (Fig. 8, A–P). Our data indicate that IAAld likely acts in an auxin-mediated signaling pathway, either by direct binding to an auxin receptor or by its conversion to IAA, which rapidly destabilizes the AXR3 protein.

IAAld and IEt Differentially Regulate Arabidopsis Root System Architecture

To determine more closely the effects of IAAld and IEt on the architecture of the Arabidopsis root system, wild-type Arabidopsis seedlings were germinated and grown on vertically oriented agar plates containing 0.2× MS medium supplied with IAAld or IEt concentrations ranging from 0.25 to 8 μM. Under these conditions, primary root length, number of lateral roots, and lateral root density were quantified. After 10 d of growth, it was observed that concentrations of IAAld greater than 1 μM inhibited primary root growth in a dose-dependent way (Fig. 9A). It was observed that IAAld-treated Arabidopsis seedlings produced a highly branched root system with abundant lateral roots. A roughly 2-fold increase in lateral root number per plant was found at concentrations of IAAld from 0.25 to 2 μM when compared with solvent-treated

Figure 6. Determination of indolic compounds from underivatized samples from *T. virens* growth medium by GC-MS. A, Total ion chromatogram of IAAld and IEt from neutral ethyl acetate extract obtained from 1 L of culture medium of *T. virens*. B to E, The 70-eV electron-impact full-scan mass spectra from *m/z* 50 to 500 of IAAld identified in the extract (B), the standard IAAld (C), IEt identified in the extract (D), and the standard IEt (E). Determinations were done from at least five independent samples.
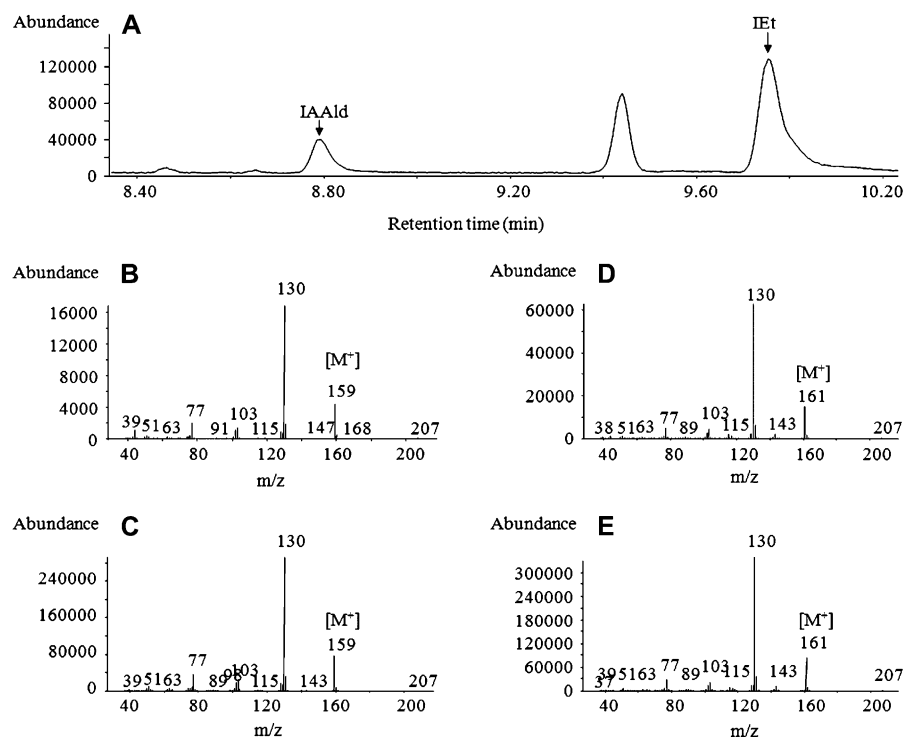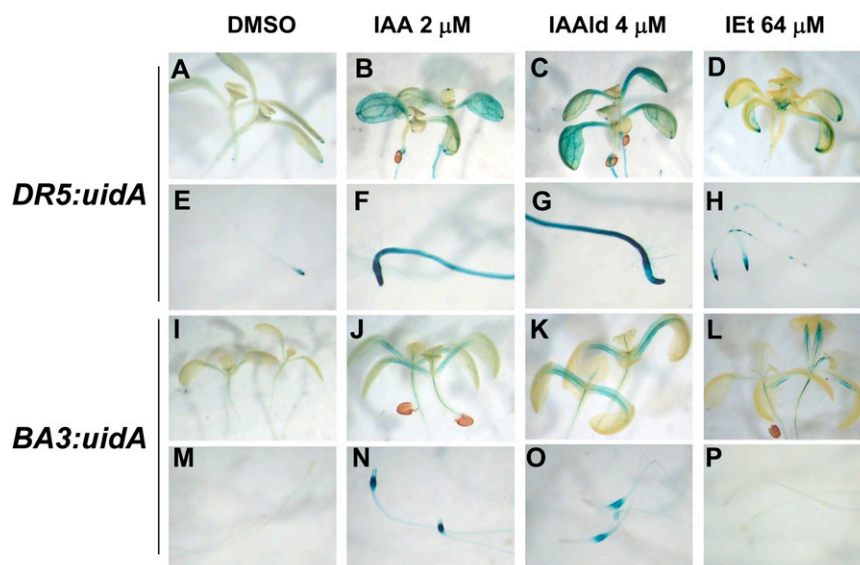
Figure 7. Effects of indolic compounds produced by *T. virens* on auxin-regulated gene expression. A to H, Twelve-hour GUS staining of *DR5:uidA* Arabidopsis seedlings grown for 6 d on agar plates containing 0.2× MS medium (A and E) and on medium supplied with 2 μM IAA (B and F), 4 μM IAAld (C and G), or 64 μM IEt (D and H). Notice the increase in GUS expression in shoots and roots in the treatments with IAAld. I to P, Twelve-hour GUS staining of *BA3:uidA* Arabidopsis seedlings grown for 6 d on agar plates containing 0.2× MS medium (I and M) and on medium supplied with 2 μM IAA (J and N), 4 μM IAAld (K and O), or 64 μM IEt (L and P). Notice the increase in GUS expression in the root elongation region in the treatments with IAA or IAAld. Photographs are representative individuals of at least 20 stained seedlings. The experiment was repeated twice with similar results. DMSO, Dimethyl sulfoxide. [See online article for color version of this figure.]

control seedlings (Fig. 9B). The density of lateral roots was also calculated by dividing the number of lateral roots by the length of the primary root to normalize for the effects of IAAld on primary root length. Lateral root density increased over 2-fold in plants treated with IAAld when compared with untreated seedlings (Fig. 9C). This increase in lateral root density was due to a stimulatory effect of IAAld on both LRP formation and lateral root emergence (Supplemental Fig. S1).

Interestingly, after 12 d of growth, IEt showed modest activity at inhibiting primary root growth (Fig. 10A) and failed to increase lateral root formation even when supplied at concentrations up to 64 μM (Fig. 10B). Lateral root density significantly increased only at 64 μM IEt concentration in the medium (Fig. 10C), indicating that this compound acts at high concentrations to activate pericycle cells. These results show that IAAld and IEt have different activity in Arabidopsis root system architecture modulation and that the effects of fungal inoculation on root development are likely due to a combined effect of all three indolic compounds, IAA, IAAld, and IEt, produced by the fungus.

IAAld Rescues the Root Hair-Defective Phenotype of the Auxin-Related *rhd6* Arabidopsis Mutant

Arabidopsis root hairs are a good system in which to study cell differentiation and morphogenesis in plants. The study of their development is also of great interest because of their putative function in water and nutrient uptake. Several auxin-related mutations have been found to alter root hair development (Parker et al., 2000). Of particular interest is the *rhd6* mutant, which is defective in root hair initiation and has been shown previously to be rescued by auxin (Masucci and Schiefelbein, 1994). We used the *rhd6* mutant as a tool to probe the mechanism of IAAld action. We compared the root hair response of Arabidopsis wild-type seedlings and *rhd6* mutants with IAA and IAAld treatments at day 5 after germination. As shown in Figure 10, treatments with 0.5 μM IAA or 4 μM IAAld stimulated root hair elongation and increased root hair formation at the primary root tip region in Arabidopsis wild-type seedlings (Fig. 11, A–C). *rhd6* mutant seedlings grown in medium without auxin were completely devoid of root hairs (Fig. 11D). Interestingly, both IAA and IAAld were found to rescue the *rhd6* root hair-defective phenotype (Fig. 11, E and F). The root hairs produced in each of these experiments exhibited normal growth and morphology. These results imply that the application of IAAld can suppress the root hair formation defects of *rhd6*.

IAA and IAAld Alter Arabidopsis Biomass Production in a Dose-Dependent Way

The fact that *T. virens*-enhanced shoot biomass production was dependent on auxin transport/signaling prompted us to determine whether exogenous auxin

Figure 8. Analysis of Aux/IAA stability with *HS::AXR3NT-GUS* fusions. Wild-type seedlings expressing the *HS::AXR3NT-GUS* constructs were heat shocked at 37°C for 2 h. After heat induction, the seedlings were treated with IAA, IAAld, or IEt for different time periods at the indicated concentrations and stained overnight for GUS activity. Notice the degradation of the fusion protein by either IAA or IAAld. A to P, Representative photographs of cotyledons (*n* = 10 stained seedlings). Similar results were obtained in two independent experiments. [See online article for color version of this figure.]
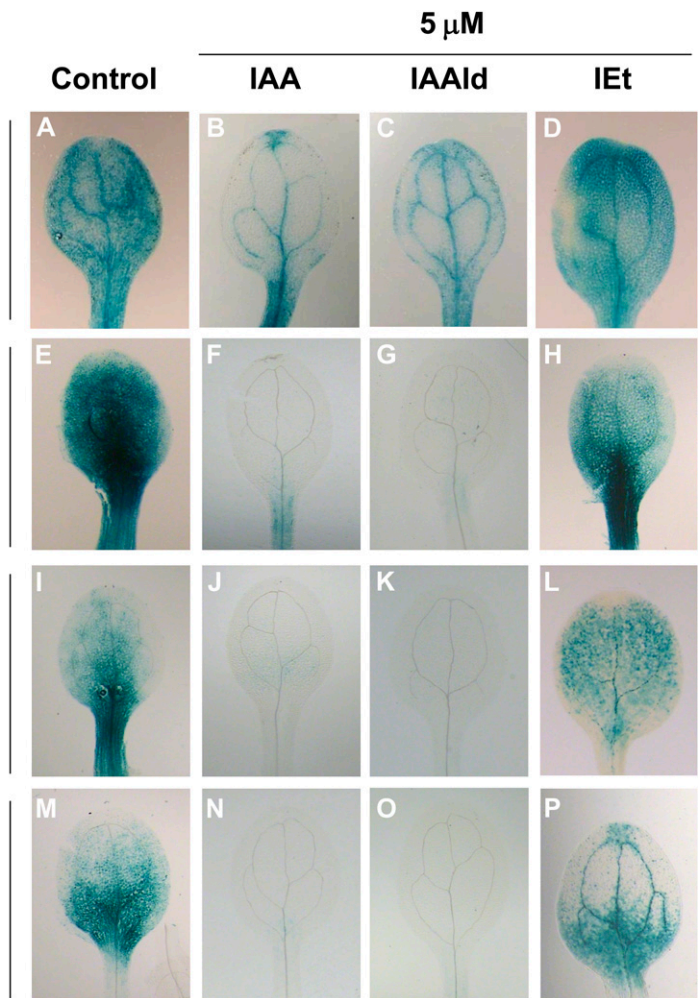
application could increase the growth of Arabidopsis seedlings. We quantified root, shoot, and total fresh weight of plants grown under varied concentrations of IAA or IAAld. Treatments of 15 to 60 nM IAA significantly increased root, shoot, and total fresh weight when compared with control plants, while concentrations of 120 to 960 nM did not affect or decreased biomass production (Fig. 12). Similar dose-dependent effects on growth were observed for IAAld-treated plants, albeit at greater concentrations than IAA (Supplemental Fig. S2).

To further define whether the effects of IAAld are mediated by auxin transport/signaling, we performed experiments to investigate the resistance of auxin-related mutants to exogenous application of IAAld. A commonly used developmental marker for auxin responses is primary root growth. Therefore, we grew wild-type plants and the auxin-related mutants *aux1-7*, *doc1*, *eir1-1*, and *axr1-3* in medium with or without 8 μM IAAld, a concentration that inhibits root growth. Our results show that *aux1-7*, *eir1-1*, and *axr1-3* are indeed very resistant to IAAld and sustained primary root

growth in an IAAld concentration that drastically inhibits growth in wild-type plants (Supplemental Fig. S3). Thus, we conclude that both auxin transport and response are important for root developmental responses to IAAld.

DISCUSSION

T. virens Promotes Arabidopsis Growth and Development through an Auxin-Dependent Mechanism

Trichoderma species are naturally occurring soil fungi that colonize roots and stimulate plant growth. Such fungi have been applied to a wide range of plant species for the purpose of growth enhancement, with a positive effect on plant weight, crop yields, and disease control. Their agricultural use could be expanded if the mechanisms of growth enhancement were known. A number of mechanisms for plant growth promotion by *Trichoderma* have been proposed (Harman et al., 2004a). Among these, fungal interaction with auxin
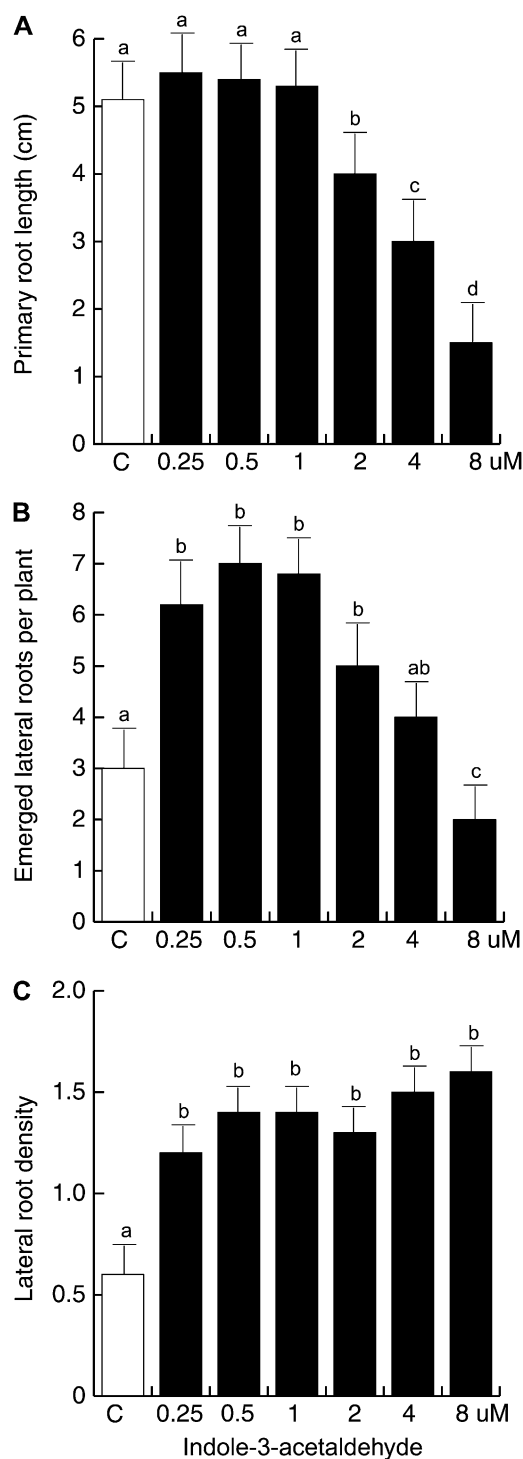
Figure 9. Effects of IAAld on Arabidopsis root architecture. Wild-type Col-0 seedlings were grown for 10 d under increasing IAAld concentrations on vertically oriented agar plates. Data are given for the length of the primary root (A), lateral root number (B), and lateral root density (C). Values shown represent means of 30 seedlings ± sd. Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

signaling has not been examined, despite auxin being a central plant growth-regulating substance.

It was noticeable that inoculation with *Trichoderma* affected lateral root development in Arabidopsis wild-type plants in a way that suggests that the effects are mediated by auxin (Figs. 1 and 2). IAA is a molecule that is synthesized by plants and a few microbes (Woodward and Bartel, 2005). In plants IAA plays a key role in root and shoot development. The hormone moves from one part of the plant to another by specific transporter systems that involve auxin importer (*AUX1*) and efflux (*PIN1-7*) proteins. IAA is a key regulator of lateral root development and root hair development (Casimiro et al., 2001). Expression studies of the auxin-inducible marker *DR5:uidA* suggested that *T. virens* inoculation increases the auxin response in Arabidopsis seedlings (Fig. 3). To further elucidate some of the aspects of auxin transport/perception involved in the Arabidopsis response to *T. virens*, we analyzed the growth and development of Arabidopsis mutants with defects in the auxin signal transduction pathway. We found that the auxin transport mutants (*aux1-7*, *eir1*, and *doc1*) have a reduced response to the fungus in terms of growth promotion (Fig. 4A) and lateral root development (Fig. 4B). In particular, the *doc1* mutant, which shows defects in lateral root initiation that can be complemented by nutrient deficiency (López-Bucio et al., 2005b), showed null induction of lateral roots when inoculated with *T. virens* (Fig. 4B). These results indicate that normal auxin transport is important for plant responses to *T. virens*. The finding that the auxin-resistant *axr1-3* mutant also shows a reduced response to inoculation suggests that the corresponding wild-type gene is required in Arabidopsis for increased growth and lateral root formation when inoculated with the fungus (Fig. 4). *AXR1* encodes a protein related to the ubiquitin-activating enzyme E1 (Leyser et al., 1993). These results indicate that plant growth promotion by *T. virens* operates through the classical auxin response pathway.

T. virens Produces IAA, IAAld, and IEt

In this study, we determined the presence of IAA (Fig. 5) and of two substances structurally related to IAA, namely IAAld and IEt, in *T. virens* growth medium (Fig. 6). When Trp was added to the growth medium of *T. virens*, an increased production of all three metabolites was evident (Table I). Although it is widely accepted that plants use several pathways to synthesize IAA, none of the pathways are yet defined to the level of knowing each relevant gene, enzyme, and intermediate. Several Trp-dependent pathways have been proposed: the indole-3-pyruvic acid (IPA) pathway, the indole-3-acetamide pathway, the tryptamine pathway, and the indole-3-acetaldoxime pathway (Woodward and Bartel, 2005). The IPA pathway (Trp → IPA → IAAld → IAA) is important in some IAA-
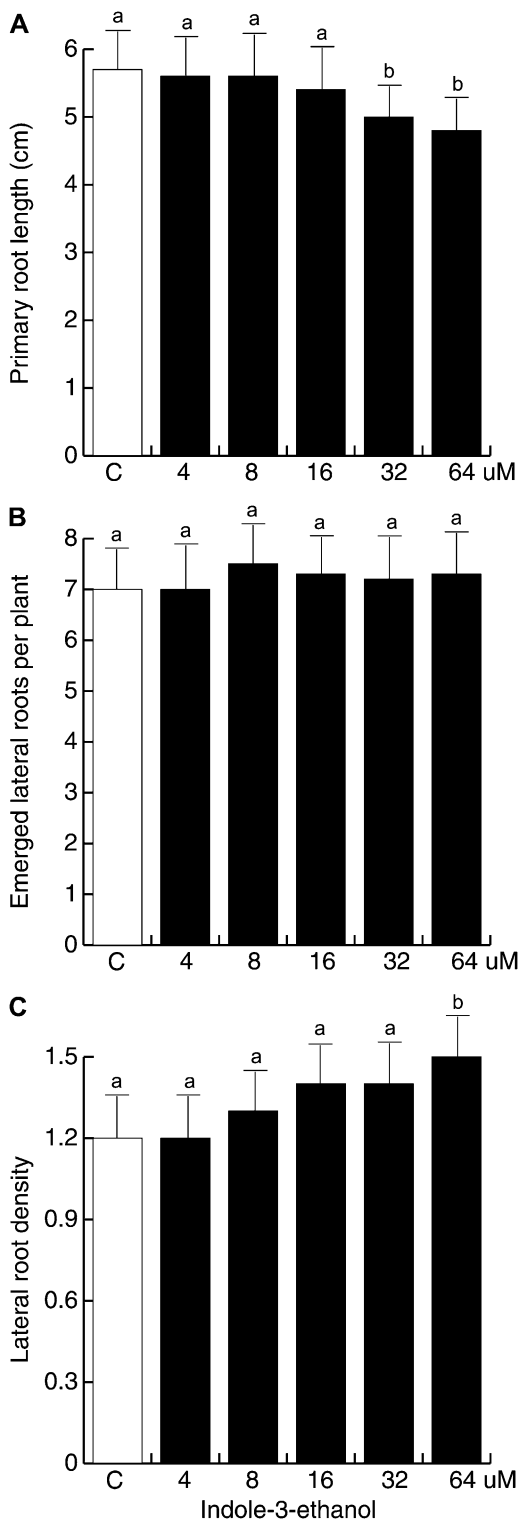
Figure 10. Effects of IEt on Arabidopsis root architecture. Wild-type Col-0 seedlings were grown for 12 d under increasing IEt concentrations on vertically oriented agar plates. Data are given for the length of the primary root (A), lateral root number (B), and lateral root density (C). Values shown represent means of 30 seedlings ± SD. Different letters represent means statistically different at the 0.05 level. The experiment was repeated three times with similar results.

synthesizing microorganisms (Koga, 1995), and recently it was demonstrated that it operates in plants as well (Stepanova et al., 2008; Tao et al., 2008). The final enzyme in the proposed IPA pathway is an IAAld-specific aldehyde oxidase (AAO1) that has increased activity in the IAA-overproducing *superroot1* mutant (Seo et al., 1998). The identification of Arabidopsis AAO1 suggests that plant- and microbe-produced IAAld can be used to produce IAA in plants. Several lines of evidence support the view that the rate of auxin biosynthesis is subject to regulation, with several IAA precursors acting as storage compounds. IAAld can be converted to IEt by an indole acetaldehyde reductase enzyme. This enzyme has been characterized in cucumber (*Cucumis sativus*) seedlings, where it plays an important role in auxin biosynthesis (Brown and Purves, 1980). Both IAAld and IEt occur naturally in plants (Purves and Brown, 1978; Magnus et al., 1982), which suggests that these compounds can act as flexible storage pools for IAA. Although IEt does show a modest auxin-like activity in activating the auxin-regulated gene markers *DR5:uidA* and *BA3:uidA* (Fig. 7), conversion of IEt to IAA has been already demonstrated in cucumber seedling shoots (Rayle and Purves, 1967).

Relatively little information is available on IAA biosynthesis in fungi. Production of IAA through the IPA pathway was identified in the fungus *Colletotrichum acutum* (Chung et al., 2003). HPLC analysis and chromogenic stains after a fluorescence thin-layer chromatography separation unambiguously identified IAA, IEt, IAAld, and IPA from cultures supplemented with Trp. Interestingly, increasing Trp concentrations drastically increased the levels of IEt but not IAA (Chung et al., 2003). It has been suggested that in this case IEt may be the end product of Trp metabolism rather than a side product of the IPA pathway. In contrast, our results show that IAA levels dramatically increase in Trp-supplied cultures of *T. virens* (Table I); it is tempting to speculate that Trp supply to *T. virens* cultures increases IAA accumulation as a direct product of its metabolism.

IAAld Shows Auxin-Like Activity in Arabidopsis

To maximize the capability of an organ to expand or elongate, or to establish a particular developmental program such as lateral root formation, plants have evolved mechanisms tightly coupled to the perception of biotic and abiotic stimuli. Many of the plant responses to environmental factors are mediated by phytohormones, such as auxin.

IAA has been found to be the typical auxin in plants, mainly evaluated by cell elongation tests in hypocotyls and primary root growth responses (Woodward and Bartel, 2005). However, the chemical space, which encompasses the term "auxin," is actually not easily achieved, since many compounds were found to exhibit an auxin-like activity in several different bioassays (Ferro et al., 2007). Our compar-
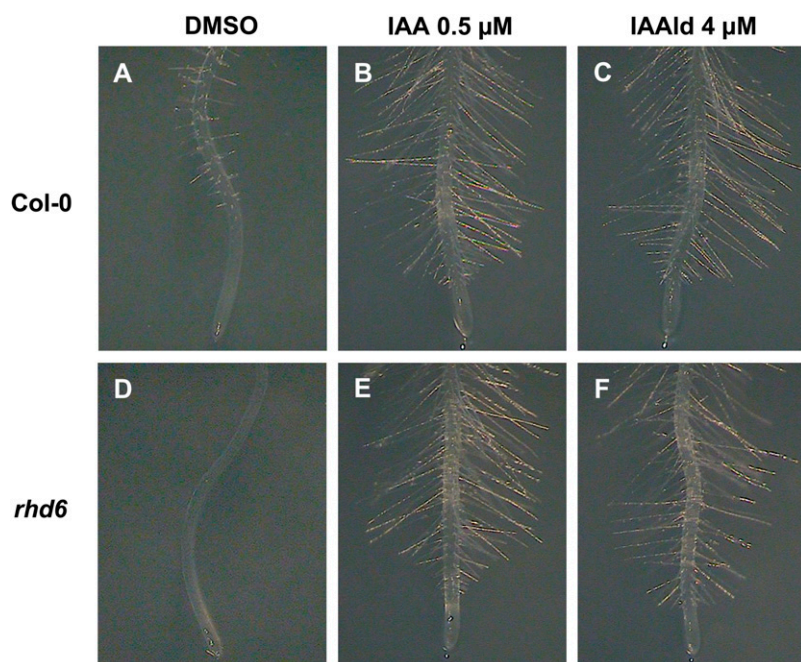
Figure 11. IAAld rescues the *rhd6* mutant phenotype. A, Wild-type Col-0 Arabidopsis root with normal root hair formation. B and C, Root hair formation in response to IAA (B) or IAAld (C) treatment. D, A typical *rhd6* Arabidopsis mutant root showing a reduction in root hair formation. E and F, Formation of root hairs in *rhd6* roots in response to IAA (E) or IAAld (F) treatment. The experiment was repeated three times with similar results. DMSO, Dimethyl sulfoxide. [See online article for color version of this figure.]

ative analysis of auxin activity for IAA, IAAld, and IEt (Fig. 7) identified IAAld, an IAA precursor in the IPA pathway, as an active auxin. Three additional lines of evidence indicate that IAAld acts as an auxin: (1) the effect of the compound on Aux/IAA stability using the Arabidopsis *HS*::*AXR3NT-GUS* line; (2) the regulation of root system architecture by its exogenous application to the seedlings; and (3) the rescue of the root hair-defective phenotype of the *rhd6* mutant of Arabidopsis when exogenously supplied to the growth medium. Treatment with IAA or IAAld showed enhanced degradation of the fusion protein *HS*::*AXR3NT-GUS* in a similar way, but IEt failed to induce degradation of the fusion protein even after 60 min of treatment (Fig. 8). These data indicate that IAAld likely acts in an auxin-mediated signaling pathway. Interestingly, exogenously supplied IEt was found to inhibit primary root growth and to increase lateral root density at a 64 μM concentration (Fig. 10), a much higher concentration than that required for IAA or IAAld to affect the same developmental traits. Compelling evidence that IAAld shows auxin-like activity came from the analysis of the root hair response in wild-type and *rhd6* mutant seedlings to this compound. The reported association between auxin and the *rhd6* mutation indicated that the *RHD6* gene product could be used as a tool to probe the mechanism of action of auxin-like compounds (Masucci and Schiefelbein, 1994). Treatment with IAAld was found to rescue the root hair phenotype of the *rhd6* mutant in a similar way to that of IAA (Fig. 11). Inoculation with *T. virens* or application of *T. virens* extracts also induced normal formation of root hairs in the *rhd6* mutant (data not shown), suggesting

that developmental effects of fungal inoculation in Arabidopsis likely occur by the production of an auxin, presumably IAA or IAAld.

Role of Auxin Signals in *Trichoderma*-Plant Interactions

The importance of auxins for plant development has been long recognized, and redundancy for IAA biosynthesis is widespread in plants and among plant-associated microorganisms. Accumulation of auxins or increased responses to auxins might lead to diverse outcomes on the plant side, varying from pathogenesis to growth promotion. *T. virens* and *T. atroviride* were found to stimulate the growth of Arabidopsis plants in vitro (Fig. 1), suggesting that these fungi likely act as plant growth-promoting microorganisms. It was previously reported that *Trichoderma* was able to colonize the entire root system of maize plants and to persist for the entire lifespan of this crop (Harman et al., 2004b). Fungal colonization stimulated plant growth by factors including increased root size and rooting depth, which aid in nutrient uptake (Yedidia et al., 2001; Harman et al., 2004a).

To further investigate whether IAA and IAAld produced by *T. virens* could have a positive effect on Arabidopsis growth, we quantified biomass production in plants treated with varied concentrations of these compounds. Both compounds showed a dose-dependent effect on growth by increasing biomass production in small amounts but repressing growth at higher concentrations (Fig. 12; Supplemental Fig. S2). Thus, the effect of inoculation with *Trichoderma* strains in plants under natural conditions may depend
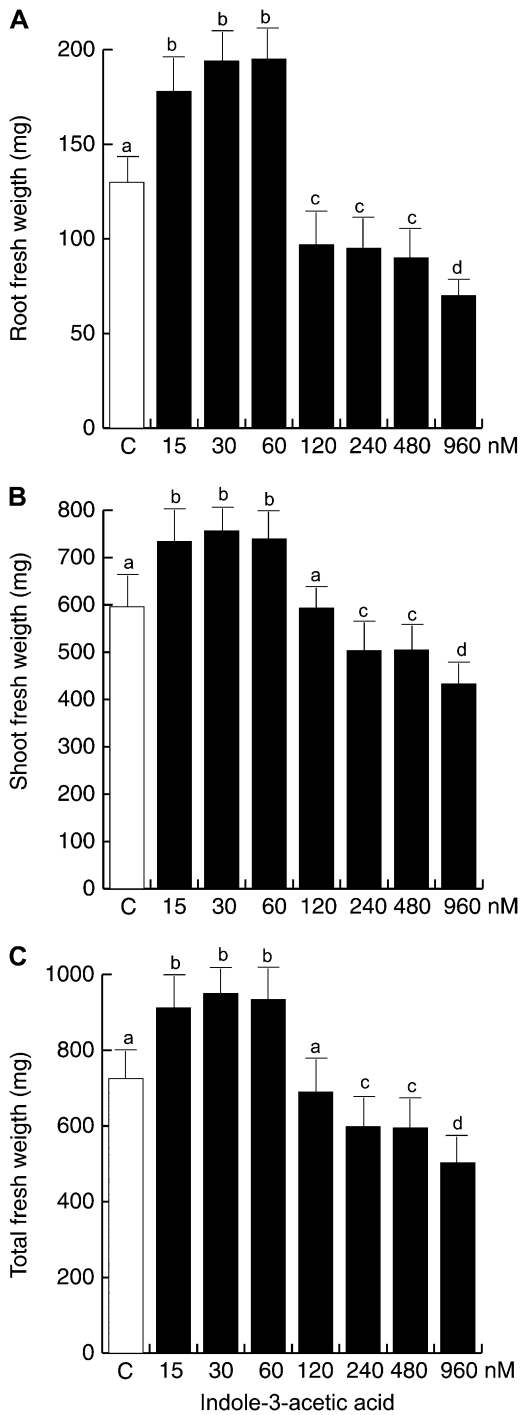
on the type and concentration of auxins being produced by the fungi.

Little is known about the molecular determinants involved in the interaction of *T. virens* with plants. We hypothesize that auxin production by this fungus promotes the interaction with roots by circumvention of basal plant defense mechanisms, as recently reported by Navarro et al. (2006), who showed that repression of auxin signaling restricts *Pseudomonas syringae* growth, implicating auxin in disease susceptibility and RNA-mediated suppression of auxin signaling in resistance. The fungus can also produce auxins as part of its colonization strategy, as published information indicates that fungus-produced IAA induces adhesion and filamentation of *Saccharomyces cerevisiae* (Prusty et al., 2004).

Although we cannot exclude the possibility that IAAld could be converted to IAA and in this way exert its biological action, the concerted action of all three indolic compounds identified may account for the plant growth-promoting properties of *T. virens* (Fig. 13). In the plant partner, alteration in lateral root formation may provide a greater root surface area for fungal colonization. In turn, increased absorptive surface by branched roots may increase water and nutrient uptake capacity of plants. It is tempting to speculate that production of auxins by *Trichoderma* may benefit plant hosts by initiating or reinforcing symbiotic behaviors with fungal partners in the rhizosphere.

The data presented in this work suggest an important role for auxin signaling in plant growth regulation by *T. virens*. Our results show great promise for the use of *Trichoderma* species as inoculants for plant improvement under controlled and field conditions.

Figure 12. Effects of IAA on Arabidopsis biomass production. Wildtype Col-0 seedlings were grown for 14 d under increasing IAA concentrations on vertically oriented agar plates. Data are given for the mean root fresh weight (A), shoot fresh weight (B), and total fresh weight (C). Plants were excised at the root/shoot junction, and fresh weights were determined on an analytical scale for four groups of 25 plants. Different letters represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results.

Figure 13. Arabidopsis growth responses to *T. virens* and their regulation. *T. virens* induces lateral root proliferation and enhances biomass accumulation by production of IAA and IAAld. IAAld can be converted to IAA by plant enzymes or can directly regulate auxin-inducible gene expression, possibly by interacting with auxin receptors. IEt did not show clear auxin-like activity, but it can act as a storage form for other active indolic compounds such as IAAld or IAA.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana* Col-0), the Arabidopsis transgenic lines *HS::AXR3NT-GUS* (Gray et al., 2001), *DR5:uidA* (Ulmasov et al., 1997), and *BA3:uidA* (Oono et al., 1998), and the mutant lines *eir1-1* (Roman et al., 1995), *doc1* (Li et al., 1994), *axr1-3* (Lincoln et al., 1990), *aux1-7* (Picket et al., 1990), and *rhd6* (Masucci and Schiefelbein, 1994) were used for the different experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing $0.2\times$ MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma. Phytagar (commercial grade) was purchased from Gibco-BRL. Plates were placed vertically at an angle of 65° to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L) with a photoperiod of 16 h of light/8 h darkness, light intensity of 300 μmol m^2 s^{-1}, and temperature of 22°C.

Fungal Growth and Indolic Compound Determinations

The following strains were used in this work: *Trichoderma virens* Gv. 29-8 and *Trichoderma atroviride* (formerly *Trichoderma harzianum*) IMI 206040. The strains of *Trichoderma* were grown and maintained on potato dextrose agar medium (Difco).

For the production of indolic compounds, an active inoculum of 1×10^6 spores of *T. virens* was added to 1 L of potato dextrose broth (Difco) and grown for 3 d at 28°C with shaking at 200 rpm. To evaluate the effect of Trp supply on indolic compounds, the medium was supplemented with L-Trp (Merck) at a concentration of 100 mg L^{-1}. For IAAld and IEt determinations, the fungal culture was filtered and the supernatant was adjusted to pH 7 using 2 N NaOH. Indolic compounds in supernatant solutions were extracted three times with 1 L of ethyl acetate. The extracts were combined and evaporated to dryness under a stream of nitrogen and then diluted in 1 mL of ethyl acetate.

For IAA determination, the fungal culture was filtered and the supernatant was adjusted to pH 3 using 1 N HCl. IAA from supernatant solutions was extracted three times with 1 L of ethyl acetate, and the extracts were combined, evaporated to dryness under a stream of nitrogen, and diluted in 1 mL of ethyl acetate. IAA was methyl esterified with 600 μL of acetyl chloride in 2 mL of dry methanol, sonicated for 15 min, and heated at 75°C for 1 h. The IAA methyl ester was evaporated under a stream of nitrogen and redissolved in 1 mL of ethyl acetate. The sample was diluted 1:10 (v/v) without L-Trp in the medium and 1:100 (v/v) with L-Trp before GC-MS analysis.

The indolic compounds were analyzed in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and a 30-m $\times$ 0.2-μm $\times$ 0.25-mm, 5% phenyl methyl silicone capillary column (HP-5 MS). Operating conditions used 1 mL min^{-1} helium as carrier gas, detector temperature of 300°C, and injector temperature of 250°C. The volume of the injected sample was 1 μL. The column was held for 3 min at 80°C and programmed at 6°C min^{-1} to a final temperature of 230°C for 5 min. Indolic compounds were identified by comparison with a mass spectra library (National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health; Chem Station; Hewlett-Packard). The identities of the indolic compounds were further confirmed by comparison of the retention time in the fungal extract with samples of the pure IAAld, IEt, and IAA standards (Sigma). A selected ion monitoring analysis was used to verify the presence of these indolic compounds in the samples. The molecular ions were monitored after electron impact ionization (70 eV). For IAAld, mass-to-charge ratios (m/z) were m/z 144, m/z 116, and m/z 89; for IEt, they were m/z 161, m/z 130, m/z 103, and m/z 77; and for IAA methyl ester, they were m/z 189, m/z 130, m/z 103, and m/z 77. To estimate the amount of compounds produced by *T. virens*, we constructed individual calibration curves for all three standards using concentrations from 40 to 400 μg for IAAld, 30 to 300 μg for IEt, and 0.5 to 5 μg for IAA.

Inoculation Experiments

T. virens and *T. atroviride* were evaluated in vitro for their plant growth-promoting ability using the Arabidopsis Col-0 ecotype. Fungal spore densities

of 1×10^6 spores were inoculated by placing the spores at the opposite ends of agar plates containing 4-d-old germinated Arabidopsis seedlings (10 seedlings per plate). Plates were sealed with Parafilm and arranged in a completely randomized design. The seedlings were cultured for different time periods in a Percival AR95L growth chamber. Plants were sectioned at the root/shoot interface to quantify shoot weight. The fresh weight was measured on an analytical scale immediately after plant harvest, stem and root lengths were measured with a ruler, and lateral roots were counted and measured with a dissection microscope.

Determination of Developmental Stages of LRP

LRP were quantified at day 5 after fungal inoculation. Seedling roots were first cleared to enable LRP at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are as follows. Stage I, LRP initiation. In the longitudinal plane, approximately 8 to 10 "short" pericycle cells are formed. Stage II, the formed LRP is divided into two layers by a periclinal division. Stage III, the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV, LRP with four cell layers. Stage V, the LRP is midway through the parent cortex. Stage VI, the LRP has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII, the LRP appears to be just about to emerge from the parent root.

Histochemical Analysis

For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated overnight at 37°C in a GUS reaction buffer (0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997). For each marker line and for each treatment, at least 10 transgenic plants were analyzed. A representative plant was chosen and photographed using Nomarski optics on a Leica DMR microscope.

Aux/IAA Protein Degradation Assay

Six-day-old *HS::AXR3NT-GUS* Arabidopsis transgenic seedlings were incubated on liquid $0.2\times$ MS medium for 2 h at 37°C, followed by transfer of the seedlings into liquid $0.2\times$ MS medium supplied with the different indolic compounds for 5, 10, 20, or 60 min at 22°C. The seedlings were washed with fresh $0.2\times$ MS medium and, 12 to 14 h later, histochemically stained for GUS activity.

Data Analysis

Arabidopsis root systems were viewed with an AFX-II-A stereomicroscope (Nikon). All lateral roots emerging from the primary root and observed under the $3\times$ objective were taken into account for lateral root number data. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS). Univariate and multivariate analyses with Tukey's posthoc test were used for testing differences in growth and root developmental responses in wild-type and mutant plants. In the figures, different letters are used to indicate means that differ significantly ($P < 0.05$).

Supplemental Data

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

Supplemental Figure S1. Effects of IAAld on Arabidopsis lateral root development.

Supplemental Figure S2. Effects of IAAld on Arabidopsis biomass production.

Supplemental Figure S3. Effects of IAAld on primary root growth in wild-type (Col-0) plants and auxin-related Arabidopsis mutants.

ACKNOWLEDGMENTS

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