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2,4-Diacetylphloroglucinol Alters Plant Root Development

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Pseudomonas fluorescens **isolates containing the *phlD* gene can protect crops from root pathogens, at least in part through production of the antibiotic 2,4-diacetylphloroglucinol (DAPG). However, the action mechanisms of DAPG are not fully understood, and effects of this antibiotic on host root systems have not been characterized in detail. DAPG inhibited primary root growth and stimulated lateral root production in tomato seedlings. Roots of the auxin-resistant *diageotropica* mutant of tomato demonstrated reduced DAPG sensitivity with regards to inhibition of primary root growth and induction of root branching. Additionally, applications of exogenous DAPG, at concentrations previously found in the rhizosphere of plants inoculated with DAPG-producing pseudomonads, inhibited the activation of an auxin-inducible *GH3 promoter::luciferase reporter* gene construct in transgenic tobacco hypocotyls. In this model system, supernatants of 17 *phlD+ P. fluorescens* isolates had inhibitory effects on luciferase activity similar to synthetic DAPG. In addition, a *phlD*$^-$ mutant strain, unable to produce DAPG, demonstrated delayed inhibitory effects compared with the parent wild-type strain. These results indicate that DAPG can alter crop root architecture by interacting with an auxin-dependent signaling pathway.**

Some strains of *Pseudomonas fluorescens*, a bacterium native to soils around the world, inhibit the growth of diverse pathogens that cause soilborne crop diseases such as damping off, root rot, take-all, and other wilting diseases (McSpadden Gardener 2007). The biocontrol activity of many *P. fluorescens* isolates has been linked to production of the secondary metabolite 2,4-diacetylphloroglucinol (DAPG) (Dwivedi and Johri 2003; Weller et al. 2002). Although DAPG exhibits clear antifungal activity (Keel et al. 1992), the effects of this compound on roots remain largely unknown.

DAPG toxicity to a broad spectrum of organisms, such as bacteria, fungi, protists, and nematodes, suggests that its targets may include basic cellular processes. In the fungus *Pythium ultimatum* var. *sporangiiferum*, DAPG causes alterations of the plasma membrane, vacuolization, and the disintegration of cell contents (de Souza et al. 2003), suggesting that it impedes the maintenance of membrane integrity. In bacteria, DAPG may cause lysis by a novel antibiotic mechanism. For instance, methicillin-resistant *Staphylococcus aureus* will lyse within 2 h of exposure to 5 μM DAPG, whereas *Vibrio parahaemolyticus* lyses more slowly and in response to higher DAPG concentrations of 114 μM (Kamei and Isnansetyo 2003). Differential DAPG sensitivity of these gram-positive and gram-negative bacteria suggests that the structure of the cell wall may influence DAPG uptake or responsiveness. Interestingly, exogenously supplied DAPG-containing supernatants are nontoxic to phagotrophic protists such as the ciliate *Colpoda steinii* and the flagellate *Neobodo designis*, although ingestion of DAPG-producing *Pseudomonas* strains rapidly poisons these organisms (Jousset et al. 2006). By contrast, the osmotrophic amoeba *Vahlkampfia* sp. exhibits cell lysis within 1 h of DAPG exposure, with loss of motility occurring within minutes (Jousset et al. 2006), suggesting that DAPG sensitivity of protists is also determined by DAPG permeability of the cell surface. Despite its stability under temperature and pH conditions found in the mammalian digestive tract, DAPG at up to 100 mg/kg surprisingly fails to elicit acute toxicity in mice (Kamei and Isnansetyo 2003).

DAPG inhibits growth and seed germination in a variety of crop plants (Keel et al. 1992; Maurhofer et al. 1995), but the mechanisms underlying these effects are not understood. Increased root mass and lateral root formation of pea plants inoculated with DAPG-producing *Pseudomonas fluorescens*, compared with a non-DAPG-producing mutant, led to the suggestion that DAPG may act as a plant-hormone-like substance (De Leij et al. 2002), and the specificity of DAPG effects for broad-leaf species can resemble that of the auxin-herbicide 2,4-dichlorophenonxyacetic acid (2,4-D) (Reddi et al. 1969). However, to our knowledge, potential interactions between DAPG and auxin signaling have not been examined. A DAPG-induced blocking of amino acid uptake by alfalfa roots has been hypothesized to be linked to an inhibition of proton secretion across the plasma membrane (Phillips et al. 2004), which is also an auxin-like effect (Coenen et al. 2002). However, effects of DAPG on root proton secretion have not been investigated directly.

Some DAPG-producing *Pseudomonas* strains change host root length or induce the formation of supernumerary lateral roots (Dubeikovsky et al. 1993; Patten and Glick 2002; Suzuki et al. 2003). Although this alteration in host root physiology has been attributed to the bacteria producing the plant hormone auxin, indole-3-acetic acid (IAA) (Dubeikovsky et al. 1993), the role of IAA as the sole agonist of changes in root architecture in the presence of *P. fluorescens* has not yet been determined conclusively. Because the growth media used in experiments demonstrating IAA synthesis by *P. fluorescens* in vitro commonly contain high concentrations of the IAA precursor

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tryptophan (Oberhänsli et al. 1991; Patten and Glick 2002; Suzuki et al. 2003), it is unclear whether such rhizosphere bacteria typically produce sufficient amounts of IAA in situ to elicit root morphology changes. At least one non-auxin-volatile compound, 2R,3R,butanediol, secreted by *P. chlororaphis* O6, can promote host plant growth (Han et al. 2006), and volatiles produced by *Bacillus subtilis* GB03 promote growth through enhancing auxin synthesis and transport in *Arabidopsis* (Zhang et al. 2007). The effects of nonauxin secreted *P. fluorescens* products such as DAPG on auxin response pathways have not been previously investigated.

Auxin exerts its effects on plant cells by upregulating transcription of a wide variety of genes. Activation of auxin-induced genes occurs within minutes (McClure and Guilfoyle 1987) and is mediated by auxin binding to a soluble, intracellular receptor protein, SCF^{TIR1} (Dharmasiri et al. 2005a; Kepinski and Leyser 2005), or to one of its homologs (Dharmasiri et al. 2005b). Auxin binding allows these receptor proteins to bind transcriptional repressor proteins of the AUX/IAA protein family and target them for degradation through the 26S proteasome pathway (Gray et al. 2001; Kepinski and Leyser 2004). One of the most rapidly auxin-inducible genes activated through this mechanism is the *GH3* gene originally isolated from soybean (Hagen et al. 1984). The *GH3* gene is specifically activated by the natural auxin IAA and all active auxin analogs tested; it does not respond to nutrients, other phytohormones, or structurally related compounds (Hagen and Guilfoyle 1985). Because the *GH3* gene is auxin-inducible in nearly all plant tissues (Gee et al. 1991; Hagen et al. 1991), the *GH3* promoter provides an excellent tool for detection of auxin activity.

The role of auxin in determining root architecture has been elucidated in part through the use of auxin-resistant mutants such as the *diageotropica* (*dgt*) mutant of tomato. The *dgt* mutant lacks lateral roots and demonstrates an inability to grow upward (Zobel 1972); it is auxin-resistant with regards to hypocotyl-elongation (Kelly and Bradford 1986), root growth (Muday et al. 1995), and auxin-inducible gene expression (Coenen et al. 2003; Mito and Bennett 1995; Nebenführ et al. 2000). The *DGT* gene encodes a cyclophilin protein whose exact role in auxin signaling is presently unclear (Oh et al. 2006). Absence of lateral roots in *dgt* seedlings has been attributed to selective abolishment of auxin-induced cell division in developing lateral root primordia without inhibition of other cell divisions in the root necessary for lateral root initiation (Ivanchenko et al. 2006).

To better understand the effects of DAPG-producing *P. fluorescens* bacteria on root growth and development, we investigated the effects of DAPG on tomato seedling roots and potential interactions between DAPG and auxin signaling pathways. Here, we provide a more detailed description of the general phytotoxic effects of DAPG on plant root development. We demonstrate that DAPG alone, without the presence of added auxin, induces auxin-like effects on tomato seedling root development and that DAPG effects require a DGT-dependent auxin-signaling pathway.

RESULTS

DAPG effects on tomato seedling development.

At 50 μM, exogenous application of DAPG inhibited primary root growth and induced primary root tip necrosis as evidenced by browning (Fig. 1A). Hypocotyls and cotyledons demonstrated no visible necrosis; however, purple coloration of the hypocotyls indicated a stress response to 50 μM DAPG. This concentration also inhibited opening of the hypocotyl hook (Fig. 1A) and induced exaggerated hook curvature upon longer exposure (Fig. 1B). At 10 μM, DAPG stimulated lateral root production in tomato seedlings without damaging the primary root (Fig. 1B). Lateral and adventitious roots produced at 50 μM DAPG did not show visible necrosis (Fig. 1B). Root hair development was not obviously affected by DAPG (absence of root hairs on some of the plants shown in Figure 1 is an artifact of poor resolution of hairs formed on roots growing into the agar surface). The effects of DAPG on primary root growth appeared to be irreversible, because transferring plants from DAPG onto fresh media failed to rescue primary root growth (data not shown).

Concentrations of 50 μM DAPG or higher completely inhibited primary root growth at all timepoints of a 4-day exposure (Fig. 2A). Roots treated with 10 and 20 μM DAPG showed growth inhibition during the initial 24 h of exposure but grew at rates similar to those of untreated roots during the remaining exposure time. The absence of inhibitory effects of low DAPG concentrations during later timepoints of prolonged exposure coincided with the slowing of growth in control seedlings (Fig. 2A). In the 4-day-old seedlings used for this experiment, lateral root formation was inhibited by as little as 1 μM DAPG during the initial 72 h of treatment (Fig. 2B). However, the inhibitory effects of DAPG concentrations <100 μM were no longer detectable after an additional 24 h of treatment (Fig. 2B).

Although DAPG was found to be unstable in the light (Supplementary Fig. 1), its effects on primary root growth were similar for both light- and dark-incubated plants (Fig. 3A). In light-incubated plants, 5 to 20 μM DAPG increased lateral root number in the 3.5-day-old seedlings used for this experiment (Fig. 3B). Because primary root growth was not inhibited at these lower concentrations, this stimulation could not be explained by a simple carbon reallocation effect from primary to lateral root growth. In contrast, lateral root number in dark-incubated seedlings increased only at a concentration (i.e., 30 μM) that inhibited primary root growth.

DAPG has been hypothesized to inhibit root proton secretion (Phillips et al. 2004), so it could inhibit primary root growth through this mechanism. To test directly whether DAPG inhibits proton secretion, we incubated tomato seedlings on bromocresol purple indicator agar for 2 h. Under these conditions, application of 10 μM DAPG to the roots failed to inhibit root proton secretion, whereas 50 μM completely inhibited this process (Table 1).

Interaction between IAA and DAPG in root growth regulation.

The overlap of DAPG effects with typical auxin responses, such as the inhibition of primary root growth, stimulation of lateral root formation, and the inhibition of proton secretion by root tips, raises the possibility that the two substances use partially shared mechanisms of action. To test whether IAA and DAPG show antagonistic or synergistic effects on primary root growth, plants were simultaneously exposed to both IAA and DAPG (Fig. 4). Increasing concentrations of IAA neither increased nor rescued root growth inhibition by DAPG, suggesting that there was no obvious synergism or antagonism between these two compounds.

To test more directly whether DAPG inhibits primary root growth and stimulates lateral root production through an auxin-dependent signaling pathway, we compared DAPG effects on wild-type plants with those on the auxin-resistant *dgt* mutant of tomato (Fig. 5). Primary roots of *dgt* seedlings were slightly less sensitive to DAPG than wild-type primary roots, requiring approximately 50% higher DAPG concentrations for similar growth inhibitory effects (i.e., >20 μM DAPG). Low concentrations of DAPG also failed to stimulate lateral root formation in *dgt*. However, *dgt* seedlings produced lateral roots in response to mechanical wounding of the root tip (data not shown) as well as

in response to DAPG concentrations that caused primary root tip necrosis (Fig. 5). Therefore, high DAPG concentrations (i.e., >20 μM DAPG) may have stimulated lateral root production through pathways similar to wounding of the primary root tip, which are functional in *dgt*. Lack of stimulation of lateral root production by ≤20 μM DAPG (Fig. 5B) indicated that this response may require a DGT-dependent auxin signaling pathway.

Inhibition of luciferase activity by DAPG and culture supernatants of DAPG-producing *Pseudomonas* spp.

To assess DAPG interactions with auxin signal transduction, we characterized the effects of pure DAPG on luciferase activity driven by the selectively auxin-responsive *GH3* promoter. Despite its auxin-like effects on root development, application of DAPG alone failed to induce the auxin-responsive *GH3* promoter (data not shown). However, when applied prior to auxin treatment, DAPG fully inhibited auxin-induced *GH3* promoter activation at a concentration of 20 μM (Fig. 6A), which is in the natural range of DAPG concentrations reported in the rhizosphere (Bonsall et al. 1997).

Similar to synthetic DAPG, supernatants from *phlD*+ *P. fluorescens* cultures completely inhibited luciferase activity in *GH3::Luc* tobacco hypocotyl segments treated with 10 μM IAA (Fig. 6B). This pattern of suppression occurred with representative strains of three different *phlD* restriction fragment length polymorphism-defined genotypes (i.e., A, S, and D; data

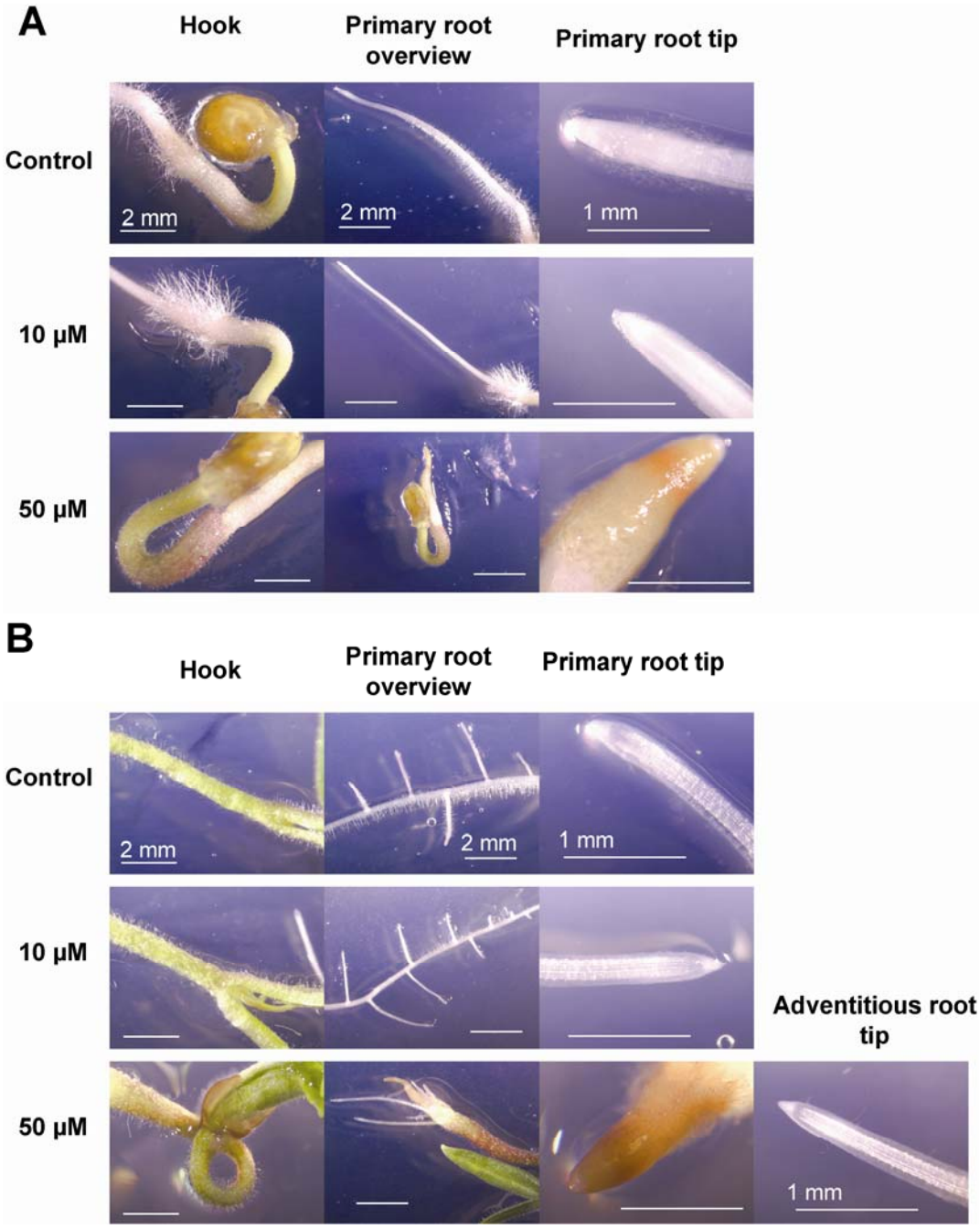

Fig. 1. 2,4-Diacetylphloroglucinol (DAPG) effects on root development in tomato seedlings. Representative examples of tomato seedlings incubated in the light for **A,** 1 and **B,** 4 days on plates containing the indicated concentrations of DAPG. Seedlings were photographed using a digital camera with Spot RT software attached to a dissecting microscope. Seedling organs were photographed at magnifications of ×1.5 (hypocotyl hook), ×0.75 (primary root overviews), or ×6 (root tips). Note that root hairs on some of the roots shown here are not visible due to growth of the root into the agar surface, so that root hair development cannot be interpreted based on this illustration.

not shown). The inhibition of luciferase activity was already apparent at the first timepoint of the preincubation period (Fig. 6B), approximately 19 min after supernatant addition. Inhibition kinetics of auxin-induced gene expression also were assessed by adding DAPG or supernatants from *P. fluorescens* strain Wayne 1R after the auxin response was already initiated (Fig. 7). The time course of the culture supernatant-induced inhibition was similar to the inhibition caused by addition of 30 μM DAPG.

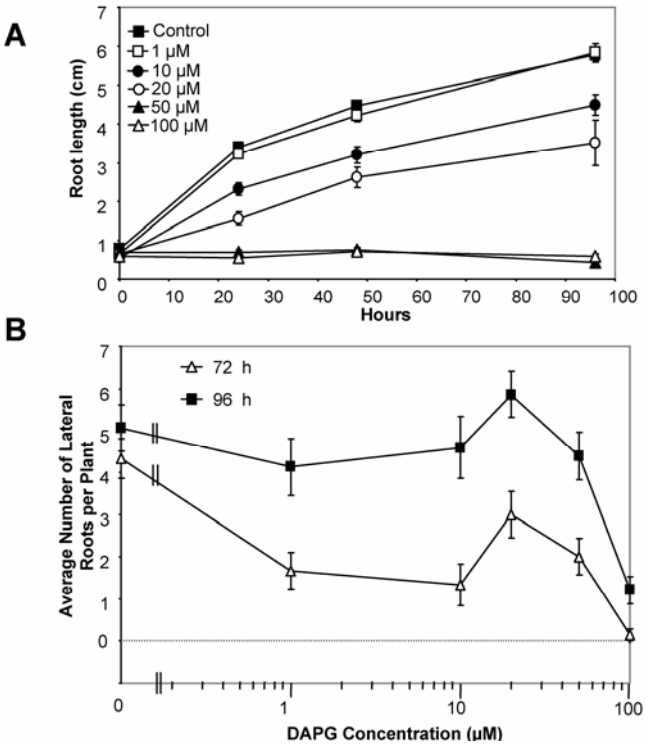

Fig. 2. Time effects on 2,4-diacetylphloroglucinol (DAPG) inhibition of tomato seedling root growth. **A,** Primary root growth and **B,** lateral root number per plant. Four days after sowing, tomato seedlings with roots 5 to 10 mm in length were moved to plates containing the indicated concentrations of DAPG and incubated for 4 days in the light. Three plates containing five seedlings each were used for each treatment, and each plate was scanned once every 24 h to determine root lengths using APS Assess software. Data points represent the average primary root length at each measuring timepoint. Lateral roots were counted by viewing plants at ×8 magnification through a Nikon dissecting microscope at 72 and 96 h after start of exposure to DAPG. Error bars represent standard error of the mean (n = 15). This experiment was repeated three times with similar results, and one representative experiment is shown here.

Table 1. Proton secretion by tomato roots exposed to 2,4-diacetylphloroglucinol (DAPG)[a]

DAPG (μM)	No. of seedlings secreting protons/ no. of seedlings scored	Color of root tip
0	4/4	White
1	4/4	White
10	4/4	White
20	3/5	White
30	2/5	White
50	0/5	Brown
100	0/5	Brown

[a] Tomato seedlings were pregerminated for 4 days until root tips were 2 to 10 mm in length, moved to half-strength Murashige and Skoog salt plus 0.8% tissue culture grade agar plates containing bromocresol purple and the indicated concentrations of DAPG, and incubated at room temperature for 4 h. Seedlings were scored for proton secretion based on yellow coloration of indicator dye immediately surrounding root tips and root hair zone.

Because *P. fluorescens* is known to produce a wide range of secondary metabolites in addition to DAPG, we compared the effects of supernatants from the fully sequenced strain Pf-5 with those of a *phlD*⁻ mutant of this strain. Supernatants from both Pf-5 and mutant cultures rapidly inhibited luciferase activity (Fig. 8), indicating that DAPG is not the sole inhibitor in these supernatants. However, two independent experiments confirmed that Pf-5 supernatant effects were significantly more rapid than those of supernatants from mutant cultures: One hour after replacement of the incubation media with culture supernatants, the average inhibition relative to the last preaddition timepoint was significantly greater for Pf-5 supernatants than for *phlD*⁻ supernatants. (experiment I: Pf-5 average inhibition = 78.9 ± 2.3%, n = 16, *phlD*⁻ average inhibition = 65 ± 2.8%, n = 15; experiment II: Pf-5 average inhibition = 95.6 ± 0.7%, n = 24, *phlD*⁻ average inhibition = 77.7 ± 1.6%, n = 24). These results were found to be highly significant, with $P < 0.002$ for experiment I and $P < 0.0001$ for experiment II. Because thin-layer chromatography (TLC) analysis of supernatants from mutant cultures confirmed loss of DAPG synthesis in the mutant (not shown), these experiments indicate that at least one other *P. fluorescens* metabolite has inhibitory activity in this system.

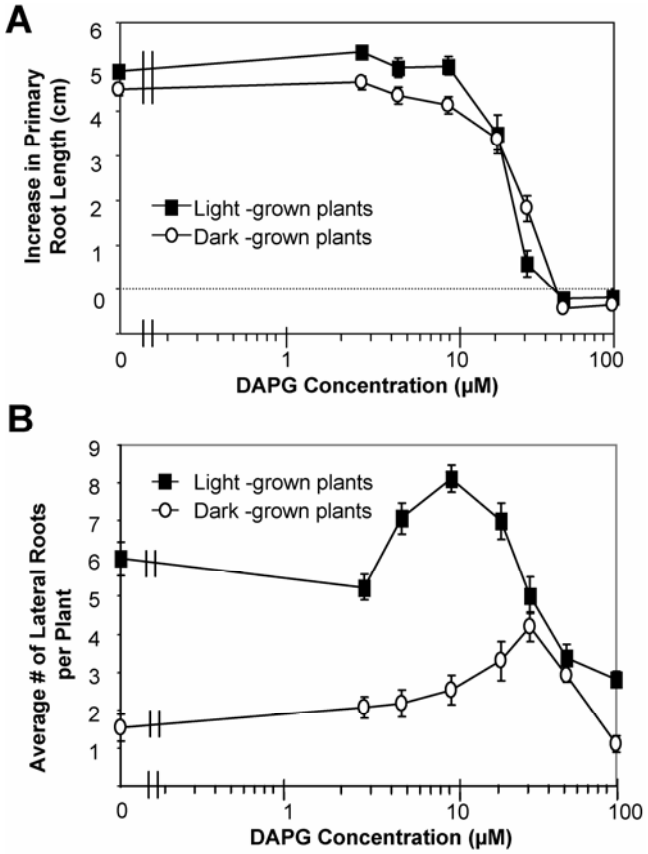

Fig. 3. Light modulation of 2,4-diacetylphloroglucinol (DAPG) effects on primary root growth and lateral root number in tomato plants after 96 h of exposure. Seedlings with roots approximately 2 to 6 mm in length (3.5 days after sowing) were moved to plates containing the indicated concentrations of DAPG and incubated in either light (16 h of light and 8 h of darkness) or continuous darkness for 96 h. Squares and circles represent light- and dark-grown plants, respectively. Root lengths were measured by ruler immediately after transfer to treatment plates and after 96 h; the increase in length of each root during the time of DAPG exposure was calculated and averaged. Six plates containing five seedlings each were used for each DAPG treatment, and error bars represent the standard error of the mean (n = 30). This experiment was repeated three times with similar results; one representative experiment is shown here.

DISCUSSION

At concentrations reported to exist in situ (Bonsall et al. 1997) and that can induce plant host defenses (Iavicoli et al. 2003), we observed that DAPG caused drastic changes in root physiology and morphology. These changes included the development of brown and shriveled root tips and swollen root zones, inhibition of primary root growth, and stimulation of root branching (Figs. 1 through 3). Such changes in root tissues may also indirectly stimulate host defense pathways. Although multiple pseudomonad factors may induce such responses in plant hosts (Bakker et al. 2007; Meziane et al. 2005), both DAPG-producing pseudomonads and 10 to 100 μM DAPG applied exogenously to *Arabidopsis thaliana* have been reported to induce resistance to foliar infections by *Peronospora parasitica* (Iavicoli et al. 2003). An interaction between DAPG and auxin signaling may partly explain the resistance-inducing properties of this compound, because modification of auxin balance in host plant tissues is associated with the development of disease symptoms (Chen et al. 2007).

Although it is unclear if all crop plants respond to DAPG by altering their root architecture, similar DAPG sensitivities for a wide range of crop species (Keel et al. 1992) may indicate that DAPG's effects on plant roots may be universal. Here, DAPG concentrations causing half-maximal inhibition of primary root growth in tomato (20 to 30 μM DAPG) (Fig. 3) were close to the minimal DAPG concentrations reported to inhibit overall tomato growth by 50% (76 μM) (Keel et al. 1992), suggesting that growth inhibition in the primary root is only partially offset by increased lateral root production over the lengthier incubation periods reported in the earlier experiments.

Restriction of DAPG-induced necrosis to specific zones of the primary root indicates that DAPG sensitivity or response mechanisms differ between specific cell types or developmental stages of the root. The distinct local confinement of necrosis to meristematic regions of primary root tips (Fig. 1) indicates that DAPG toxicity may primarily occur in rapidly dividing cells. This localized sensitivity was further supported by the fact that the more systemic responses of shoots and elsewhere on the root were consistent with non-necrotic stress responses. Although DAPG producers may represent approximately 1 to 10% of the root-colonizing pseudomonads (McSpadden Gardener et al. 2005), they have been estimated to colonize no more than 0.1% of the root surface, and DAPG concentrations may vary widely as a result (Haas and Keel 2003). Therefore, local DAPG concentrations produced by *phlD*+ pseudomonads likely vary dramatically across the root, and bacterially produced DAPG concentrations in natural systems may rarely reach toxic levels in the most sensitive root zones (i.e., primary root tip). Although the typical distribution of DAPG producers on field grown roots remains unclear, Aino and associates (1997) demonstrated that two strains could move toward tomato root tips with growth of the root and form colonies as well as DAPG crystals inside and around the roots. This indicates that at least some *phlD*+ *Pseudomonas* strains may be able to produce sufficiently high DAPG concentrations near the root tip to inhibit growth and, subsequently, stimulate secondary root growth. Whether such a response would generally benefit plant health or vigor remains unclear. However, the abundance of DAPG producers often has been positively correlated with stands and yields of corn (Rotenberg et al. 2007), a crop species that relies heavily on a frequently branching root system to access nutrients and is known to harbor relatively high populations of DAPG-producing pseudomonads (McSpadden Gardener et al. 2005). It would be interesting to see whether the abundance

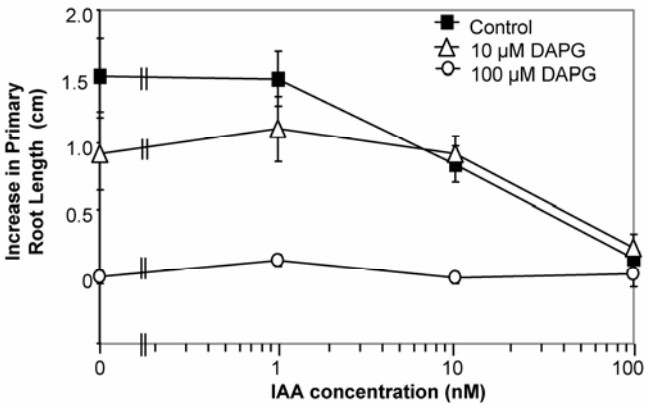

Fig. 4. Primary root growth in tomato seedlings exposed to 2,4-diacetylphloroglucinol (DAPG) and indole-3-acetic acid (IAA). Seedlings with roots approximately 2 to 6 mm in length (3.5 days after sowing) were moved to plates containing the indicated concentrations of DAPG and IAA. Root length was measured with the help of APS Assess software immediately after transfer to treatment plates and after 24 h of incubation at 26°C under a light and dark cycle of 16 and 8 h, respectively. Error bars represent standard error of the mean for the increase in root length exhibited by fifteen seedlings grown on three separate plates. The experiment was repeated three times with similar results; one representative experiment is shown here.

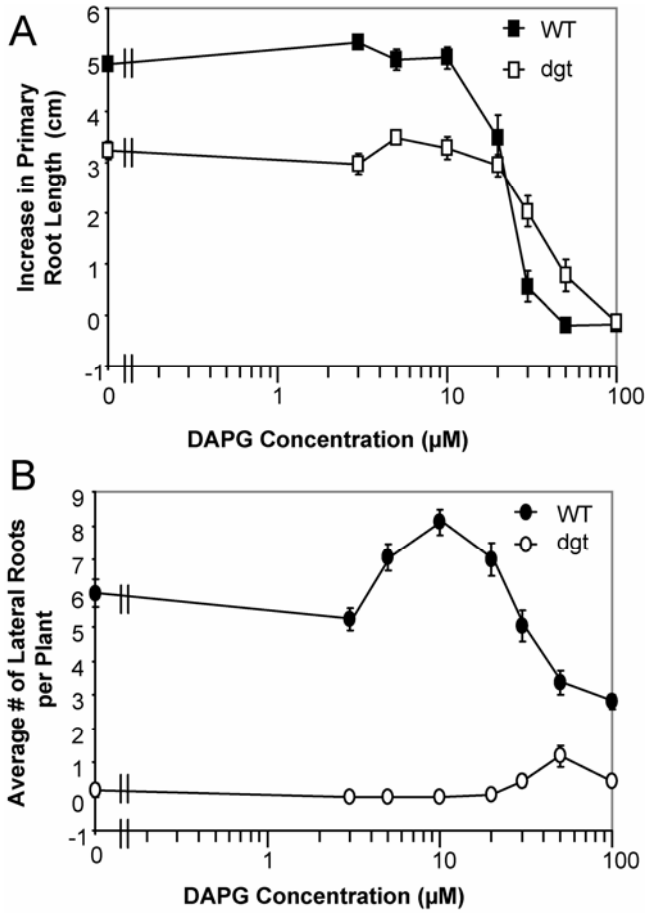

Fig. 5. 2,4-Diacetylphloroglucinol (DAPG) effects on root growth and lateral root initiation in *diageotropica* (*dgt*) tomato seedlings. **A,** Primary root growth and **B,** lateral root number; WT = wild type. Tomato seedlings with roots approximately 2 to 6 mm in length were moved to plates containing the indicated concentrations of DAPG. After 96 h at 26°C and a light and dark cycle of 16 and 8 h, respectively, the number of lateral roots was counted and primary root lengths were measured by ruler. Six plates containing five seedlings each were used for each DAPG treatment, and error bars represent the standard error of the mean. This experiment was repeated three times with similar results; one representative experiment is shown here.

of DAPG producers, the amount of root branching, and crop vigor are also correlated in field-grown tomato and other dicot crops.

DAPG concentrations too low to inhibit pathogen growth directly are known to change gene expression in both bacteria and plants (Haas and Keel 2003) and, thus, may profoundly influence development. In tomato, DAPG-induced changes in root physiology were complex and dependent on developmental stages of roots, suggesting that DAPG may act through multiple mechanisms. The increased initiation and growth of lateral roots in response to DAPG (Figs. 1 and 3) indicated that DAPG does not act exclusively by inducing cell death. Consistent with increased sensitivity of rapidly growing or dividing cells to DAPG action, the effects of DAPG on both primary root growth and lateral root number were less evident as incubation time on DAPG increased and growth of control roots slowed (Fig. 2A and B). In addition, stimulation of lateral root production in the slightly older plants used in this experiment was less pronounced than in experiments using younger seedlings (compare Figs. 2B and 3B), indicating that root sensitivity to DAPG may be under developmental control. After 96 h of exposure, lateral root meristems may have adapted to the presence of DAPG (Fig. 2B), an interpretation that is supported by absence of necrosis in lateral root tips. Alternatively, DAPG concentrations in the media may have fallen below inhibitory levels prior to initiation or emergence of the lateral roots. Loss of primary root growth inhibitory effects of DAPG-containing media after 4 days of incubation in the light and the loss of lateral root inhibition between 72 and 96 h post DAPG application (Fig. 2B) support this latter interpretation.

Several lines of evidence indicate that DAPG effects on plant tissues are rapid. Auxin-induced luciferase activity of tobacco hypocotyl segments decayed within minutes of DAPG application (Fig. 7), root tip browning (Fig. 1) became evident within hours of DAPG application, and inhibition of proton secretion by root tips (Table 1) and root growth (Fig. 2) were evident at the earliest timepoint at which these responses were assessed (4 and 24 h, respectively). This rapid action of DAPG likely explains why this compound affected root growth in

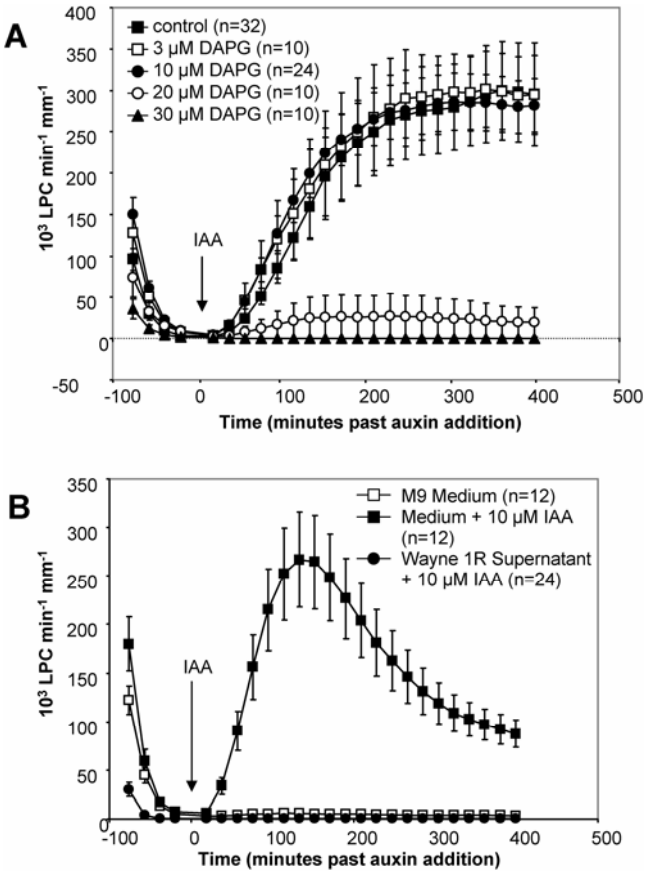

Fig. 6. Effects of 2,4-diacetylphloroglucinol (DAPG) and supernatants from *Pseudomonas fluorescens* cultures on luciferase activity in tobacco hypocotyls carrying the *GH3::Luc* construct. **A,** Concentration dependence of DAPG effects on luciferase activity. Excised *GH3::Luc* hypocotyls were incubated in a phosphate buffer containing 1 mM luciferin, 10 mM potassium phosphate, and the indicated concentrations of DAPG. Indole-3-acetic acid (IAA) was added to a final concentration of 10 µM after 80 min (time 0). Data shown were compiled from two separate experiments, and sample size (*n*) for each treatment is listed in the legend. **B,** *P. fluorescens* supernatant effects on luciferase activity. Excised *GH3::Luc* tobacco hypocotyls were preincubated in 75% M9 media containing 1 mM luciferin. IAA was added to a final concentration of 10 µM after 80 min (time 0). Luminescence counts were repeated every 19 min. Error bars represent the standard error of the mean. This experiment was repeated six times with similar results. LPC = luminescent photon counts.

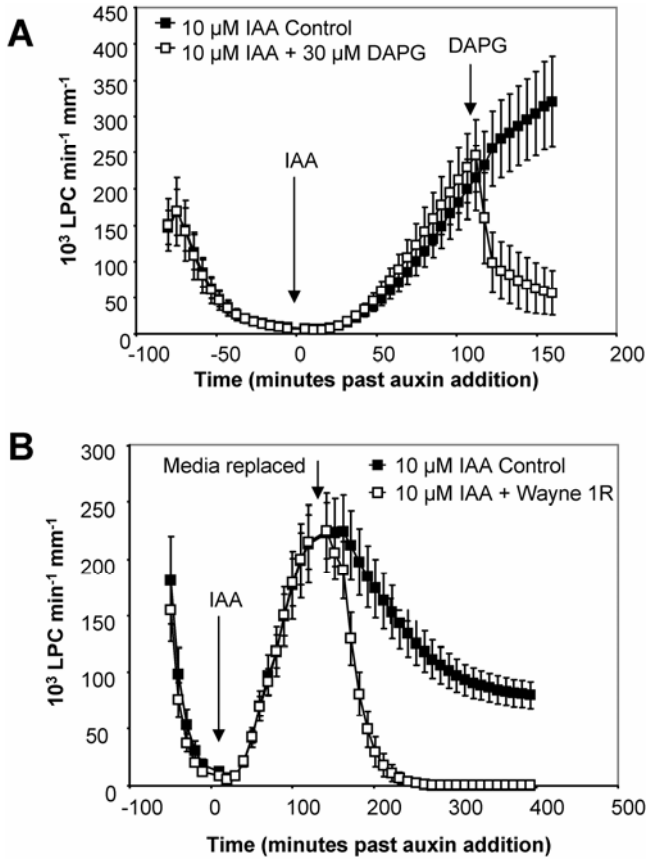

Fig. 7. Inhibition kinetics of luciferase activity by 2,4-diacetylphloroglucinol (DAPG) and *Pseudomonas fluorescens* supernatants. **A,** Time course of DAPG action. Excised *GH3::Luc* hypocotyls were incubated in buffer containing 1 mM luciferin and 10 mM potassium phosphate. Indole-3-acetic acid (IAA) was added to a final concentration of 10 µM after 80 min (time 0), and DAPG was added to a final concentration of 30 µM 120 min after indole-3-acetic acid (IAA) addition. Luminescence counts were repeated every 4 min. This experiment was repeated twice with similar results, $n = 12$ for both treatments as shown in this representative experiment. **B,** Time course of *P. fluorescens* effects. Excised *GH3::Luc* hypocotyls were incubated in 75% M9 medium containing 1 mM luciferin. IAA was added to a final concentration of 10 µM after 80 min (time 0), and media were replaced 120 min later with either 1 mM luciferin and 10 µM IAA in 75% M9 medium or 1 mM luciferin and 10 µM IAA in 75% supernatant from *P. fluorescens* strain Wayne 1R. Luminescence counts were repeated every 10 min. Error bars represent the standard error of the mean. The experiment was repeated twice with similar results; for the representative experiment shown here, $n = 11$ and 10 for the control and supernatant treatments, respectively. LPC = luminescent photon counts.

light- and dark-incubated seedlings similarly (Fig. 3), despite its ability in the light. Loss of DAPG effects in light-incubated medium also explains release of roots from the inhibition of growth (Fig. 2A) and lateral root formation (Fig. 2B) during prolonged incubation.

Induction of lateral root primordia is usually associated with the accumulation of auxin in pericycle cells. If DAPG stimulates lateral root initiation via an auxin-dependent signaling pathway, this response should be absent in the auxin-resistant tomato mutant *dgt*, which does not show auxin-induced lateral root formation (Ivanchenko et al. 2006). Primary root growth of *dgt* seedlings was less sensitive to DAPG than wild-type root growth, requiring 50% higher concentrations for a similar effect (Fig. 5), suggesting a link between auxin signaling and DAPG sensitivity. Low concentrations of DAPG failed to stimulate lateral root development in *dgt*, indicating that the cyclophilin protein encoded by the DGT gene product (Oh et al. 2006) is required for this DAPG response. Because *dgt* roots initiate new lateral roots in response to mechanical wounding of the primary root tip (data not shown), stimulation of root branching by DAPG concentrations that kill the primary root tip could be mediated by pathways similar to those elicited by a wound response. Although simultaneous application of DAPG and IAA to growing tomato seedlings demonstrated no obvious synergism or antagonism between the effects of these two compounds on tomato primary root growth (Fig. 4), the DGT dependence of DAPG effects on root development suggest an interaction between DAPG and auxin signaling.

Auxin is known to block proton secretion at the tomato root tip (Coenen et al. 2002), and DAPG has been speculated to block root proton secretion as one possible mechanism underlying its effects on amino acid secretion (Phillips et al. 2004). Although root proton secretion on plates containing the pH indicator dye bromocresol purple was indeed blocked by DAPG (Table 1), the concentrations required for this response also elicited necrosis, making it impossible to determine whether the inhibition of proton secretion was the cause or consequence of root cell death. Because auxin inhibition of proton secretion is not accompanied by necrosis of the root tip, it seems unlikely that DAPG and IAA use similar mechanisms to effect this response.

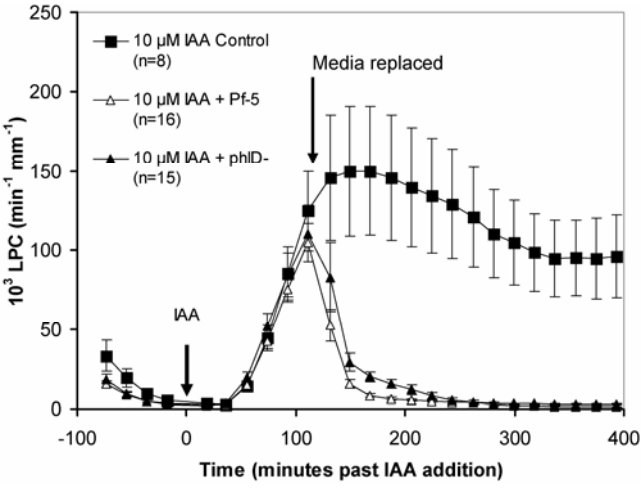

Fig. 8. Inhibition kinetics of luciferase activity by supernatants from 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas fluorescens* cultures and from a *phlD*⁻ mutant. Excised *GH3::Luc* hypocotyls were incubated in 75% M9 medium containing 1 mM luciferin. Indole-3-acetic acid (IAA) was added to a final concentration of 10 µM after 80 min (time 0), and media were replaced 120 min later with 1 mM luciferin and 10 µM IAA in 75% M9 medium, 1 mM luciferin and 10 µM IAA in 75% supernatant from *P. fluorescens* strain Pf-5, or 1 mM luciferin and 10 µM IAA in 75% supernatant from the *phlD*⁻ mutant of *P. fluorescens* strain Pf-5. Luminescence counts were repeated every 19 min. Error bars represent the standard error of the mean. The experiment was repeated twice with similar results; one representative experiment is shown. LPC = luminescent photon counts.

Although early studies suggested that DAPG effects may be 2,4-D-like (Reddi et al. 1969), DAPG failed to stimulate the activity of the *GH3* promoter (not shown), which is 2,4-D responsive (Hagen et al. 1991). Although DAPG's failure to activate the *GH3* promoter suggests that it does not act via the same signal transduction pathways as other auxins, it could elicit auxin-like effects on root development by altering cellular auxin sensitivity or auxin transport, either directly, as recently shown for volatiles emitted by a plant growth promoting strain of *Bacillus subtilis* (Zhang et al., 2007), or through stimulation of ethylene synthesis. Hook curling exhibited by tomato seedlings in response to 50 µM DAPG (Fig. 1B) is part of the classic ethylene triple response, and ethylene is a known regulator of root auxin synthesis (Stepanova et al. 2005) and transport (Lee et al. 1990). Ethylene effects on patterns of auxin transport may be due to ethylene-induced flavonoid synthesis (Buer et al. 2006). However, DAPG-induced increases in root branching occurred at a lower DAPG concentration than DAPG-induced hook curling and anthocyanin synthesis (Fig. 1), suggesting that mediation of DAPG effects through ethylene could be limited to high DAPG concentrations.

Although DAPG's antibiotic effects on plant pathogens are well documented (de Souza et al. 2003; Schouten et al. 2004) and consistent with the biocontrol activity of *phlD+ Pseudomonas* isolates (Keel et al. 1992), the complete spectrum of effects conferred by DAPG producers has yet to be fully characterized. Our finding that DAPG may interact with auxin signaling to modify root architecture suggests that DAPG may exert direct growth regulatory effects in addition to its antibiotic activity toward plant pathogens. The pleiotropic, complex, and developmentally dependent effects of DAPG on tomato roots suggest that its action mechanisms may involve multiple molecular targets. We expect that the mechanisms underlying DAPG's activities related to both plant protection and plant development are varied and context dependent. Comprehensive genomic analyses, such as those now being undertaken with strain Pf-5 (Loper et al. 2007) and other biocontrol pseudomonads, will likely lead to a much more comprehensive description of the varied metabolic activities that contribute to biocontrol and plant growth promotion by DAPG-producing strains.

MATERIALS AND METHODS

Plant materials and culture.

Seed of the *dgt* mutant of tomato (*Lycopersicon esculentum*) and its parent cultivar, *Ailsa Craig*, were originally obtained from Charles Rick at the University of California, Davis. Seed used in this study were from plants grown in the Allegheny College greenhouse. Seed were surface sterilized in 20% household bleach for 20 min, rinsed with at least 700 ml of sterile distilled water, and plated on half-strength Murashige and Skoog salt (Murashige and Skoog 1962) and 0.8% tissue culture grade agar (both from PhytoTechnology Labs, Lenexa, KS, U.S.A.) (MSO) medium. Seed were incubated at 26°C with a light and dark cycle of 16 and 8 h, respectively, for 72 to 96 h, until root tips were 2 to 10 mm in length.

Tobacco (*Nicotiana plumbaginifolia)* plants transformed with gene constructs containing the auxin-inducible *GH3* promoter (Hagen et al. 1991) linked to a luciferase reporter gene were generated by ligating promoter-*luciferase* cassettes into binary vectors derived from pBin19 (Bevan 1984), which were already carrying selectable kanamycin markers in their T-DNA region and had been modified by insertion of a pUC18 poly-

linker site. Following *Agrobacterium*-mediated transformation of the T-DNA into leaf disk cells (Fillatti et al. 1987), several independent, kanamycin-resistant shoots from the explants were regenerated into plants. A T_2 line (64E) of seeds homozygous for the transgene construct was used in all experiments. Seeds were surface sterilized with 35% household bleach for 25 min while shaking continuously, rinsed five times with sterile double-distilled water, and incubated overnight in 1 mM gibberellic acid at 4°C. After several rinses to remove adhering gibberellic acid solution, seeds were plated on MSO medium and placed in a light incubator at 26°C, with a light and dark cycle of 16 and 8 h, respectively, until the root tip barely protruded from the seed coat (approximately 72 h). Seedlings were then incubated in darkness at 26°C for an additional 48 h to permit hypocotyls to elongate.

Bacterial culture.

Strains of DAPG-producing *Pseudomonas fluorescens* (Cosh1R, Clinto1R, Crawf1R, Delaw1R, Darke1R, Henry1R, Huron1R, Preble1R, Wayne1R, Wood3R, Wyand2R, Wood1R, Clarke1R, Wayne1RB, Wood1RB, and Eaton 2) isolated from fields in Ohio (McSpadden Gardener et al. 2005), as well as the *phlD*$^-$ mutant and its isogenic parent strain, Pf-5 (Loper et al. 2007), were cultured initially on half-strength tryptic soy agar plates (EMD Chemicals, Gibbstown, NJ, U.S.A.) at 27°C, and single colonies were used to inoculate half-strength tryptic soy broth (tryptic soy extract at 15 g liter^{-1}). For experiments assessing effects of supernatants from the Ohio strains on luciferase activity in tobacco hypocotyl segments, 25 µl of 24-h-old broth cultures were used to inoculate 3 ml of M9 broth supplemented with 500 µM tryptophan. For experiments on Pf-5 and the *phlD*$^-$ mutant, 4 ml of half-strength tryptic soy broth were inoculated with single colonies. All broth cultures were grown in the dark at 26°C while shaking at 200 rpm.

Construction of the *phlD*$^-$ mutant of Pf-5.

The construct used to generate the *phlD*$^-$ mutant was created using an overlap extension polymerase chain reaction (PCR) method modified from Choi and Schweizer (2005). All PCR was performed using KOD Hot Start DNA polymerase (Novagen, Madison, WI, U.S.A.). In the first round of PCR, 5′ and 3′ *phlD* gene fragments were amplified using the following primers: phlD UpF-*Hin*d, 5′-GACGAAGACGAAGCTTCCTA CAGATTCCTGTGCCTA-3′; phlD UpR-FRT, 5′-TCAGAGC GCTTTTGAAGCTAATTCGACACGATCAGTACGTGGTT G-3′; phlD DnF-FRT, 5′-AGGAACTTCAAGATCCCCAATT CGAGAGCGAGCACTACATCAAG-3′; and phlD DnR-*Hin*d, 5′-GTGGTTGTGGAAGCTTTGGTTGGCATTCCAGTGTT C-3′. Amplification of the gentamycin resistance gene cassette from pPS856 (Hoang et al. 1989) was performed with the Gm-F and Gm-R primers essentially as described (Choi and Schweizer 2005). In the second-round PCR, 50 ng each of the phlD Up, phlD Dn, and *FRT*-gentamycin-*FRT* cassette PCR products were used to perform three rounds of amplification without added primers. The phlD UpF-*Hin*d and phlD DnR-*Hin*d primers were added halfway through the third cycle extension. Amplification of the full-length product was completed with an additional 25 cycles of PCR. The second-round PCR product was digested with *Hin*dIII and cloned into the *Hin*dIII site of pEX18Tc (Hoang et al. 1989). This construct was transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA, U.S.A.) and confirmed by sequencing.

The deletion construct was transformed into the mobilizing strain *E. coli* S17-1 (Simon et al., 1983) and introduced into Pf-5 via conjugation. Pf-5 transconjugants were selected on King's medium B (KMB) (King et al. 1954) with streptomycin (100 µg/ml, innate resistance of Pf-5) and gentamycin (40 µg/ml). Colonies were grown for 3 h without selection in Luria-Bertani (LB) broth and then plated on LB medium with gentamycin (40 µg/ml) and 5% sucrose to favor growth of resolved merodiploids. Sucrose-resistant colonies were patched onto KMB containing tetracycline (200 µg/ml) to confirm resolution of merodiploids. Tetracycline-sensitive, gentamycin-resistant clones were considered to be putative mutants. To remove the gentamycin resistance gene, the plasmid pFLP2Km expressing Flp recombinase was introduced into the Pf-5 mutant by conjugation. pFLP2Km was constructed through introduction of the kanamycin resistance cassette from pUC4K (Vieira and Messing 1982) into the unique *Sca*I site of pFLP2 (Hoang et al. 1989) (D. Mavrodi and L. Thomashow, *personal communication*). Transconjugants were selected on KMB with streptomycin (100 µg/ml) and kanamycin (50 µg/ml). Colonies were patched onto KMB containing gentamycin (40 µg/ml) to screen for loss of the gentamycin resistance gene. Gentamycin-sensitive colonies were patched onto LB with 5% sucrose to promote loss of the pFLP2Km plasmid. Sucrose-resistant colonies were patched onto KMB kanamycin plates to confirm loss of the plasmid. The resulting mutant, containing only an FRT scar at the site of the deletion, was confirmed by performing PCR across the deletion site (including all flanking sequence introduced by recombination) and sequencing the resultant product.

Analysis of Pf-5 DAPG production by TLC.

Loss of DAPG production in the *phlD*$^-$ mutant of Pf-5 was confirmed by TLC essentially as described (Whistler et al. 2000). Wild-type and mutant cultures were grown in duplicate in nutrient broth (Difco Laboratories, Detroit) containing 2% glucose at 27°C. Overnight cultures were diluted to an optical density at 600 nm = 0.1 and grown for an additional 24 h. Cultures were centrifuged 5 min at 5,000 × *g*. The culture supernatants were adjusted to pH ≤2.0 with 1 M HCl and extracted twice with 2 ml of ethyl acetate. The organic phases were combined and dried under vacuum. Extracts were dissolved in 100 µl of methanol and separated on TLC plates (silica gel 60 F254; EM Science, Gibbstown, NJ, U.S.A.) in chloroform/acetone (9:1, vol/vol). Compounds were visualized under shortwave UV and compared with authentic standards. The *phlD*$^-$ mutant showed no detectable DAPG or monoacetylphloroglucinol production (not shown).

DAPG effects on root physiology.

Tomato seedlings were transferred to MSO plates containing various concentrations of DAPG (Toronto Research Chemicals, Toronto). The seedlings were incubated on vertically positioned DAPG plates at 26°C, in either light (light and dark cycle of 16 and 8 h, respectively) or darkness. After 24 and 48 h, the plates were scanned on a Hewlett Packard DeskJet scanner and primary root lengths were measured using the APS Assess Imaging software (American Phytopathological Society Press, St. Paul, MN, U.S.A.). After 72 and 96 h, primary and lateral root lengths were determined by ruler.

To assess root growth recovery from DAPG exposure, tomato seedlings were removed from DAPG plates after 24 to 48 h of treatment, transferred to fresh MSO plates containing no DAPG, and incubated for an additional 24 to 48 h. Root length was assessed before and after incubation on DAPG and after incubation on fresh plates.

Root proton secretion (Coenen et al. 2002) responses to DAPG were examined by placing tomato seedlings on MSO medium (0.6% agar) containing various concentrations of DAPG and bromocresol purple indicator dye at 400 mg liter^{-1}. Proton secretion was determined after 4 h by assessing color change of the bromocresol purple.

Auxin-induced *GH3* promoter activity.

Hypocotyls of tobacco seedlings were excised using a scalpel under a dissecting microscope equipped with a green light and placed into individual drops of sterile distilled water on a petri dish. Hypocotyl segments were photographed with a Pixera camera attached to a Nikon SMZ1000 dissecting microscope (0.8 magnification) and segment lengths were determined using the Pixera Pro image-capturing software and measurement tool. Hypocotyls were then transferred under green light, one every 20 s, to individual wells of a 96-well plate (Wallac, 1450-105 8X12) containing 100 µl of a preincubation solution containing 1 mM luciferin (Gold BioTech Inc., St. Louis) dissolved in M9 medium or phosphate buffer. Luminescence measurements for each hypocotyl segment were repeated for the indicated intervals over the time course of each experiment using a Micro Beta TriLux Luminometer (PerkinElmer, Salem, MA, U.S.A.). IAA was added from ethanolic stocks to the indicated concentrations. Ethanol concentrations within all experiments were adjusted to the same final concentration (≤1%, vol/vol).

In experiments characterizing the effects of DAPG on auxin-induced gene expression, the preincubation solutions consisted of 10 mM potassium phosphate (pH 6.7), 1 mM luciferin, and various concentrations of DAPG. After 80 min, IAA was added to each well to a final concentration of 10 µM, and luminescence measurements were continued. To determine the time course of DAPG effects, DAPG was omitted from the preincubation buffer and instead added 120 min after IAA addition. The responses of plant tissue were followed over an additional 60 to 80 min.

To assess inhibition of auxin-inducible gene expression by supernatants of *P. fluorescens* cultures, the preincubation buffer contained 1 mM luciferin dissolved either in 75% M9 media or in 75% supernatants from *P. fluorescens* grown in M9 media for 4 days. After 80 min, IAA was added to a final concentration of 10 µM, and luminescence counts were continued. To determine the time course of *P. fluorescens* supernatant effects, hypocotyls were preincubated in 75% M9 media containing 1 mM luciferin and incubation solutions were replaced with 1 mM luciferin dissolved in 75% M9 media or 75% *P. fluorescens* supernatants 120 min after IAA addition.

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