Auxins Upregulate Expression of the Indole-3-Pyruvate Decarboxylase Gene in *Azospirillum brasilense*

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Transcription of the *Azospirillum brasilense ipdC* gene, encoding an indole-3-pyruvate decarboxylase involved in the biosynthesis of indole-3-acetic acid (IAA), is induced by IAA as determined by *ipdC-gusA* expression studies and Northern analysis. Besides IAA, exogenously added synthetic auxins such as 1-naphthaleneacetic acid, 2,4-dichlorophenoxypropionic acid, and *p*-chlorophenoxyacetic acid were also found to upregulate *ipdC* expression. No upregulation was observed with tryptophan, acetic acid, or propionic acid or with the IAA conjugates IAA ethyl ester and IAA-L-phenylalanine, indicating structural specificity is required for *ipdC* induction. This is the first report describing the induction of a bacterial gene by auxin.

Auxins constitute a class of phytohormones that play important roles in the coordination of plant growth and development. Indole-3-acetic acid (IAA), the most abundant naturally occurring auxin, has been implicated in regulating a variety of developmental and cellular processes such as cell extension, cell division, vascular differentiation, root formation, apical dominance, and tropisms (18). Regulation of these processes by auxin is believed to involve auxin-induced changes in gene expression (1, 31). Over the past 10 years, a number of plant genes that are transcriptionally induced by auxin, and that may play roles in one or more of these processes, have been cloned and further characterized (28, 31). The signal transduction pathways leading to the auxin-mediated gene induction in plants, however, are still not well understood (13, 26).

Besides plants, many soil and rhizosphere bacteria, including phytopathogenic, epiphytic, and plant growth-stimulating bacteria, also produce IAA. IAA biosynthesis in these bacteria has been shown to occur through different biosynthetic pathways (6, 20). By means of feeding experiments with tritiated putative IAA precursors, we have previously demonstrated the existence of multiple routes for IAA biosynthesis in the plant growth-promoting rhizobacterium *Azospirillum brasilense* (24). One of the pathways was identified as the indole-3-pyruvic acid (IPyA) pathway (L-tryptophan [Trp]→IPyA→indole-3-acetaldehyde→IAA) by cloning of the *A. brasilense ipdC* gene encoding an IPyA decarboxylase (5). In addition, two amino acid aminotransferases that catalyze the transamination of Trp, the first step of this pathway, were also purified (29). An *A. brasilense ipdC* knockout mutant was found to synthesize less than 10% of the level of wild-type IAA production, indicating that the IPyA decarboxylase is a key enzyme for IAA biosynthesis in this bacterium (24). In this study, we show that the expression of the *A. brasilense ipdC* gene is upregulated by IAA and other auxins.

Construction of the translational *ipdC-gusA* fusion pFAJ64. To study the expression of the *A. brasilense ipdC* gene, we constructed a translational *ipdC-gusA* fusion. First, the promoter region of the *A. brasilense ipdC* gene was amplified by means of PCR with primers annealing approximately 350 bp upstream of the *ipdC* start codon (avbgltx1, 5′-CGCC<u>GGAT</u> <u>CC</u>AAAGACGCCCATCAGGCGTC-3′) and at codons 1 to 7 of the *ipdC* gene (avbipdC1, 5′-AGGG<u>AGATCT</u>CACCCGG AATCCCGAACATG-3′). The proofreading Vent DNA polymerase (New England Biolabs, Beverly, Mass.) was used to increase the fidelity of DNA synthesis. The amplified fragment was flanked by *Bam*HI (avbgltx1) and *Bgl*II (avbipdC1) recognition sites (underlined in the sequences of the primers). Following digestion with *Bam*HI and *Bgl*II, the fragment was then cloned into the *Bam*HI site of pFAJ1171, a pUC18 derivative containing the promoterless *gusA* gene from pBI101.3 (15) on a 2-kb *Bam*HI-*Eco*RI fragment. The orientation of the insert in the transformants was determined by PCR with the primer avbgltx1 and an internal *gusA* primer (5′-GATTTCACGGGT TGGGGTTTCT-3′). The DNA sequence of the 350-bp promoter fragment was verified by DNA sequence analysis using the primers avbgltx1 and avbipdC1. Nucleotide sequence analysis using the internal *gusA* primer confirmed that the fusion was in frame. Finally, the *Bam*HI-*Eco*RI restriction fragment of approximately 2.4 kb, carrying the entire *ipdC-gusA* fusion, was inserted in the corresponding sites of the broad-host-range plasmid pLAFR3 (30), yielding pFAJ64. pFAJ64 was then transferred to *Escherichia coli* S17.1, from which it was mobilized to *A. brasilense* Sp245 and to the Sp245 IpdC⁻ derivative FAJ009.

Expression of *ipdC* is cell density dependent. It was observed previously that the level of IAA biosynthesis in *A. brasilense* varies during bacterial growth and that the highest IAA production level is obtained during the stationary-growth phase (24). In a first experiment, expression of the *ipdC-gusA* fusion, pFAJ64, in the wild-type strain Sp245 and in the IpdC⁻ mutant FAJ009 was measured quantitatively during growth in complex medium (Luria-Bertani medium supplemented with 2.5 mM MgSO₄ and 2.5 mM of CaCl₂ [LB* broth]). As shown in Fig. 1, expression of the *ipdC-gusA* fusion in both strains clearly increases with cell density and reaches its maximum when the cells enter the stationary-growth phase. Strikingly, the maximum level of *gus* expression in FAJ009 was found to be reduced to approximately 60% of that of the wild-type level. Growth phase and cell density-dependent induction of bacterial genes have often been shown to be mediated by small diffusible signal molecules. In *E. coli*, for instance, accumulation of weak acids in stationary-phase cultures serves as a signal to activate the expression of the sigma factor, RpoS, which in turn is required for the activation of other growth phase-specific sets of genes (27). Additionally, a variety of

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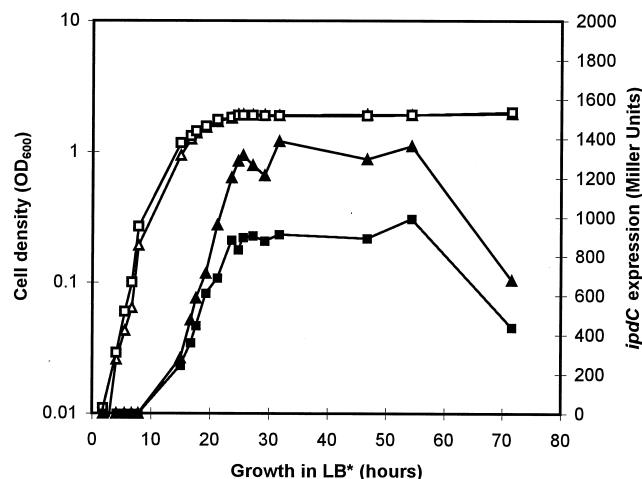
FIG. 1. Growth and expression of *ipdC* in *A. brasilense* Sp245 and in the IpdC⁻ mutant FAJ009. Cultures of Sp245(pFAJ64) and FAJ009(pFAJ64) were grown in LB* broth. Periodically, samples were taken to determine the expression of the *ipdC-gusA* fusion, pFAJ64. Quantitative analysis of β-glucuronidase activity was carried out in microtiter plates with *p*-nitrophenyl-β-D-glucuronide as substrate (14). Growth was monitored by measuring the OD_{600}. The data presented are from one representative experiment, which was confirmed twice in additional independent experiments. Triangles, Sp245(pFAJ64); squares, FAJ009(pFAJ64); open symbols, cell density (OD_{600}); solid symbols, β-glucuronidase activity in Miller units (17).

FIG. 2. Induction of the *ipdC-gusA* fusion by spent culture supernatants. An exponential-phase culture of Sp245(pFAJ64) (OD_{600} = 0.6) grown in LB* broth was centrifuged and resuspended in fresh LB* broth. Two-milliliter samples of this culture were then transferred to sterile test tubes, supplemented with different amounts of spent culture supernatant (ranging from 0 to 2 ml), and diluted up to a total volume of 4 ml with fresh LB* broth. After 2.5 h of growth at 30°C, the cultures were assayed for β-glucuronidase activity as detailed in the legend of Fig. 1. The spent culture supernatants were obtained from late-stationary-phase cultures (OD_{600} = 2.0) of Sp245 (solid bars) or FAJ009 (stippled bars) grown in LB* broth. Open bar, control culture to which no supernatant was added. All data are the means from three replicates. Error bars denote the standard deviations.

cellular processes in bacteria, including bioluminescence in *Vibrio fischeri* (4, 7, 9), production of exoenzymes and antibiotics in *Erwinia carotovora* (2, 16), and plasmid conjugal transfer in *Agrobacterium tumefaciens* (22, 34), are switched on by diffusible compounds, termed autoinducers and identified as *N*-acyl homoserine lactones (AHLs). Activation of the specific target genes occurs when a required threshold of AHLs is attained and in this way is only achieved at high cell densities. Therefore, the observed growth phase-dependent expression of the *A. brasilense ipdC* gene prompted us to investigate whether an extracellular product accumulating in spent medium is involved in *ipdC* induction. To test this possibility, different amounts of filter-sterilized culture supernatant (0.22-μm-pore size) obtained from a stationary-growth-phase culture of *A. brasilense* Sp245 were added to exponentially growing cells of Sp245(pFAJ64), and β-glucuronidase activity was determined after 2.5 h of additional growth. As can be concluded from Fig. 2, *ipdC* expression increases with increasing amounts of the stationary-phase culture supernatant. A twofold dilution of the reporter strain (2 ml of culture plus 2 ml of supernatant) resulted in a fivefold increase in β-glucuronidase activity compared to the activity detected in control cultures to which no supernatant was added.

When this experiment was repeated with spent medium of a stationary-phase culture of the IpdC⁻ strain FAJ009, only a slight effect on *ipdC* expression was observed (Fig. 2). Only when large amounts of supernatant of a FAJ009 culture were applied was pFAJ64 induction significantly greater than in the control cultures.

Expression of *ipdC* is upregulated by IAA. The difference in *ipdC* expression levels between the wild type and the IpdC⁻ mutant and the observation that spent culture supernatant of the IpdC⁻ mutant does not efficiently induce *ipdC* expression led us to speculate that the end product of the biosynthetic pathway, IAA, could be the inducing compound. It has been shown previously that IAA accumulates in the supernatant of wild-type culture (up to 200 μM in a stationary-growth-phase
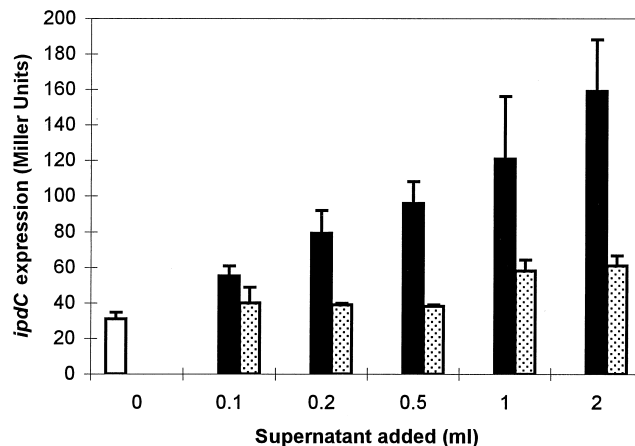
culture in LB* broth) but is reduced to 10% of the wild-type level in the supernatant of the IpdC⁻ mutant (24). The idea of IAA as inducing compound was further tested by examining the effect of exogenously added IAA on *ipdC* expression. Sp245(pFAJ64) and FAJ009(pFAJ64) cultures at different growth phases (starter cultures) were supplemented with 1 mM IAA and, after 3.5 h of additional growth, compared for *ipdC-gusA* induction with cultures to which no IAA was added. As can be concluded from Fig. 3, in all growth phases, both for Sp245 and FAJ009, a higher expression level of pFAJ64 was found in cells exposed to exogenously added IAA compared to the level in untreated cells. As expected, upregulation by exogenously added IAA (i.e., the difference in the expression levels between a culture supplemented with IAA and an untreated culture) was most obvious in the IpdC⁻ mutant background and was growth phase dependent. The difference in the extent of upregulation at the various growth phases and between the mutant and the wild type may be due to differences in endogenous IAA levels in the starter cultures. For Sp245, a threefold upregulation was detected in early-exponential-phase cells (optical densities at 600 nm [OD_{600}] of 0.1 and 0.4). Upregulation then gradually decreased in the mid and late-exponential phases (1.4-fold increase at OD_{600} = 1.1 and 1.4) and stationary-phase cultures (1.1 fold increase at OD_{600} = 1.9) because of the higher endogenous IAA production in the corresponding starter cultures. In contrast to Sp245 and concomitant with the lower IAA production capacity of the IpdC⁻ mutant, a fourfold upregulation could still be detected in late-exponential-phase FAJ009 culture (OD_{600} = 0.9). The lowest concentration of exogenously added IAA for which significant *ipdC* induction was observed in an exponential-phase culture of FAJ009 was 10 μM (Table 1).

The observed induction of the *ipdC-gusA* fusion in cells growing in the presence of IAA was confirmed by quantitative Northern analysis. RNA of *Azospirillum* cells was isolated essentially as described by Eggermont et al. (8). RNA concen-
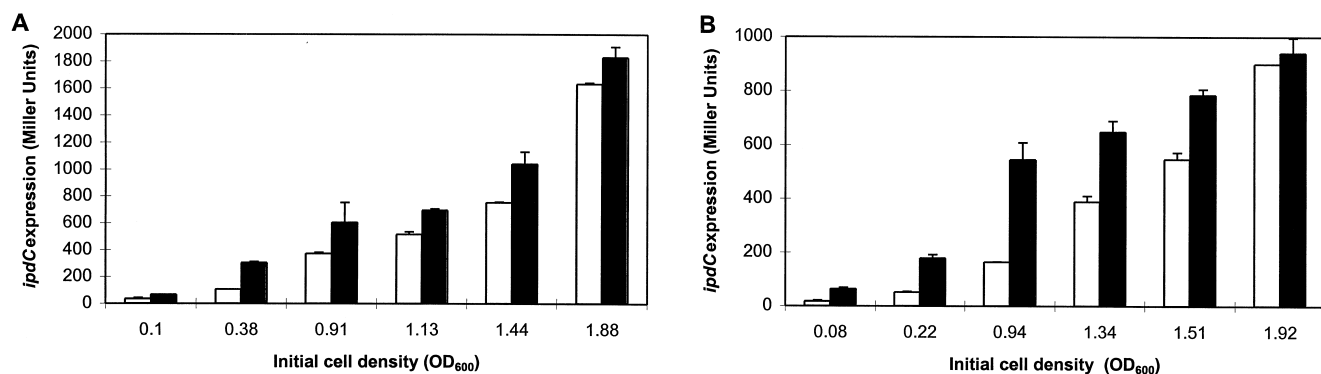
FIG. 3. Effect of exogenously added IAA on *ipdC* expression in Sp245 and the IpdC⁻ mutant, FAJ009. To obtain cultures at various cell densities of Sp245(pFAJ64) (A) and FAJ009(pFAJ64) (B) different flasks containing 100 ml of LB* medium were inoculated with different volumes of preculture and grown overnight at 30°C. From each culture, six samples of 3 ml were transferred to sterile test tubes. Three test tubes of each series were supplemented with IAA to a final concentration of 1 mM (solid bars); to the remaining three test tubes no IAA was added (open bars). After 3.5 h of additional growth at 30°C, β-glucuronidase activity was assayed as detailed in the legend of Fig. 1. Data are the means from the three replicates. Error bars denote the standard deviations.

trations were determined fluorimetrically after staining with SYBR Green II in accordance with the manufacturer's protocols (Molecular Probes). A total of 10 μg of each RNA sample was separated on a formaldehyde-agarose gel, transferred to a positively charged nylon membrane (Boehringer Mannheim), and hybridized as described previously (8). To verify equal loading and transfer of RNA, the loading buffer was supplemented with 50 μg of ethidium bromide/ml, allowing visualization of RNA in the gel and on the blot when illuminated with UV light (8) (Fig. 4A). To prepare the *ipdC* riboprobe, a 1.8-kb *Sma*I fragment containing approximately 200 bp of the *ipdC* promoter and almost the entire coding region was cloned into pBlueScriptIISK+ vector (Stratagene). The digoxigenin-labeled antisense transcript was then prepared by in vitro run-off transcription with the Dig RNA labeling kit from Boehringer Mannheim. Figure 4B shows the presence of a single transcript of approximately 2.6 kb hybridizing with the *ipdC*

probe in RNA isolated from wild-type cells, which corresponds to the predicted size of a putative dicistronic mRNA product of the *ipdC* gene (1,635 bp) and a downstream located open reading frame (ORF) of 669 bp. The deduced amino acid
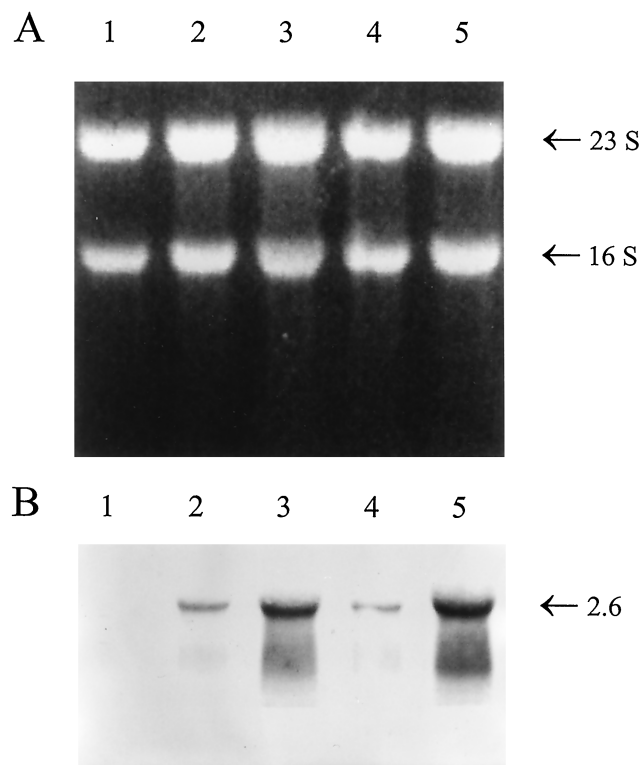

FIG. 4. Northern blot analysis of RNA extracted from cells of *A. brasilense* wild-type and IpdC⁻ mutant strains. (A) Blot of total RNA (10 μg per lane) isolated from *A. brasilense* IpdC⁻ mutant (lane 1) and wild-type strains (lanes 2 to 5). RNA was visualized by epi-illumination with UV light. Cells were harvested at an OD₆₀₀ of 0.9 (lanes 1 and 3), 0.5 (lane 2), or 0.4 (lanes 4 and 5). To measure the effect of IAA on the transcription of the *ipdC* gene, the wild-type culture at OD₆₀₀ of 0.4 was divided into two and further incubated for 3 h at 30°C in the absence (lane 4) or presence of exogenously added IAA at 1 mM (lane 5). The positions of the 16S and 23S ribosomal RNAs are indicated at the right. (B) RNA blot analysis of the same samples as in panel A after hybridization with a digoxygenin-labeled *ipdC*-derived riboprobe. The size of the *ipdC* transcript (in kilobases) is indicated at the right and was determined relative to RNA standards that were electrophoresed in the same gel.

TABLE 1. Effect of auxins on expression of translational *gusA* fusions with either *ipdC* (pFAJ64) or a constitutive *A. brasilense* promoter (pFAJ31.13)

Substrate added (final concn)[a]	Mean β-glucuronidase activity[b] in:	
	FAJ009(pFAJ64)	FAJ009(pFAJ31.13)
None	152 (5)	296 (31)
IAA (1 mM)	466 (18)	304 (26)
IAA (0.1 mM)	353 (15)	261 (24)
IAA (0.01 mM)	222 (30)	ND[c]
NAA (1 mM)	439 (14)	252 (17)
NAA (0.1 mM)	367 (8)	254 (7)
2,4-DP (1 mM)	473 (28)	ND
4-CPA (1 mM)	509 (39)	ND
IBA (1 mM)	137 (10)	ND
Trp (1 mM)	103 (6)	ND
Acetic acid (1 mM)	154 (8)	ND
Propionic acid (1 mM)	77 (7)	ND
IAA ethyl ester (1 mM)	113 (23)	ND
IAA-L-phenylalanine (1 mM)	175 (26)	ND

[a] Samples (3 ml) of exponential-phase cultures of FAJ009(pFAJ64) and FAJ009(pFAJ31.13) (OD₆₀₀ = 0.4) grown in LB* broth were transferred to sterile test tubes and supplemented with the various substrates to the final concentrations indicated in parentheses. After 4 h of additional growth at 30°C, the cultures were assayed for β-glucuronidase activity as detailed in the legend of Fig. 1.
[b] Standard deviations of three replicates are given in parentheses.
[c] ND, not determined.

sequence of this ORF is homologous to the *E. coli* anti-sigma cross-reacting protein (SCRP-27A [32]) (57% identity, unpublished results). The message was absent in the IpdC⁻ mutant, proving the specificity of the *ipdC* probe (Fig. 4B, lane 1). In agreement with the results of the *ipdC-gusA* expression analysis, the level of *ipdC* mRNA in wild-type cells increased during growth (Fig. 4B, lanes 2 and 3) and was clearly higher in cells grown in the presence of exogenously added IAA (Fig. 4B, lanes 4 and 5).

Different auxins induce *ipdC* expression. IAA-inducible genes in higher plants have been shown to respond to, besides IAA, synthetic auxins such as naphthaleneacetic acid (NAA) and chlorophenoxy acids (28). To evaluate whether the *A. brasilense ipdC* gene could also be induced by other auxins, exponential-growth-phase cultures of FAJ009(pFAJ64) (OD$_{600}$ = 0.4) were supplemented with the naturally occurring auxin indole-3-butyric acid (IBA) or with the synthetic auxins NAA, 2,4-dichlorophenoxypropionic acid (2,4-DP), or *p*-chlorophenoxyacetic acid (4-CPA) and compared for *ipdC* induction. As indicated by the data in Table 1, the three tested synthetic auxins, NAA, 2,4-DP, and 4-CPA, were found to upregulate *ipdC* expression. Under the conditions tested, induction of the *ipdC-gusA* fusion by all three synthetic auxins was as strong as the induction observed with IAA. In contrast, addition of IBA to the cultures did not affect *ipdC* expression. In plants, the conversion of IBA to IAA has been reported (10, 11), and it is not yet clear whether IBA is itself an auxin or whether it exerts its auxin activity through its conversion to IAA (3). The observation that the *A. brasilense ipdC* gene is not upregulated by IBA might therefore be attributed to the absence of enzymes catalyzing the conversion of IBA into IAA in *Azospirillum*. The addition of the two IAA conjugates, IAA ethyl ester and IAA-L-phenylalanine, to the FAJ009(pFAJ64) culture had no effect on *ipdC* expression (Table 1). This might be due to the lack of intracellular transformation of these compounds into active auxins, although it cannot be excluded that these conjugates fail to induce *ipdC* gene expression because they are not taken up by *A. brasilense* cells. Treatment of the FAJ009(pFAJ64) culture with similar concentrations of Trp, acetic acid, and propionic acid also did not enhance *ipdC* induction (Table 1), indicating that the upregulation of *ipdC* expression by IAA and the synthetic auxins is auxin specific and not only caused by the acid group of the auxin molecules. In control experiments, incubation of FAJ009 containing pFAJ31.13, a pLAFR1 derivative carrying a fusion between a constitutive *A. brasilense* promoter and *gusA* (33), with IAA or NAA did not affect β-glucuronidase activity (Table 1).

General conclusion. Evidence is presented here for the upregulation of expression of the *A. brasilense* IAA biosynthetic gene, *ipdC*, by IAA and synthetic auxins. This positive feedback regulation by IAA is responsible for the increasing *ipdC* transcription levels during growth of an *A. brasilense* culture, with the highest expression level of the *ipdC* gene observed in the stationary-growth phase. Reduced IAA accumulation in stationary-phase cells of the IpdC⁻ mutant strain results in a lower *ipdC* expression maximum in this strain compared to that of the wild type. In contrast to negative transcription control of biosynthetic genes by the end product formed (19, 23), autoinduction of a biosynthetic pathway is rather exceptional. Other genes, besides the *A. brasilense ipdC* gene, that have been demonstrated to be autoinduced are the AHL biosynthetic genes found in various gram-negative bacteria (12) and the genes involved in the production of the siderophores yersiniabactin in *Yersinia enterocolitica* (21) and pyochelin in *Pseudomonas aeruginosa* (25). Until now auxin-responsive genes have only been identified in plants (1, 28, 31). The role of many of these auxin-inducible genes in plant developmental processes is not yet fully understood. This study presents data

indicating for the first time the induction of a bacterial gene by auxin. This finding suggests that proteins involved in IAA perception and signal transduction are present not only in higher plants but also in bacteria.

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