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Research Notes

Cloning of the Genes for Indoleacetic Acid Synthesis from *Pseudomonas syringae* pv. *syringae*

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Genes for indole-3-acetic acid (IAA) biosynthesis in *Pseudomonas syringae* pv. *syringae*, the causal agent of brown spot disease in bean, were identified and cloned by using a DNA fragment that contained the *iaaM* and *iaaH* genes from *P. s.* pv. *savastanoi* as a probe. A 2.8-kb *Eco*RI fragment was detected in DNA from six strains of *P. s.* pv. *syringae* that were originally isolated from bean and pear. Strains of *P. s.* pv. *syringae* from tomato, wheat, and corn, and two strains of *P. s.* pv. *pisi* were negative. The results suggest that the supbgroup of *P. s.* pv. *syringae* strains from bean plants and possibly strains from pears synthesize IAA via the *iaaM/iaaH* pathway.

Plant pathogenic bacteria synthesize a variety of plant growth regulators (Gross and Cody 1985; Morris 1986), and the ability to produce the auxin indole-3-acetic acid (IAA) is particularly widespread among plant pathogenic bacteria (Sequeira and Williams 1964; Kosuge *et al.* 1966; Fett *et al.* 1987). In a number of cases from molecular and physiological studies, a role for IAA in disease symptomatology has been indicated. In *Pseudomonas syringae* pv. *savastanoi* (Smith) Young *et al.*, *Agrobacterium tumefaciens* (Smith and Townsend) Conn, and *A. rhizogenes* (Riker *et al.*) Conn, the production of IAA via the indoleacetamide pathway is involved in gall formation (Smidt and Kosuge 1978; Akiyoshi *et al.* 1983; Surico *et al.* 1985; White *et al.* 1985; Cardarelli *et al.* 1987) and is suspected to be involved in other diseases involving tissue hyperplasia (Iacobellis *et al.* 1988).

IAA is also produced by pathogens where the direct consequence of synthesis is not clear (Fett *et al.* 1987; Sequeira and Williams 1964), indicating that IAA may have other effects in host/pathogen interactions in addition to tissue hyperplasia and that IAA may even serve as a pathogen metabolite irrespective of the host. In studying the effect of microbial auxin biosynthesis in disease, we reasoned that a homologue of the *iaa* operon from *P. s.* pv. *savastanoi* might be found in other species that are represented in the diverse *P. syringae* group, many or most of which do not cause galls on the respective host plants. Genomic DNA samples from a variety of pathovars of *P. syringae* were tested for sequence relatedness to a probe from the *iaa* region of *P. s.* pv. *savastanoi* (Zeigler *et al.* 1987). Hybridization was detected with a number of pathovars, and one of the pathovars with a strong hybridization signal was a strain of *P. s.* pv. *syringae* (van Hall) Dye, the causal agent of brown spot disease of bean.

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To further investigate the presence of the *iaa* biosynthetic genes in *P. s.* pv. *syringae*, genomic DNA from strains of *P. s.* pv. *syringae* from diverse host plants (Table 1) were screened for sequence relatedness to pLUC1, which contained the proximal portion of the *iaa* operon of *P. s.* pv. *savastanoi* (Yamada *et al.* 1985). DNA from six strains of *P. s.* pv. *syringae* were determined to contain a 2.8-kilobase (kb) *Eco*RI fragment with sequence relatedness to pLUC1 (Table 1). DNA from four strains, B76, B64, B61, and 176 that were originally isolated from tomato, wheat (two strains), and corn, respectively, did not hybridize with the probe (Table 1). Because hybridization with *iaaM* to DNA from a strain of *P. s.* pv. *pisi* had been reported previously (Zeigler *et al.* 1987), two strains of *P. s.* pv. *pisi,* G28-6 and pisi-4 (Table 1), were also included. DNA from both strains did not give detectable hybridization with the probe. DNA from an additional four strains of *P. s.* pv. *syringae* that were originally isolated from bean plants were also found to contain the 2.8-kb fragment (Table 1).

In addition to the hybridization with pLUC1, culture fluids of strains were analyzed qualitatively for IAA by thin-layer chromatography (TLC) after partial purification from 5 ml of late-log cultures. With culture supernatants from strains Y30 and B86-7, radioactivity was detected at the position expected for ^{3}H-IAA (Fig. 1; lanes 3 and 4). Strain EW2009 of *P. s.* pv. *savastanoi* was used as a positive control. The strain produced large amounts of IAA in the culture supernatant (Fig. 1, lane 2), whereas strain EW2009-3 of *P. s.* pv. *savastanoi*, which had been previously cured of the plasmid harboring the *iaa* operon, was negative (Fig. 1, lane 5). Strains Y30 and B86-7 of *P. s.* pv. *syringae* were both positive for IAA production, although the levels were lower than for *P. s.* pv. *savastanoi* (Fig. 1, lanes 3 and 4). Strains B64 of *P. s.* pv. *syringae* and G28-6 of *P. s.* pv. *pisi* produced comparatively little, if any, IAA (Fig. 1, lanes 6 and 7, respectively). All strains were similarly analyzed and scored as positive for IAA if the spot was clearly visible and negative if the amount of IAA was either extremely low or undetectable by TLC (Table 1). Strains that had the 2.8-kb *Eco*RI fragment were positive, whereas all other strains tested were negative.

A cosmid library was constructed by ligation of *Sau*3A partial digestion products of DNA from *P. s.* pv. *syringae* Y30 into the *Bam*HI site of pHC79 (Hohn and Collins 1980), and clones with the 2.8-kb *Eco*RI fragment were selected by colony hybridization with the purified fragment from pLUC1 as the probe (Ausubel *et al.* 1987). The *iaa* region of one clone, pY305, was mapped by restriction enzyme analysis (Fig. 2).

To determine whether the putative *iaa* genes could direct the synthesis of IAA, pY305 was subcloned into the wide-host-range plasmid pLAFR3 (Staskawicz *et al.* 1987). Two clones that overlapped the putative *iaa* region were obtained (Fig. 2, pY305-4 and pY305-10). These two clones were subjected to Tn*5* mutagenesis (Ruvkun and Ausubel 1981). Two clones of pY305-4 were identified with insertions in the 2.8-kb fragment. The vector pLAFR3 and the clones pY305-10, pY305-4, pY305-4::Tn*5*-1, and pY305-4::Tn*5*-2 were then transferred to the Iaa$^-$ strain EW2009-3rif of *P. s.* pv. *savastanoi* by mobilization using *Escherichia coli* strain 17-1 (Simon *et al.* 1983).

The clones pY305-4 and pY305-10 conferred on EW2009-3rif the ability to synthesize detectable amounts of IAA as indicated by colorimetric assays (Table 2). The insertions of Tn*5* into the 2.8-kb *Eco*RI fragments resulted in reduced levels of IAA in the supernatants compared with the levels in EW2009-3rif(pY305-4) (Table 2). The supernatants of strains that harbored pY305-4 and pY305-10 were also examined for IAA by TLC and observed to contain a com-

Table 1. Bacterial strains used in this study

Designation[a]	Relevant characteristics	IAA[b]	*Eco*RI[c] (2.8 kb)	Reference or source
Pseudomonas syringae pv. *savastanoi*				
EW2009	Wild-type, oleander (*Nerium oleander* L.) *iaa* on plasmid pIAA1	+	+	Comai and Kosuge 1980
EW2009-3	Cured of pIAA1	−	−	Comai and Kosuge 1980
TK1050	Wild-type, olive (*Olea europa* L.) chromosomal *iaa*	+	+	Glass and Kosuge 1980
EW2009-3rif	Rif^r EW2009-3	+	+	This study
pv. *syringae*				
Y30	Wild-type, bean (Wisconsin)	+	+	D. Legard and M. Schroth[d]
B86-7	Wild-type, snapbean (New York)	+	+	D. Legard
B86-1	Wild-type, snapbean (New York)	+	+	D. Legard
BBS 6-3	Wild-type, snapbean (New York)	+	+	D. Legard
BBS 102-6	Wild-type, snapbean (New York)	+	+	D. Legard
PS955	Wild-type, pear	+	+	Currier and Morgan 1983
B76 (132)	Wild-type, tomato	−	−	T. Denny[e]
B64 (138)	Wild-type, wheat	−	−	T. Denny
B61 (144)	Wild-type, wheat	−	−	T. Denny
176	Wild-type, corn	−	−	T. Denny
pv. *pisi*				
G28-6 (pisi-1)	Wild-type, pea (New York)	−	−	D. Legard
pisi-4	Wild-type, pea (Wisconsin)	−	−	D. Legard

[a] Original designation if known, otherwise the designation of source was used. Designation in parentheses is strain collection number from source.
[b] Presence (+) or absence (−) of IAA determined by thin-layer chromatography.
[c] Presence of 2.8-kilobase (kb) *Eco*RI fragment determined by Southern blot analysis with the pLUC1 probe.
[d] New York State Agricultural Experiment Station, Cornell University, Geneva, and University of California, Berkeley, respectively.
[e] University of Georgia, Athens.

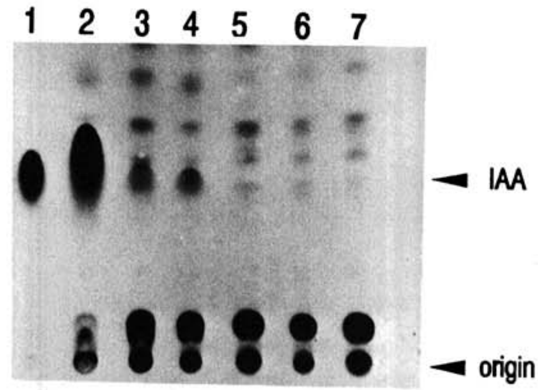

Fig. 1. Thin-layer chromatography analysis of culture supernatants from pathovars of *Pseudomonas syringae*. Lane 1, 0.25 μCi ^{3}H-IAA in 10 μmoles of indole-3-acetic acid as a standard; lane 2, EW2009; lane 3, Y30; lane 4, B86-7; lane 5, EW2009-3; lane 6, B64; lane 7, G28-6. *P. syringae* pathovars were grown in King's B (King *et al.* 1954) that contained 2.5 mM tryptophan or 0.36 μM [5-^{3}H]L-tryptophan (10 μCi; Amersham, Arlington Heights, IL), and pelleted by centrifugation for 5 min at 10,000 rpm. The supernatant was collected, adjusted to pH 3.0 by dropwise addition of 1 N HCl and extracted twice with 5 ml of ethyl acetate. The ethyl acetate was removed by vacuum evaporation, and the residue was resuspended in methanol. A 4 μl-sample was applied to a precoated silica gel 60 plate (HPTLC, EM Science, Cherry Hill, NJ) and developed in 9:1 $CHCl_3$/methanol. Equal amounts of supernatant were applied in lanes 3–7. Extract from EW2009 was diluted twofold. Plate was sprayed with En^{3}Hance (New England Nuclear, Boston, MA) and exposed to X-ray film for 5 days at −70° C.

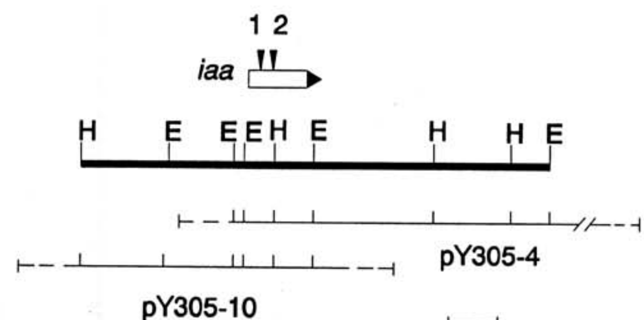

Fig. 2. Map of the *iaa* region of *Pseudomonas syringae* pv. *syringae*. The open box designates the position of the *iaa* operon as determined by sequence relatedness to *iaaM* and *iaaH* of *P. s.* pv. *savastanoi* (data not shown). Arrow at left indicates the direction of transcription. Arrows 1 and 2 indicate the approximate positions of Tn*5* in pY305-4::Tn*5*-1 and pY305-4::Tn*5*-2. Fragments less than 0.4 kb were not mapped. E, *Eco*RI; H, *Hin*dIII.

Table 2. Indole-3-acetic acid (IAA) production directed by *iaa* region of *Pseudomonas syringae* pv. *syringae*

Strain	μM IAA[a]
EW2009-3rif (pLAFR3)	0
EW2009-3rif (pY305-4)	33 ± 6
EW2009-3rif (pY305-10)	61 ± 4
EW2009-3rif (pY305-4::Tn*5*-1)	2 ± 4
EW2009-3rif (pY305-4::Tn*5*-2)	7 ± 3
EW2009	65 ± 4

[a] IAA concentration in culture supernatants of the transconjugants grown in liquid King's medium was determined by a colorimetric assay (Gordon and Weber 1951). A dilution series of authentic IAA was used as standards. Absorbance at 530 nm of culture supernatant of EW2009-3rif was assumed to be due to other reactive indoles or IAA via other pathways. The background absorbance of EW2009-3rif (pLAFR3) was subtracted from all values for IAA concentration that were also normalized to cell culture density as determined by absorbance at 600 nm. Values in the table were derived by linear regression analysis of absorbance values from three culture supernatants of each strain.

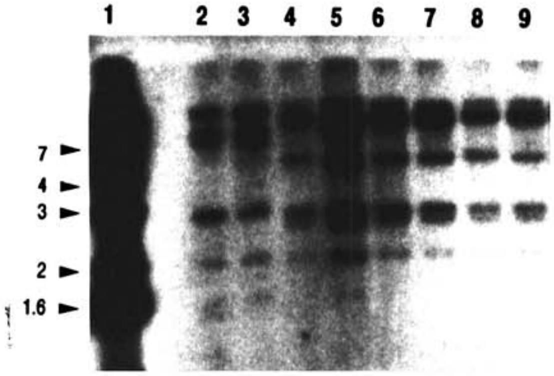

Fig. 3. Filter hybridization analysis of DNA from strains of *Pseudomonas syringae* using ^{32}P-labeled pY305 as probe. Lane 1, 1-kb standard; lane 2, TK1050; lane 3, EW2009; lane 4, Y30; lane 5, B86-7; lane 6, B86-13; lane 7, BBS 6-3; lane 8, BBS 102-6; lane 9, PS955. Bacterial DNA isolation and hybridization procedures were as described by Leach et al. (1990). DNA was digested with *Eco*RI. Arrows at left indicate selected size standards in kilobases.

pound that comigrated with the authentic IAA standard, while supernatants from strains that contained pY305-4::Tn*5*-1 and pY305-4::Tn*5*-2 had little or no IAA (data not shown). The results indicate that the genes and gene products are active in the closely related strain EW2009-3rif, and, although the presence of indoleacetamide was not specifically assayed in this study, some IAA in *P. s.* pv. *syringae* is produced via the *iaaM*/*iaaH* pathway. Fett *et al.* (1987) have identified IAM in culture supernatants of one strain of *P. s.* pv. *syringae*.

The strains from bean have been grouped into different subgroups based on restriction fragment polymorphism (RFLP) analysis, and, with the exception of one strain from pear, the strains in this study represented various bean subgroups. When pY305 was used as a hybridization probe, RFLP analysis of the six IAA positive strains revealed identical patterns (Fig. 3). The results suggest that the bean strains have relatively high sequence conservation in this portion of the genome. The *iaa* genes may then represent important functions for adaptation to beans. More strains of *P. s.* pv. *syringae* from pear will have to be examined before general conclusions can be drawn regarding the similarity of pear isolates in general to strains from bean. If strain 955 is typical, the data indicate that the pear strains are also members of the bean subgrouping, and the possible involvement of IAA in bacterial blossom blast of pear may warrant further examination.

The results presented here have identified a subgroup of strains of *P. s.* pv. *syringae* that contain apparent homologues of *iaaM* and *iaaH* genes of *P. s.* pv. *savastanoi*. Hybridization was detected only in the strains of *P. s.* pv. *syringae* that were isolated from bean (*Phaseolus vulgaris* L.) and pear. Of the strains examined, the presence of the genes was strictly correlated with relatively high levels of IAA in the culture supernatants. Other pathovars and strains may harbor *iaaM* and *iaaH* homologues whose sequences have diverged to the degree that hybridization is not readily detected. Whereas IAA levels were low or undetectable by TLC in strains of *P. s.* pv. *syringae* from corn, wheat, and tomato and in two strain of *P. s.* pv. *pisi* from pea under liquid culture conditions, the possibility remains that larger amounts of IAA are produced under other culture conditions and that the same pathway may be used as for *P. s.* pv. *savastanoi*. Homologues of *iaaM* are known to exist in the T-DNA regions of *A. tumefaciens* and *A. rhizogenes* that were not detected in hybridization studies with the respective T-DNA probes (Levesque *et al.* 1988). Low-level signals were observed for DNA from a variety of pathovars using pLUC1, and each warrants further investigation (Zeigler *et al.* 1987).

In disease, IAA has often been implicated in the expression of symptoms involving tissue hyperplasia (Akiyoshi *et al.* 1983; Comai and Kosuge 1980; Inze *et al.* 1984). IAA may have effects beyond the stimulation of plant cell growth. Inhibition in the hypersensitive reponse on tobacco after inoculation with *P. s.* pv. *phaseolicola* and *A. tumefaciens* or *P. s.* pv. *savastanoi* was recently shown to depend on the presence of the genes for IAA biosynthesis (Robinette and Matthysse 1990), and strains of *P. s.* pv. *savastanoi* with mutations in *iaaL* were found to have increased IAA levels, reduced virulence, and suppressed growth within host tissue (Glass and Kosuge 1988). Studies of *P. s.* pv. *savastanoi* can be confounded by the difficulty of tests that involve oleander and olive, both woody species that are difficult to culture axenically. *P. s.* pv. *syringae* may prove to be an excellent subject for the study of IAA involvement in host/pathogen interactions. The isolation of the *iaa* operon from *P. s.* pv. *syringae* represents the first step toward this goal.

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