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Indole-3-Acetic Acid Production in *Pseudomonas fluorescens* HP72 and Its Association with Suppression of Creeping Bentgrass Brown Patch

Shino Suzuki, Yuxi He, Hiroshi Oyaizu

Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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Abstract. *Pseudomonas fluorescens* HP72, which suppresses the brown patch disease on bentgrass, produces several secondary metabolites, 2,4-diacetylphloroglucinol (2,4-DAPG), HCN, siderophore, and indole-3-acetic acid (IAA). In this study, IAA biosynthesis in strain HP72 was investigated. After several repeated subcultures, the spontaneous IAA low-producing mutant HP72LI was isolated. The IAA low production of the strain HP72LI was due to the low tryptophan side chain oxidase (TSO) activity. Colonization of strain HP72 on the bentgrass root induced root growth reduction, while strain HP72LI did not induce such growth reduction. The colonization ability of strain HP72 on the bentgrass root is higher than that of strain HP72LI. However, as for biocontrol ability, a significant difference in both strains was not detected. IAA production by strain HP72 may play a role in the construction of short root systems and take advantage of root colonization, but does not contribute to the biocontrol properties of *P. fluorescens* HP72.

Because increasing fungicide inputs to highly managed golf course turf has a high potential to cause contamination of drinking water, biological control is an attractive means of reducing these inputs [4, 19]. Certain root-associated strains of fluorescent *Pseudomonas* are known to suppress soil-borne plant pathogens [24]. In order to control brown patch disease caused by *Rhizoctonia solani*, which is one of the most destructive pathogens of turfgrass, the bacterium *Pseudomonas fluorescens* HP72 was isolated from roots of creeping bentgrass on brown patch-suppressive soil [16]. Strain HP72 has been selected as a biocontrol agent for the following reasons: (i) having strong antifungal activity in vitro to several pathogens, *Rhizoctonia solani* AG2-2 IIIB, *Sclerotinia homoeocarpa*, *Pythium aphanidermatum*, and *Gaeumannomyces graminis*; (ii) suppressing brown patch disease caused by *Rhizoctonia solani* AG2-2 IIIB as effective as that provided by fungicide in natural soil in a growth chamber; and (iii) colonizing and maintaining a high population rate on bentgrass roots in both sod test in the laboratory and on the turfgrass in the field.

The analysis of secondary metabolites from strain HP72 revealed that strain HP72 produced the major known antifungal factors 2,4-diacetylphloroglucinol (2,4-DAPG), HCN, and fluorescent compound [17]. In this study, we focused on the plant growth regulator indole-3-acetic acid (IAA), which was one of the secondary metabolites produced by strain HP72, and we attempted to reveal the relationship between the IAA production and good root colonization ability in strain HP72.

Many root-associated bacteria have been reported to produce IAA in culture media [6, 10, 21]. Several pathways for the biosynthesis of IAA from L-tryptophan have been investigated. The indole acetamide (IAM) pathway was found in several bacterial pathogens, i.e., *Pseudomonas syringae* pv. *savastanoi*, *Agrobacterium tumefaciens* [5, 25, 28], and the indole-3-pyruvic acid (IpyA) pathway, whose key enzyme, indole-3-pyruvate decarboxylase, was found in the plant growth-promoting rhizobacteria (PGPR) *Enterobacter*, *Azospirillum*, and *Pseudomonas putida* [7, 13, 27]. The 2,4-DAPG producing biocontrol strains of *Pseudomonas fluorescens* CHA0 and *Pseudomonas fluorescens* Pf-5 synthesize the tryptophan side chain oxidase (TSO) [20, 26]. In strain CHA0, the TSO pathway has been reported to be one of the major pathways to produce IAA [20].

In gall-forming pathogenic bacteria such as *Pseudo-*

Correspondence to: S. Suzuki.

monas syringae pv. *savastanoi*, IAA has been demonstrated to have an important role in the production of disease symptoms [14, 21]. However, the role of IAA production by non-pathogenic bacterial species in plant-bacteria association remains unclear.

From the viewpoint of colonization ability, IAA produced by non-pathogenic *Erwinia herbicola* 299R has been reported to contribute to the colonization on bean and tobacco leaves and pear flowers [2, 3]. In terms of biocontrol efficacy, IAA overproduced by the biocontrol strain *P. fluorescens* CHA0 does not improve the protection of cucumber against *Pythium* root rot [1].

In this study, we focus on the IAA-synthesizing pathway of the biocontrol *P. fluorescens* HP72. In addition, we assess the effect of IAA production on the root colonization ability, which is one of the important factors of PGPR, and on the growth of bentgrass root and its relationship to biocontrol ability by comparing the IAA low-producing mutant with its parental strain.

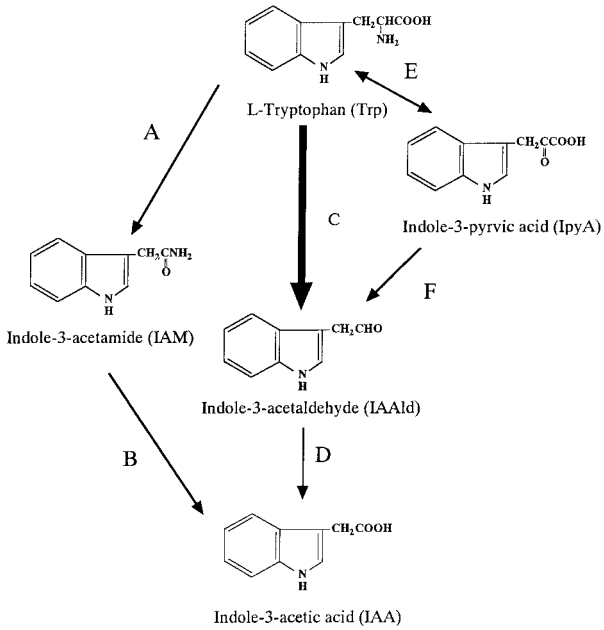

Fig. 1. Pathways in the bacterial biosynthesis of indole-3-acetic acid (IAA): indole-3-acetamide pathway (A-B), indole-3-pyruvic acid pathway (E-F-D), and tryptophan side chain oxidase pathway (C-D). A, tryptophan monooxygenase; B, indoleacetamide hydrolase; C, tryptophan side chain oxidase; D, indole-3-acetaldehyde oxidase; E, tryptophan transferase; F, indole-3-pyruvate decarboxylase.

Materials and Methods

Bacterial strains, culture conditions, and plant growth condition. *P. fluorescens* strain HP72 was isolated from the root of bentgrass for the purpose of suppressing the brown patch disease on turfgrass [16]. After several repeated subcultures, the spontaneous IAA low-producing mutant of *P. fluorescens* HP72 was isolated and named strain HP72LI. Cultures of the *Pseudomonas* strains were grown at 30°C in King's B broth [12] containing nalidixic acid (25 μg/mL) (Nal) for routine purposes. Strain HP72 spontaneously was resistant to Nal. For IAA extraction and detection, *Pseudomonas* strains were grown at 30°C in M9 minimal broth [15] with Nal (25 μg/mL) and 0.3 mM tryptophan (Trp) in the dark. Creeping bentgrass (*Agrostis palustris Huds*., cv. Pencross) was grown in an autoclaved sand soil mixture (95% sand and 5% vermiculite clay) at 28°C with a 12-h photoperiod. At the start of each experiment, sterile water was added to the plants, and after 2 days, sterile plant nutrient solution (PNS), containing 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and micronutrients [11], was added to plants, and this condition was repeated.

Detection of IAA and other indole compounds. Strains HP72 and HP72LI were grown in M9 medium with 0.3 mM Trp for 96 h, and twice the volume of Salkowsky reagent (0.01 M $FeCl_2$ in 35% $HClO_4$) was added to each bacterial culture [9]. IAA was visually detected by the color development. To confirm IAA production in the culture supernatant and to detect other intermediate indole compounds, strain HP72 was grown in 200 mL of M9 medium supplemented with 0.3 mM Trp. The culture supernatant was adjusted to pH 2 with HCl and was extracted twice with equal volumes of ethyl acetate. The solvent phase was dried by using a rotary evaporator. The extract was trimethylsilylated with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (GL Sciences Inc., Japan) according to the manufacturer's instructions and was analyzed by gas chromatography–mass spectrometry (GC–MS). The GC–MS analysis was performed on a GCMS–QP5050 (Shimadzu) equipped with a DB-1 column in which the temperature was increased from 90°C to 280°C at 7°C per min.

Resting cell reaction and enzyme assay. Strains HP72 and HP72LI were grown for 18 h in King's B broth. The cells were harvested by centrifugation at 3000 *g* for 10 min and washed twice with 50 mM potassium phosphate (pH 6.0). The cells were suspended at a concentration having an OD_{600} value of 20.0 in the same potassium phosphate supplemented with 0.3 mM Trp, IpyA, indole-3-acetaldehyde (IAAld), or IAM (Sigma Chemical Co.) as a substrate (Fig. 1). The suspension was incubated in a test tube at 30°C for 6 h at 120 strokes/min in the dark. IAA and the other indole derivatives in the reaction mixture were analyzed by C18 reverse-phase high performance liquid chromatography (HPLC) (4.6 × 250 mm inertsil ODS-3, GL Sciences Inc., Japan) by using 33% aqueous methanol containing 5% acetic acid as the mobile phase (detection at 280 nm). The IAA was quantified by reference to the peak area obtained for the respective authentic standards (Sigma Chemical Co.) of a known concentration. The HPLC fraction with a retention time of 19–20 min was dried with a rotary evaporator and was confirmed by GC–MS as described above. Analysis of the indole compound was also performed on TLC with silica-gel (Merck KGaA) in a chloroform–methanol–water (84:14:1) solvent [9]. The chromatograms were developed by spraying with Salkowski's reagent, followed by heating at 200°C. The TSO activities were measured as already described [18, 26].

Determination of IAA sensitivity of bentgrass. Creeping bentgrass seeds were sterilized with 70% ethanol with shaking for 30 min, washed with sterilized distilled water three times, and air-dried at room temperature. The surface-sterilized seeds were planted on the surface of plant nutrient agar (PNS containing 0.4% agar) supplemented with various concentrations of IAA. After 10 days, the root length was measured.

Assay of root colonization and root growth in autoclaved sand soil pot. *P. fluorescens* HP72 was grown for 24 h in King's B broth. Cells were harvested by centrifugation at 3000 *g* for 10 min and washed twice with phosphate-buffer saline (PBS). The cells were suspended in

PBS containing 0.5% methylcellulose (OD_{600} = 2.0). The sterilized seeds were shaken in the cell suspension for 2 h. Thirty mg (dry weight) of seeds were transferred to a 50-mL sand soil pot. Ten days after inoculation of the seeds, the root length and root colonizing cells were measured. The colonized cells of strains HP72 and HP72LI were isolated from the roots that were washed with PBS and were homogenized. The colonies were counted on the King's B medium containing Nal.

Disease suppression. *P. fluorescens* HP72 and HP72LI were bacterized on bentgrass seeds with the same procedure as described above, and 100 mg (dry weight) of seeds was inoculated onto 200 mL sand soil pots. Twenty days after inoculation, 0.5 g of cultivated *Rhizoctonia solani* AG2-2 IIIB [16] was added to the pot. *R. solani* AG2-2 IIIB was cultivated on a sterile solid material, which consisted of equal weights of bran and vermiculite and contained 8% peptone yeast extract medium (5% peptone, 5% yeast extract). The cultures, aftes 5 days at 28°C, were used as the inoculum for the pot disease suppression test. To grow *Rhizoctonia solani* on turfgrass, the plants in a pot were covered for 2 days with a plastic bag that had many small holes. One week after inoculation of *Rhizoctonia solani*, the disease severity was estimated by the surviving area percentage of the plants.

Results and Discussion

Isolation and characterization of IAA low-producing mutant. *P. fluorescens* HP72 was cultivated for 96 h in M9 medium supplemented with Trp. IAA was visually detected by the color development after the addition of Salkovsky's reagent to the bacterial cultures. After several repeated subcultures of strain HP72, a bacterial culture HP72LI, which showed much less IAA color development, was isolated. Figure 2 shows the HPLC profiles of the products from Trp in the resting cell. The amount of IAA by the mutant from Trp was 6.8% of that by the wild-type strain. The growth rate of this spontaneous low IAA-producing mutant was slightly increased in King's B broth, but was significantly different in the production of the other secondary metabolites, as 2,4-DAPG, HCN, fluorescent siderophore were not detected (data not shown).

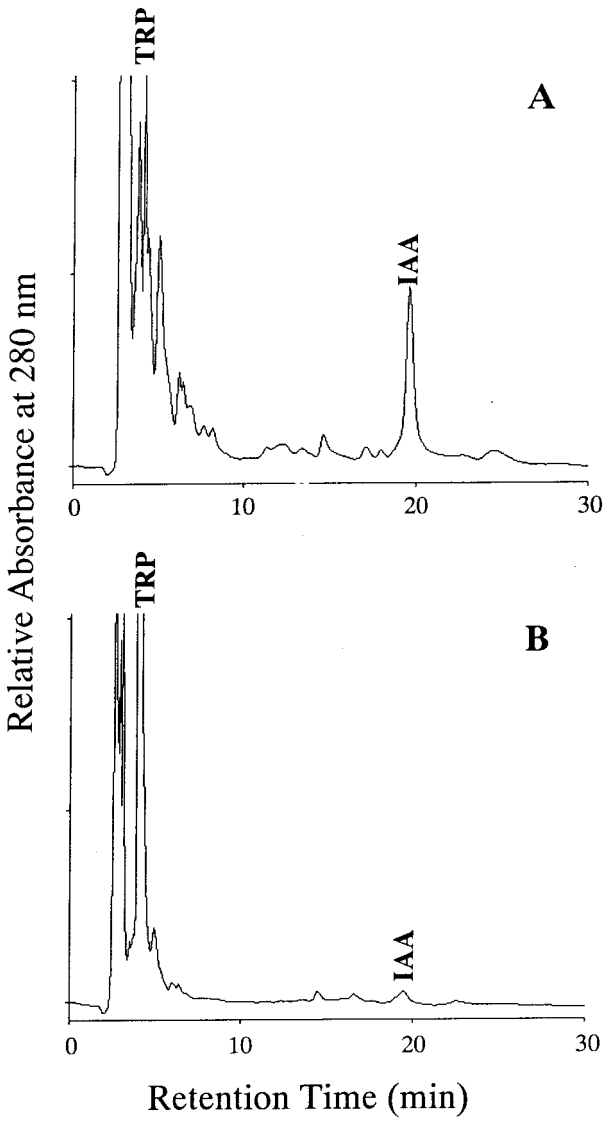

Fig. 2. HPLC profiles of compounds produced by *P. fluorescens* HP72 wild-type (A) and IAA low-producing mutant HP72LI (B) in the resting cell reaction supplemented with 0.3 mM Trp.

Pathway of IAA synthesis. To determine the IAA biosynthetic pathway of strain HP72, an extract of the culture supernatant was analyzed by GC–MS. The GC–MS analysis of the strain HP72 culture supernatant extracts confirmed the presence of IAA; however, other indole compounds were not detected (data not shown). Therefore, we analyzed the product derivatives from the series of Trp and other IAA biosynthetic intermediates IpyA, IAAld, and IAM in the resting cell reactions (Table 1 and Fig. 1). The IAA produced by each resting cell reaction was determined and quantified by HPLC. The HPLC fraction was confirmed by comparing the R_f value with that of authentic IAA by TLC and/or the spectrum by GC–MS. IAA was not detected in the incubation of authentic Trp, IpyA, IAAld, and IAM in the assay solution without cells. The wild-type strain HP72 produced IAA from Trp, IpyA, or IAAld, but not from IAM. By comparison of the wild-type strain HP72 with the IAA low-producing mutant HP72LI, the amount of IAA produced by HP72LI from Trp was approximately 1/15 of that by the wild-type strain. When IAAld was added to the cells, both strains produced IAA and did not show a substantial difference in the amount of IAA produced. IAA from IpyA was detected by using the wild-type strain HP72, but was not detected with the strain HP72LI. However, IAA from IpyA was less than that from Trp with the wild-type strain HP72, and IpyA was mainly and immediately converted to Trp in the resting

Table 1. Quantification of IAA produced by *P. fluorescens* HP72 wild-type and low IAA-producing mutant from various indole compounds in resting cell reactions

Substrate	Strain	IAA(μg/mL)[a]
Trp	HP72	11.8
	HP72LI	0.8
IpyA	HP72	1.6
	HP72LI	ND[b]
IAAld	HP72	36.9
	HP72LI	29.7
IAM	HP72	ND
	HP72LI	ND

[a] IAA was quantified by HPLC.
[b] ND, not detected.

Table 2. TSO activity of cell extract of *P. fluorescens* wild-type HP72 and low IAA-producing mutant HP72LI

Strain	TSO activity (U/10^9 CFU)
HP72	1093 ± 107
HP72LI	94 ± 21

Mean from three replicate cultures. One unit is defined as the amount of enzyme that catalyzed the formation of 1 μmol of *N*-acetyl-α, β-didehydrotryptophanamide/min [18].

cell reaction in both strains. The IAA from IpyA could possibly be produced from Trp which is converted from IpyA. IpyA might be the precursor of IAA, but not the intermediate compound in the major pathway from Trp to IAA in strain HP72. The result of the resting cell reactions indicated that the IAA production deficiency from Trp in strain HP72LI was not due to the low activity of the conversion of IpyA or IAAld. After the restriction cell reactions, a TSO activity assay was carried out. The TSO activity assay revealed that strain HP72LI has approximately 1/12 the activity of the wild-type (Table 2). These results indicated that strain HP72LI is impaired in TSO activity, and this is the reason that strain HP72LI produces a much lower amount of IAA in its bacterial culture and in the resting cell reaction than that of the wild-type strain. As a result of the resting cell reaction and TSO activity assay, TSO is revealed to be the key enzyme for the conversion of Trp to IAA in strain HP72. The TSO activity has been reported to be detected in other 2,4-DAPG-producing *P. fluorescense* strains, i.e., *P. fluorescense* CHA0 and *P. fluorescense* Pf-5 [20, 26]. In 2,4-DAPG-producing fluorescent *Pseudmonas*, the TSO pathway may be a common IAA biosynthetic pathway.

The effect of IAA low production of strain HP72 on root colonization, on its root growth, and on suppression ability of brown patch. After 10 days of cultivation of the bentgrass seeds treated with the wild-type strain HP72 or mutant strain HP72LI, the populations of each strain on the root were counted (Table 3). Strain HP72LI was 4.5-fold impaired in the colonization of the bentgrass root compared with the wild-type strain. Bentgrass bacterized with the wild-type HP72 was inhibited in its root growth, but that treated with HP72LI was not (Table 3). In order to determine the effect of the exogenous IAA on the bentgrass, plants were grown on plant nutrient agar supplemented with various concentrations of IAA (Fig. 3). At least 0.01 mM of the exogenous IAA reduced the significant growth of the bentgrass roots the same as was reported for some other plants [3]. These observations suggested that the root growth inhibition by strain HP72 is attributed to the IAA produced by this strain.

Brandl and Lindow [3] have reported that the IAA-deficient mutant of the non-pathogenic, epiphytic *Erwinia herbicola* 299R, which produces IAA via the in-

Table 3. Effect of wild-type and IAA low-producing *P. fluorescens* HP72 on colonization ability and root length

Bacterial strain added	Colonization ($\log_{10}$ CFU/g)[a]	Root length (mm)[b]
None		37.9 ± 2.7 x
HP72	7.41 ± 0.67 x	25.7 ± 2.2 y
HP72LI	6.75 ± 0.43 y	42.2 ± 2.8 x

[a] Numbers of HP72 and its derivative on roots ($\log_{10}$ CFU per 1.0 g of fresh roots) were measured from roots grown in five independent pots, and the middle three of five values were used for statistical analysis).
[b] Root lengths were measured in five independent pots (n = 20).
[a, b] Data represent the mean ± SE. Values in the same column with different letters are significantly different according to Fisher's protected least significant difference (PLSD) test at $P = 0.05$.

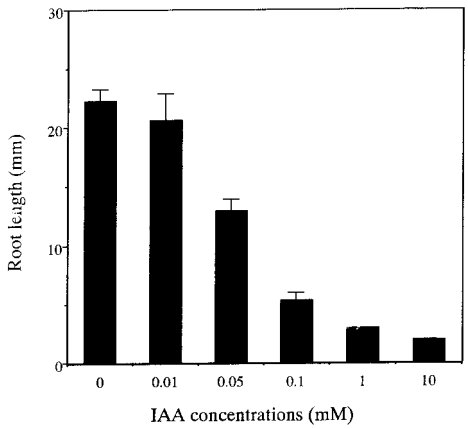

Fig. 3. Effect of IAA on the bentgrass root growth. Each value represents the mean and standard error for three roots.

Table 4. Suppression of brown patch of creeping bentgrass by *P. fluorescens* HP72 and IAA low-producing mutant HP72LI in autoclaved sand soil

Bacterial strain added	Surviving plants (%)[a]	
	R. solani not added	*R. solani* added
None	100 z	51 x
HP72	100 z	93 yz
HP72LI	100 z	89 y

[a] Data are means from three independent pots. Values with different letters are significantly different at $P = 0.05$ (Fisher's PLSD test).

dole-3-pyruvic acid pathway, does not significantly inhibit radish root elongation compared with the parent strain, and the population size of the IAA-deficient mutant on the roots is lower than that of the parental strain. Though the plant-bacteria interaction of strain 299R was similar to that of strain HP72, Patten and Glick [22] noted that canola seedling primary roots from seeds treated with the wild-type *Pseudomonas putida* GR12-2 are longer than the roots from seeds treated with the IAA-deficient mutant and the *P. putida* GR12-2 IAA mutant that can colonize the canola root to the same extent as the wild-type strain. The IAA overproducing strain of *P. fluorescens* BSP53a stimulated root development of black currant, but in sour cherry cuttings, root development was suppressed [8]. These reports suggested that bacterial IAA affected the host plant, but its effect on plants may depend on the plant sensitivity to IAA, the amount of IAA produced from plant-associated bacteria, and other indirect influences such as induction of other phytohormones [23]. In our system, IAA produced by strain HP72 affected the bentgrass root conformation and may directly cause modification of the microhabitat of strain HP72 on the root by stimulating plant cell division and proliferation. Also, this modification may take advantage of their colonization ability by supplying nutrients from the plant roots.

The suppression of brown patch disease on bentgrass by strains HP72 and HP72LI was evaluated in autoclaved sand soil (Table 4). In spite of the impairment in colonization ability, HP72LI gave almost the same suppression level as strain HP72 in terms of disease severity. This indicated that this extent of decrease in population size of strain HP72LI did not influence its biocontrol ability.

In this research, we showed that the biocontrol *P. fluorescens* HP72 mainly produced IAA via the tryptophan side chain oxidase pathway. Bacterial synthetic IAA contributed to the root colonization of strain HP72, but did not have a significant effect on the biocontrol ability against brown patch disease caused by *Rhizoctonia solani*. IAA produced by strain HP72 does not play any role in the antagonistic activity; however, it contributes to plant association ability.

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