Plant Cell Physiol. 32(1): 1–9 (1991)
JSPP © 1991

Production of Indole-3-Acetic Acid by *Bradyrhizobium japonicum*: A Correlation with Genotype Grouping and Rhizobitoxine Production

Kiwamu Minamisawa and Katsuhiko Fukai

Faculty of Agriculture, Ibaraki University, Ami, Ibaraki, 300-03 Japan

Bioassays show that rhizobitoxine-producing strains of *Bradyrhizobium japonicum* excreted another phytotoxic compound into their culture fluid. This compound was purified and identified by HPLC and mass spectrometry as indole-3-acetic acid (IAA). The levels of IAA produced by the different strains of *B. japonicum*, for which the genotype groups have been determined with respect to the degree of base substitution in and around *nifDKE*, were quantified using gas chromatography/mass spectrometry and a deuterated internal standard. Genotype II strains, which produce rhizobitoxine, excreted more than 20 μM of IAA into their culture fluid. However, no IAA was detected in the culture supernatants of genotype I strains, which do not produce rhizobitoxine. This was true even when tryptophan was added to the medium. Moreover, cells of genotypes I and II strains, which were grown under our culture conditions, did not show IAA degradation activity. These results suggest that, in wild-type *B. japonicum* strains, complete IAA biosynthesis is confined exclusively to genotype II strains that produce rhizobitoxine.

Key words: *Bradyrhizobium japonicum* — Indole-3-acetic acid — Rhizobitoxine.

In a recent survey of *B. japonicum* strains, a good correlation was found between sequence divergence in and around *nifDKE* genes, RT production, hydrogenase phenotype, and EPS composition (Minamisawa 1989, 1990). The sequence divergence in and around *nifDKE* clearly divided the *B. japonicum* strains into two different groups, designated GTI and GTII. GTI strains produced a type A EPS composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid, and no RT. Whereas, GTII strains produced RT and a type B EPS composed of rhamnose and 4-*O*-methyl glucuronic acid. Hup positive strains, possessing the *hup* structural genes, were confined exclusively to GTI. This demonstrated that GTI and GTII strains are two separate evolutionary lines that define a marked division of various phenotypes such as RT production, EPS composition and Hup.

While examining bioassay systems for RT, we found another phytotoxic compound in the cultures of RT^+ strains of *B. japonicum*. This compound inhibited the root growth of alfalfa, red clover, soybean and sorgum. With RT^- strains no inhibition of root growth was observed. We purified this phytotoxic compound, and determined by mass spectrometry and HPLC that it was the phytohormone IAA. Furthermore, unequivocal determination of IAA in culture supernatant by using GC/MS confirmed that only the GTII strains of *B. japonicum* accumulated IAA in culture.

Abbreviations: DRT, dihydrorhizobitoxine; EPS, extracellular polysaccharide; GC/MS, gas chromatography/mass spectrometry; GTI, genotype I; GTII, genotype II; REI, root elongation inhibiting; RT, rhizobitoxine; RT^+, rhizobitoxine producer; RT^-, non-producer of rhizobitoxine; SIM, selected ion monitoring; Hup, hydrogen uptake.

Materials and Methods

Bacterial strains, media and growth conditions—Strains of *B. japonicum* were obtained from Y. Sawada of the National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan (15 strains with the prefix NIAES); from T. Takahashi of the Tokachi Federation of Agricultural Cooperatives, Hokkaido, Japan (strain J1B70); from H. H. Keyser of the U. S. Department of Agriculture (USDA), Beltsville, MD, U.S.A. (5 strains with the prefix USDA); from S. Tsuru of the National Institute of Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan (strain J501).

Stock cultures were maintained on yeast extract-mannitol agar medium (Minamisawa 1989). For bioassay and IAA determinations, cells were routinely grown in the dark at 30°C in Tris-YMRT broth medium (Owens and Wright 1964b). This medium contained the following components dissolved in 1 liter of water (pH 6.8): mannitol,

10.0 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; Tris, 1.21 g; yeast extract, 0.2 g; casamino acid (acid hydrolyzed casein, Difco, Technical grade), 1.0 g. For purification of the phytotoxic compound, strain USDA 94 was grown at 30°C for 10 days in P-YMRT broth medium (pH 6.8) where the Tris contained in the Tris-YMRT medium was replaced with 2 mM potassium phosphate buffer (Owens and Wright 1964b).

Ion exchange column chromatography—The supernatant, separated from 6.6 liters of USDA 94 culture by centrifugation, was evaporated to dryness in vacuo, and resuspended in methanol. The methanol soluble extract was chromatographed on a Dowex 50 column (pyridine form, 200–400 mesh, 1.8 × 110 cm). The column was eluted with a linear gradient from 800 ml of 0.6 M pyridine buffer (pH 4.9) to 800 ml of 2.0 M pyridine buffer (pH 5.1). The pH of the buffer was adjusted with acetic acid. Flow rate and fraction size were 3 ml/min and 15 ml, respectively.

Bioassay—For the bioassay of culture supernatants, strains were grown for 7–10 days and culture supernatants were obtained by centrifugation (8,000 × *g*, 15 min). Agar powder was added to the supernatants to a final concentration of 1.5% (w/v). For the bioassay of the fractions separated by the ion exchange chromatography and HPLC, fraction aliquots were evaporated to dryness in vacuo and dissolved in 5 ml of 10 mM Tris buffer (pH 7.5) containing 1.5% (w/v) agar powder. After autoclaving the solutions at 120°C for 15 min, surface-sterilized alfalfa seeds (*Medicago sativa*) were placed on the sterilized, solidified solutions, and grown at 30°C for 4 days in the light. Root lengths of the alfalfa seedlings were measured 4 days after sowing the seeds.

UV spectrophotometry—Fractions from the ion exchange column were evaporated to dryness in vacuo, dissolved in methanol and analyzed by UV absorption sectrometry with a JASCO Ubest-50.

Mass spectrometry—The electron impact mass spectra were taken with a Shimadzu GCMS 9020-DF instrument (ionization voltage, 70 eV) interfaced with a Shimadzu GCMSPAC-1100S data system.

HPLC analysis—The fraction containing the phytotoxic compound was injected into a Nucleosil C18 column (4.6 × 250 mm, 7 μm), and eluted with a gradient from 1% (v/v) acetic acid to methanol (0–17 min, 1% acetic acid; 17–47 min, linear gradient from 1% acetic acid to methanol; 47–60 min, methanol) at a flow rate of 0.5 ml/min.

IAA determination—IAA in the culture supernatant was quantified by an isotope dilution method using deuterium-labeled IAA as an internal standard. [Methylene-$^2H_2$]IAA was accurately added to culture supernatants, and the pH adjusted to 2.1–2.8 with sulfuric acid. This mixture was partitioned against ether, and the ether phase evaporated to dryness in vacuo and dried over P_2O_5. The samples were silylated in a mixture of BSA (N,O-bis-[trimethylsilyl]acetamide) and pyridine (2 : 1, v/v) at 60°C for 30 min, and analyzed on a Shimadzu GCMS 9020-DF equipped with a solvent cut injection system for GC. Electron impact ionization (70 eV) was used. GC conditions were as follows: column, Ultra-1 fused silica capillary column (25 m × 0.2 mm × 0.33 μm film thickness, Hewlett-Packard); carrier gas, He; injection temperature, 300°C; column temperature, 230°C. The m/z 319 and 321 signals, which are the molecular ion pair of the ditrimethylsilyl derivatives of [^1H]IAA and [$^2H_2$]IAA, were monitored by the SIM system of the GC/MS. The ratio of endogenous IAA ([^1H]IAA) in the culture supernatant to the internal standard ([$^2H_2$]IAA) was calculated from the areas of the IAA peaks in the SIM chromatograms at m/z 319 and 321.

Preparation of methylene-$^2H_2$ IAA—Deuterium-labeled IAA was prepared by base catalyzed exchange in [$^2H_2$]O according to the method of Magnus et al. (1980). No significant exchange of incorporated deuterium label was found following derivatization.

IAA disappearance assay—Four representative strains were grown in 100-ml Tris-YMRT medium in the dark for 5 days. The cells were washed twice with magnesium-phosphate buffer (0.1 M sodium phosphate, 2.5 mM $MgCl_2$, pH 7.0) and suspended with 10 ml of the buffer. The reaction was started at 30°C by adding IAA (final concentration, 100 μM). Samples were taken at 0, 0.5, 1 and 2 h. The reaction was stoppted by cooling the samples to 4°C and removing the cells by centrifugation at 15,000 × *g* for 5 min. IAA concentration was determined by the method of Gordon and Weber (1951). An aliquot of the cell suspensions was sonicated for 10 min followed by centrifugation at 15,000 × *g* for 15 min. Protein concentration of the supernatants was determined by the method of Bradford (1976).

Results and Discussion

Figure 1 shows the growth responses of alfalfa grown on sterilized, solidified culture supernatants of *B. japonicum* USDA 94 and USDA 110. Alfalfa did not grow normally on the culture supernatant of USDA 94 (Fig. 1A, tube 1). The inhibition of root elongation obviously occurred even when the culture supernatant of USDA 94 was diluted to 100-fold with 10 mM Tris buffer (Fig. 1A, tube 3), where the elongation of the roots was more strongly inhibited than that of the shoots. On the other hand, alfalfa grew normally on the culture supernatant of USDA 110 and its dilutions (Fig. 1B). When we tested various strains of *B. japonicum* in our bioassay system, root elongation was always inhibited by the cultures of RT^+ strains (Table 1). However, RT does not inhibit root elongation of alfalfa seedlings, even at a final concentration of 100 μM (data not shown). Thus, RT^+

strains of *B. japonicum* must produce another phytotoxic compound that inhibits root elongation.

Figure 2 shows the ion exchange column chromatogram of the methanol soluble extract from USDA 94 culture as monitored with the alfalfa root elongation response. The phytotoxic compound eluted around fractions No 60–64, whereas RT and DRT eluted around fraction No 40. The UV spectra of fractions No 60, 61 and 62 showed a common absorption band at 280 nm (Fig. 3B, 3C and 3D). Authentic RT has no band at 280 nm (Fig. 3A).

Fractions 60, 61 and 62 were combined and termed the REI fraction. The mass spectrum of the REI fraction (Fig. 4A) indicated that the phytotoxic compound has a benzene moiety (m/z 77) in its chemical structure and that its molecular weight may be 175. Comparisons with numerous data of mass spectra show that the spectrum of IAA is very similar to that of the phytotoxic compound (Rivier 1986). Thus, authentic IAA was also analyzed (Fig. 4B) and its spectrum was found to be exactly the same as that of the REI fraction. IAA at concentrations of 1 mM–1 μM in the root cause inhibition of growth. However, lower concentrations of IAA, 0.1 μM–10 pM induce root elongation and cell division in meristemic tissues (Cadwick and Burg 1967, Elliott 1982, Harari et al. 1988). Thus, we tested the effect of IAA on the root elongation of alfalfa in our bioassay system (Fig. 5). Two micromolar of authentic IAA almost completely inhibited root elongation (Fig. 5, tube 3), although lower concentrations of IAA did not appear to enhance root elongation. This suggests that the phytotoxic compound produced by strain USDA 94 was IAA.

To determine whether IAA is the only compound in

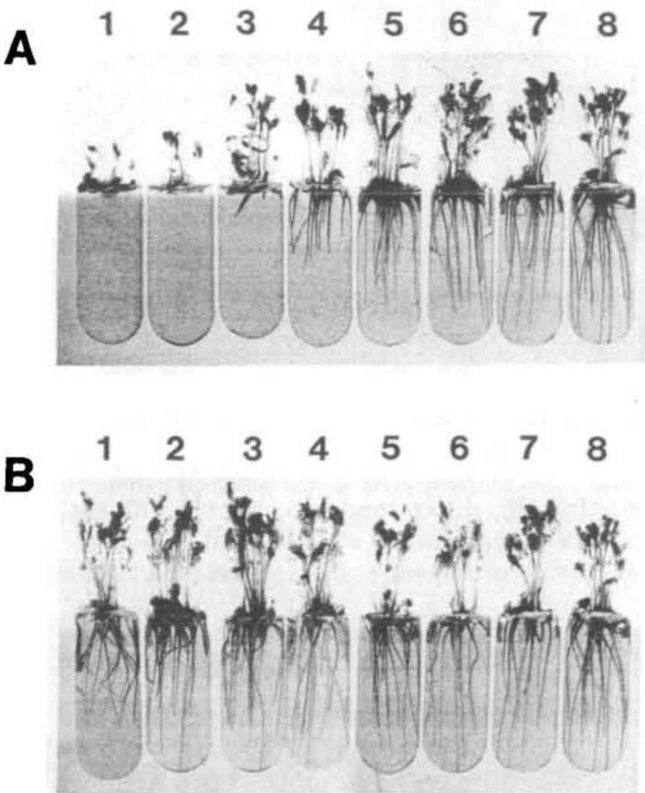

Fig. 1 Responses of alfalfa grown for 4 days at 30°C on solidified culture supernatants of *B. japonicum* USDA 94 and USDA 110. *B. japonicum* USDA 94 (RT^+ strain) and USDA 110 (RT^- strain) were grown at 30°C in the dark in Tris-YMRT broth medium. After 8 days growth of each strain, culture supernatant was obtained by centrifugation, and the supernatant was serially diluted to 10^5-fold with 10 mM Tris buffer (pH 7.5). The bioassay was conducted as described in Materials and Methods. (A) USDA 94 culture supernatant and its dilutions. (B) USDA 110 culture supernatant and its dilutions. The tubes contained the following solutions: 1, culture supernatant; 2, 10-fold dilution; 3, 10^2-fold dilution; 4, 10^3-fold dilution; 5, 10^4-fold dilution; 6, 10^5-fold dilution; 7, 10 mM Tris buffer used for the dilution; 7, Tris-YMRT medium.

Table 1 Rhizobitoxine production and growth response of alfalfa in cultures of various strains of *B. japonicum*

Strain	Rhizobitoxine production [a]	Root elongation [b]
USDA 94	+	−
USDA 31	+	−
NIAES 3126	+	−
NIAES 3136	+	−
NIAES 3158	+	−
NIAES 3168	+	−
NIAES 3172	+	−
NIAES 3193	+	−
NIAES 3196	+	−
NIAES 3202	+	−
USDA 76	+	−
NIAES 3203	+	−
USDA 110	−	+
J501	−	+
J1B70	−	+
NIAES 3127	−	+
NIAES 3154	−	+
NIAES 3160	−	+
NIAES 3178	−	+
USDA 122	−	+
NIAES 3135	−	+
NIAES 3144	−	+

[a] Phenotype with respect to rhizobitoxine production, as reported previously (Minamisawa 1989).

[b] The growth response was expressed as root elongation of alfalfa according to the following criteria: +, root elongation of alfalfa seedlings was normal; −, root elongation was inhibited entirely.

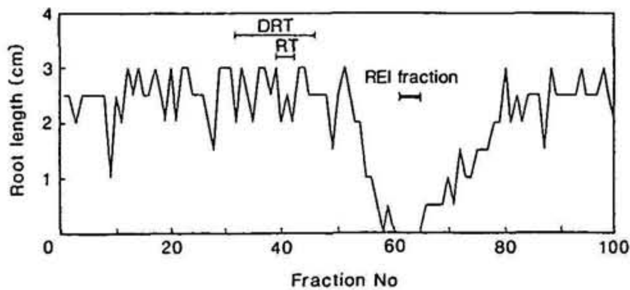

Fig. 2 Ion exchange column chromatography of methanol soluble extract from USDA 94 culture as monitored with alfalfa root elongation response. The root elongation response was expressed as average root length of alfalfa grown for 4 days. The fractions (No 60, No 61 and No 62) showing the complete inhibition of the root elongation were combined, and termed REI (root elongation-inhibiting) fraction. The fractions containing RT and DRT were determined by paper chromatography with ninhydrine (Owens et al. 1964a).

the REI fraction that causes inhibition of root elongation, the REI fraction was subjected to further HPLC analysis (Fig. 6A and 6B). In the chromatogram of the REI fraction (Fig. 6B), a large peak was detected with the same retention time as that of authentic IAA (Fig. 6C). Only one fraction correspond to the large peak inhibited alfalfa

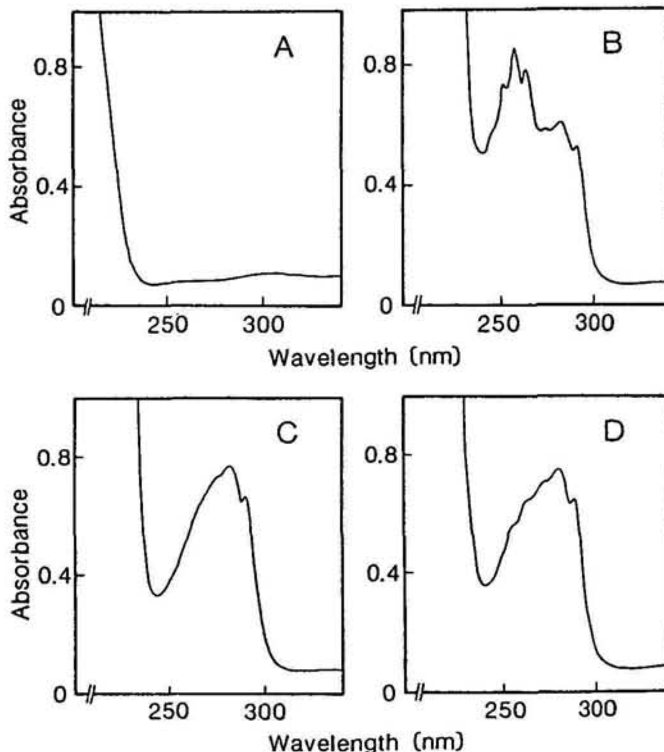

Fig. 3 UV spectra of rhizobitoxine and phytotoxic fractions separated by ion exchange column chromatography shown in Fig. 2. (A) Rhizobitoxine. (B) Fraction No 60. (C) Fraction No 61. (D) Fraction No 62.

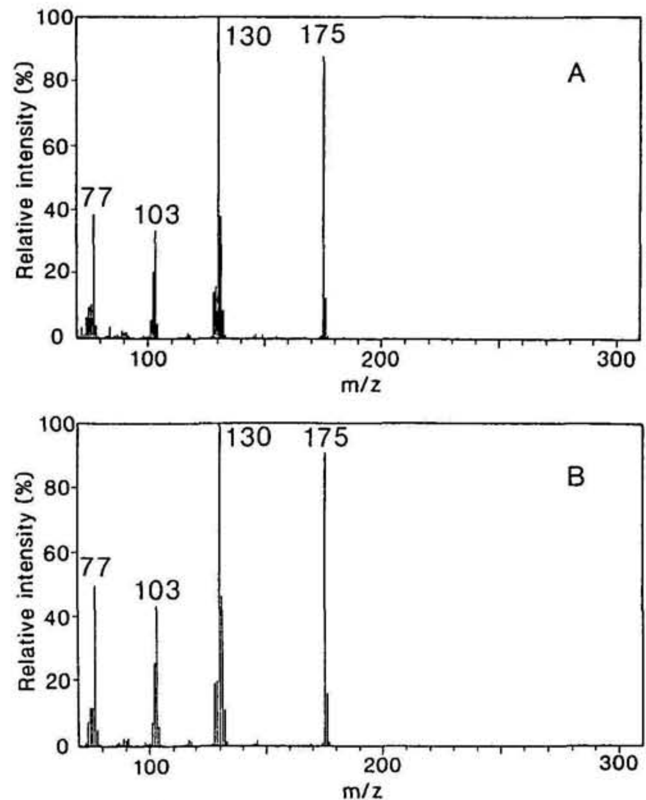

Fig. 4 Mass spectra of REI fraction from USDA 94 culture (A) and authentic IAA (B).

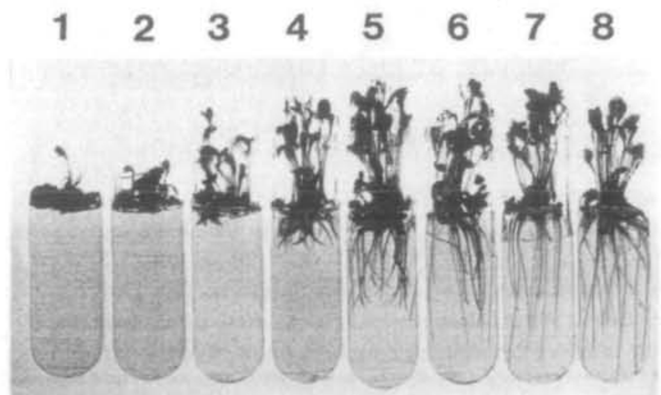

Fig. 5 Effect of IAA addition on alfalfa growth response. Authentic IAA was dissolved in 10 mM Tris buffer (pH 7.5). The bioassay was conducted as described in Materials and Methods. The tubes contained the following solutions: 1, 200 μM IAA; 2, 20 μM IAA; 3, 2 μM IAA; 4, 0.2 μM IAA; 5, 20 nM IAA; 6, 2 nM IAA; 7, 10 mM Tris buffer; 8, Tris-YMRT medium.

root elongation (Fig. 6A). Moreover, the UV spectrum of authentic IAA agreed with that of fraction No 61 (Fig. 3C).

The above results show that the phytotoxic compound produced by the RT^+ strain USDA 94 is IAA. The bioassay (Table 1) suggested that free-living RT^+ strains produced more IAA than did RT^- strains. Thus, we decided to quantitate IAA in culture supernatants by GC/MS, employing deuterium-labeled IAA as an internal standard. Table 2 shows IAA concentration in culture supernatants of various strains of *B. japonicum*. IAA concentrations with RT^+ strains were more than 20 μM, high enough to inhibit the root elongation of alfalfa seedlings in our bioassay system. On the other hand, no IAA was detected in culture supernatants of RT^- strains. Thus, we attribute the inhibitory effect of the culture supernatants on the root growth of alfalfa (Fig. 1, Table 1) to high concentrations of IAA accumulated by RT^+ strains.

Nielsen et al. (1988) observed in vivo IAA degradation activity of *B. japonicum*. Thus, with respect to the difference in IAA accumulation between GTI and GTII strains, two explanations are possible. (i) GTI strains not only produce IAA but also possess higher activity of IAA degradation, which results in no IAA accumulation in their culture fluid, while GTII strains produce IAA and possess no activity of IAA degradation. (ii) GTII strains exclusively produce IAA, whereas GTI strains did not produce IAA. To solve these questions, we examined constitutive and inducible activities of in vivo IAA degradation of GTI and GTII strains (Table 3). We could not detected the constitutive activity of IAA degradation in either GTI and GTII strains, indicating that both GTI and GTII strains did not degradate IAA under our culture conditions. When IAA was added to cultures 14 h before IAA degradation assay, only GTI strains showed inducible activity of IAA degradation, which seemed to be observed by Nielsen et al. (1988). This reversely supported that GTI strains did not produce IAA under our culture conditions, because, if GTI strains produce IAA, the degradation activity would be induced in GTI strains without IAA addition. The results indicate that the second explanation is tenable.

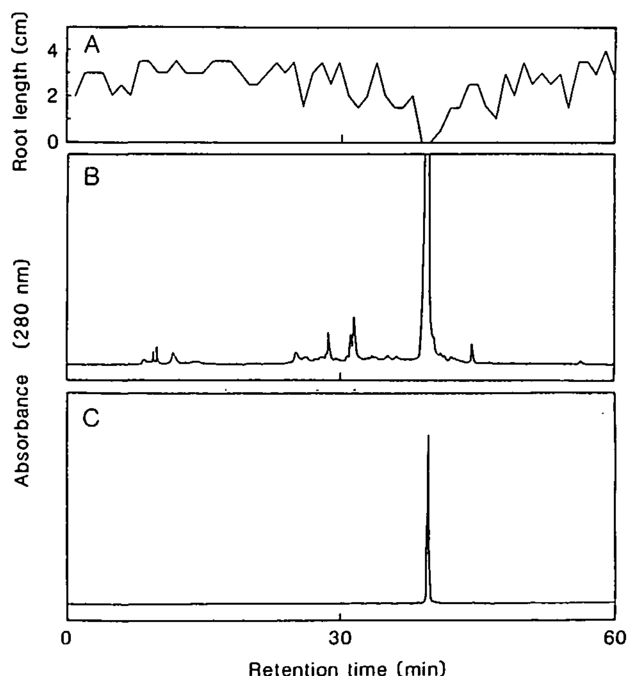

Fig. 6 HPLC chromatograms of the REI fraction from USDA 94 culture and authentic IAA as monitored with the bioassay and UV absorbance. REI fraction separated by the ion exchange column chromatography (Fig. 2) was subjected to HPLC analysis as monitored with root elongation response of alfalfa (A) and UV absorbance (B). Authentic IAA was also analyzed as monitored with UV absorbance (C). The root elongation response was expressed as average root length of alfalfa grown for 4 days.

Therefore, these results show a strict correlation between IAA production and genotype (GTI and GTII) of *B. japonicum* (Table 2). GTII strains, which produce RT and an type B EPS, excreted a large amount of IAA. On the other hand, GTI strains, which produce no RT and an type A EPS, excreted no detectable IAA. Free-living GTI strains of *B. japonicum* may lack the succesive pathway for IAA synthesis that GTII strains possess. Thus, in addition to RT production and EPS type, IAA production by free-living cells is a phenotypical characteristic that distinguishes these two evolutionary lines, GTI and GTII of *B. japonicum*.

Rhizobium can produce IAA as well as a number of IAA precursors asymbiotically (Badenoch-Jones et al. 1982a, b, Ernstsen et al. 1987, Wang et al. 1982). Similarly, it has been reported that *Bradyrhizobium* grown in tryptophan-supplemented media can produce IAA (Hunter 1987, Kaneshiro et al. 1983, Sekine et al. 1988), and that there are two conversion pathways of tryptophan to IAA via indole-3-acetamide (Sekine et al. 1988, 1989) and via indole-3-pyruvic acid (Kaneshiro et al. 1983). Since tryptophan is generally a key intermediate in IAA biosynthesis in microorganisms (Morris 1986), we tested the effect of tryptophan addition on IAA production by representative GTI and GTII strains of *B. japonicum* (Table 4). Tryptophan addition greatly increased IAA concentrations in the culture supernatants of GTII strains (USDA 94 and NIAES 3193). However, no IAA was detected in the culture supernatants of GTI strains (USDA 110 and USDA 122) even in the presence of tryptophan. In our preliminary in vivo and in vitro experiments, both GTI and GTII strains possessed the ability to convert indole-3-acetamide to IAA (data not shown). Thus, IAA biosynthetic pathway in free-living *B. japonicum* is likely to be complicated, because GTI strains, which did not produce IAA in our culture conditions, also showed the activity of

Table 2 Comparison of IAA concentration in culture supernatants of various strains of *B. japonicum* with other characteristics [a]

Strain	IAA conc (μM)	Genotype [b]	RT production [c]	EPS type [d]
USDA 94	49	II	+	B
USDA 31	25	II	+	B
NIAES 3126	29	II	+	B
NIAES 3136	40	II	+	B
NIAES 3158	38	II	+	B
NIAES 3168	57	II	+	B
NIAES 3172	21	II	+	B
NIAES 3193	36	II	+	B
NIAES 3196	39	II	+	B
NIAES 3202	55	II	+	B
USDA 76	57	II	+	B
NIAES 3203	70	II	+	B
USDA 110	<0.01	I	−	A
J501	<0.01	I	−	A
J1B70	<0.01	I	−	A
NIAES 3127	<0.01	I	−	A
NIAES 3154	<0.01	I	−	A
NIAES 3160	<0.01	I	−	A
NIAES 3178	<0.01	I	−	A
USDA 122	<0.01	I	−	A
NIAES 3135	<0.01	I	−	A
NIAES 3144	<0.01	I	−	A

[a] Each strain was grown in the dark in Tris-YMRT broth medium for 8 days. Optical density (660 nm) of cultures ranged from 1.10 to 1.28 after 8 days growth. IAA concentration in culture supernatants was determined by GC/MS.
[b] Genotype with respect to the degree of sequence divergence in and around *nifDKE* genes (Minamisawa 1990).
[c] Phenotype with respect to rhizobitoxine production (Minamisawa 1989).
[d] Phenotype with respect to extracellular polysaccharide (EPS) composition (Minamisawa 1989). Type A EPS is composed of glucose, mannose, galactose, 4-*O*-methyl galactose and galacturonic acid. Type B EPS is composed of rhamnose and 4-*O*-methyl glucuronic acid.

Table 3 IAA degradation activity of *B. japonicum*

Strain	Genotype [a]	IAA degradation activity (nmole·h^{-1}·(mg protein)$^{-1}$) [b]	
		−IAA	+IAA
USDA 94	II	ND	ND
NIAES 3139	II	ND	2ND
USDA 110	I	ND	99
USDA 122	I	ND	147

[a] Genotype with respect to the degree of sequence divergence in and around *nifDKE* genes (Minamisawa 1990).
[b] Four representative strains were grown in Tris-YMRT medium in the dark for 5 days. To mesure the inducible activity of IAA degradation, IAA was added to the 4-days-cultures at a final concentration of 100 μM 14 h before IAA degradation assay (+IAA). To measure the constitutive activity, no IAA was added to the cultures before the assay (−IAA).
ND: No activity detected (<1 nmol·h^{-1}·(mg protein)$^{-1}$).

Table 4 Effect of tryptophan and genistein addition on IAA production by *B. japonicum*[a]

Strain	Genotype[b]	IAA conc (μM)		
		Control	+ Tryptophan	+ Genistein
USDA 94	II	66	349	83
NIAES 3193	II	44	197	89
USDA 110	I	<0.1	<0.1	<0.1
USDA 122	I	<0.1	<0.1	<0.1

[a] Tryptophan and genistein were added to Tris-YMRT medium before inoculation at final concentrations of 490 μM and 5 μM, respectively. Four representative strains were grown for 8 days in the dark in Tris-YMRT medium (Control), the tryptophan-supplemented medium (+ Tryptophan) and the genistein-supplemented medium (+ Genistein). Optical density (660 nm) of cultures ranged from 1.00 to 1.18 after 8 days growth. IAA concentration in culture supernatants was determined by GC/MS. Genistein was obtained from Funakoshi Ltd. (Tokyo).

[b] Genotype with respect to the degree of sequence divergence in and around *nifDKE* genes (Minamisawa 1990).

indole-3-acetamide hydrolase that convert indole-3-acetamide to IAA. It would be necessary to investigate which pathway mainly operates in free-living GTII strains of *B. japonicum* or whether an alternative pathway is present in the GTII strains.

Hunter (1987) isolated a high-IAA-producing mutant from *B. japonicum* I-110, a substrain of USDA 110, using 5-methyltryptophan resistance. In this work, USDA 110, which falls into GTI, produced no detectable IAA, even in the presence of tryptophan (Table 2, Table 4). This suggests that the GTI strains of *B. japonicum* may possess a set of genes for IAA synthesis that are expressed only in symbiotic association with soybeans. Recently, it has been found that a special class of flavonoids induce *Rhizobium* and *Bradyrhizobium* nodulation genes that are required for an initial step of symbiotic association with legumes (Peters and Verma 1990). In soybeans, the inducing compounds are genistein and daidzein (Kosslak et al. 1987, Banfalvi et al. 1988). Thus, we tested the effect of genistein on IAA production by GTI and GTII strains of *B. japonicum* (Table 4). No IAA was detected in the cultures of GTI strains (USDA 110 and USDA 122) grown in the genistein-supplemented medium, although IAA concentrations of GTII strains (USDA 94 and NIAES 3193) tended to increase with the addition of genistein. This result suggested that the expression of IAA biosynthetic genes in GTI strains, if present, would not be subjected to the flavonoid signals. However, GTI strains may harbor IAA synthetic genes which are derepressed in a later step of the symbiotic association with soybeans or under other specific conditions. This would explain that the mutants derivered from USDA 110, a GTI strain, produced a large amount of IAA (Hunter 1987).

The involvement of IAA in root nodule symbiosis was suggested long ago (Thimann 1936); however, to date there is no direct and conclusive evidence for a specific role. With respect to the effect of endophyte IAA production on the nodulation of soybeans, two observations has been reported: IAA-producing mutants of *B. japonicum* USDA 26 showed enhanced nodulation (Kaneshiro and Kwolek 1985); while, soybeans inoculated with high-IAA-producing mutants of *B. japonicum* USDA 110 had lower nodule mass and fixed less nitrogen (Hunter 1987). These studies suggested that the bacterial endophyte must produce an optimum amount of IAA (or some related matabolite) for the best nodulation. To evaluate symbiotical and ecological roles of IAA produced by *B. japonicum*, IAA production-deficient mutants from GTII strains would be better candidates, because wild-type GTII strains always produce IAA asymbiotically.

It is known that bacterial production of the phytohormone IAA is involved in the virulence interactions between microorganisms and plants (Morris 1986). Phytopathogenic bacteria, *Pseudomonas syringae* pv. *savastanoi* and *Agrobacterium tumefaciencs*, possess a set of IAA biosynthetic genes. In *B. japonicum* establishing a nitrogen-fixing symbiosis with soybeans, GTII strains produced both IAA and RT (Table 2), which are phytohormone-related compounds active against plant growth. RT is a potent inhibitor of the formation of the phytohormone ethylene (Lieberman 1979, Owens et al. 1971). It also induces chlorosis in the new leaves of the host plants (Owens and Wright 1964a, Owens et al. 1972). Recently, RT was found also to be produced by *Pseudomonas andropogonis*, a phytopathogen that cause bacterial stripe disease (Mitchell and Frey 1988). Therefore, the genetic relationships between GTII strains of *B. japonicum* and other phytopathogenic bacteria is of great interest.

The authors are indebted to Miss. K. Kano (Ibaraki University) for the bioassay of the phytotoxic compound and Mr. H. Iino (Ibaraki University) for the measurement of IAA degradation activity. The authors are grateful to Dr. W. J. Hunter (USDA-ARS, Northern Plains Area, CO) for critical reading of the manuscript, to Mr. Tsuchiya (Ibaraki University) for his help in photography, and to Dr. T. Asami and Dr. M. Kubota (Ibaraki University) for their continuing interest and encouragement.

This work was supported in part by a Grant-in-Aid for Science and Culture.

References

Badenoch-Jones, J., Summons, R. E., Djordjevic, M. A., Shine, J., Letham, D. S. and Rolfe, B. G. (1982a) Mass spectrometric quantification of indole-3-acetic acid in *Rhizobium* culture supernatants: Relation to root hair curling and nodule initiation. *Appl. Environ. Microbiol.* 44: 275–280.

Badenoch-Jones, J., Summons, R. E., Entsch, B., Rolfe, B. G., Parker, C. W. and Letham, D. S. (1982b) Mass spectrometric identification of indole compounds produced by *Rhizobium* strains. *Biomed. Mass Spectrom.* 9: 429–437.

Banfalvi, Z., Nieuwkoop, A., Schell, M., Besl, L. and Stacey, G. (1988) Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. *Mol. Gen. Genet.* 214: 420–424.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Chadwick, A. V. and Burg, S. P. (1967) An explanation of the inhibition of root growth caused by indole-3-acetic acid. *Plant Physiol.* 42: 415–420.

Elliott, M. C. (1982) The regulation of plant growth. *In* Plant Growth Regulator Potential and Practice. Edited by Thomas, T. H. pp. 57–98. BCPC Publications, London.

Ernstsen, A., Sandberg, G., Crozier, A. and Wheeler, C. T. (1987) Endogenous indoles and the biosynthesis and metabolism of indole-3-acetic acid in cultures of *Rhizobium phaseoli.* *Planta* 171: 422–428.

Gordon, S. A. and Weber R. P. (1951) The colorimetric estimation of IAA. *Plant Physiol.* 26: 192–195.

Harari, A., Kigel, J. and Okon, Y. (1988) Involvement of IAA in the interaction between *Azospirillum brasilense* and *Panicum miliaceum* roots. *Plant Soil* 110: 275–282.

Hunter, W. J. (1987) Influence of 5-methyltryptophan-resistant *Bradyrhizobium japonicum* on soybean root nodule indole-3-acetic acid content. *Appl. Environ. Microbiol.* 53: 1051–1055.

Kaneshiro, T., Slodki, M. E. and Plattner, R. D. (1983) Tryptophan catabolism to indolepyruvic and indoleacetic acids by *Rhizobium japonicum* L-259 mutants. *Curr. Microbiol.* 8: 301–306.

Kaneshiro, T. and Kwolek, W. F. (1985) Stimulated nodulation of soybeans by *Rhizobium japonicum* mutant (B-14075) that catabolizes the conversion of tryptophan to indole-3yl-acetic acid. *Plant Sci.* 42: 141–146.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E. and Appelbaum, E. R. (1987) Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max.* *Proc. Natl. Acad. Sci. USA* 84: 7428–7432.

Lieberman, M. (1979) Biosynthesis and action of ethylene. *Ann. Rev. Plant Physiol.* 30: 533–539.

Magnus, V., Bandurski, R. S. and Schulze, A. (1980) Synthesis of 4,5,6,7 and 2,4,5,6,7 deuterium-labeled indole-3-acetic acid for use in mass spectrometric assays. *Plant Physiol.* 66: 775–781.

Minamisawa, K. (1989) Extracellular polysaccharide composition, rhizobitoxine production, and hydrogenase phenotype in *Bradyrhizobium japonicum.* *Plant Cell Physiol.* 30: 877–884.

Minamisawa, K. (1990) Division of rhizobitoxine-producing and hydrogen-uptake positive strains of *Bradyrhizobium japonicum* by *nifDKE* sequence divergence. *Plant Cell Physiol.* 31: 81–89.

Mitchell, R. E. and Frey, E. J. (1988) Rhizobitoxine and hydroxythreonine production by *Pseudomonas andropogonis* strains, and the implications to plant disease. *Physiol. Mol. Plant Pathol.* 32: 335–341.

Morris, R. O. (1986) Genes specifying auxin and cytokinin biosynthesis in phytopathogens. *Annu. Rev. Plant Physiol.* 37: 509–538.

Nielsen, S. V. S., Egebo, L. A. and Jochimsen, B. (1988) Bradyrhizobial indoleacetic acid metabolism and its significance for root nodule development. *In* Molecular Genetics of Plant-Microbe Interactions. Edited by Palacios, R. and Verma, D. P. S. p. 151–152.

Owens, L. D. and Wright, D. A. (1964a) Rhizobial-induced chlorosis in soybeans: Isolation, production in nodules, and varietal specificity of the toxin. *Plant Physiol.* 40: 927–930.

Owens, L. D. and Wright, D. A. (1964b) Production of the soybean-chlorosis toxin by *Rhizobium japonicum* in pure culture. *Plant Physiol.* 40: 931–933.

Owens, L. D., Lieberman, D. M. and Kunishi, A. (1971) Inhibition of ethylene production by rhizobitoxine. *Plant Physiol.* 48: 1–4.

Owens, L. D., Thompson, J. F., Pitcher, R. G. and Williams, T. (1972) Structure of rhizobitoxine, an antimetabolic enol-ether amino acid from *Rhizobium japonicum.* *J.C.S. Chem. Commun.* 714.

Peters, N. K. and Verma, D. P. S. (1990) Phenolic compounds as regulators of gene expression in plant-microbe interactions. *Molecular Plant-Microbe Interaction* 1: 4–8.

Rivier, L. (1986) GC-MS of auxins. *In* Gas chromatography/mass spectrometry. Edited by Linskens, H. F. and Jackson, J. F. pp. 146–188. Springer-Verlag, Berlin/Heidelberg.

Sekine, M., Ichikawa, T., Kuga, N., Kobayashi, M., Sakurai, A. and Syono, K. (1988) Detection of the IAA biosynthetic pathway from tryptophan via indole-3-acetamide in *Bradyrhizobium* spp. *Plant Cell Physiol.* 29: 867–874.

Sekine, M., Watanabe, K. and Syono, K. (1989) Molecular cloning of a gene for indole-3-acetamide hydrolase from *Bradyrhizobium japonicum.* *J. Bacteriol.* 171: 1718–1724.

Thimann, K. V. (1936) On the physiology of the formation of nodules on legume roots. *Proc. Natl. Acad. Sci. USA* 22: 511–514.

Wang, T. L., Wood, E. A. and Brewin, N. J. (1982) Growth regulators, *Rhizobium* and nodulation in peas. *Planta* 155: 345–349.

(Received April 9, 1990; Accepted October 6, 1990)