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Short Communication

The Involvement of Indole-3-Acetic Acid Produced by *Bradyrhizobium elkanii* in Nodule Formation

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IAA-deficient mutants T10, T27 and TN3 were isolated from *B. elkanii* USDA 31 by spontaneous and/or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutageneses. Inoculation with these mutants significantly reduced the nodule number on soybean roots when compared to that of the parent strain. Furthermore, exogenous IAA application restored the nodule number of soybeans inoculated with TN3 to the original level.

Key words: *Bradyrhizobium elkanii* — IAA — Nodulation — Soybean.

Soybean bradyrhizobia are comprised of two highly divergent species; *japonicum* and *elkanii* (Kuykendall et al. 1992). Although these two species differ in various phenotypic and genotypic traits, IAA production could be a criterion for the classification of soybean bradyrhizobia; *Bradyrhizobium elkanii* strains exclusively synthesize and secrete IAA in the culture, while *B. japonicum* does not produce IAA (Minamisawa and Fukai 1991). In addition, *B. elkanii* possesses the following unique features for nodulation when compared with *B. japonicum*; (1) a higher competitive ability for wild-soybean and siratro (Minamisawa et al. 1993), and (2) nodulation of "non-nodulating" rj_1 soybean plants that excludes most indigenous strains (Devine and Weber 1977, Pueppke and Payne 1987). Thus, we hypothesize that the ability to produce IAA may be responsible for these nodulation characteristics of *B. elkanii*.

Kaneshiro and Kwolek (1985) showed that IAA-producing mutants of soybean bradyrhizobia USDA 26 enhanced soybean nodulation, while soybeans inoculated with high-IAA-producing mutants of *B. japonicum* had a lower nodule mass. The application of 2,4-D (2,4-dichlorophenoxyacetate), a synthetic auxin, increased soybean nodulation (Francisco et al. 1991). In this communication, we describe further investigation into the nodulation of soybeans, *Glycine max* (L.) Merr. with *B. elkanii* wild-type strain USDA 31 and its mutants deficient in IAA production.

To obtain spontaneous IAA-deficient mutants, each single colony derived from *B. elkanii* USDA 31 was inoculated in a well of microtiter plates containing 100 μl of the Tris-YMRT medium supplemented with 0.5 mM L-tryptophan (IAA-testing broth) (Minamisawa and Fukai 1991, Minamisawa et al. 1992). After 10 days of incubation at 30°C in the dark, 100 μl of 0.01 M $FeCl_3$ in 35% $HClO_4$ was added to each well. IAA in the culture was visually semiquantified by color development for 30 min. (Minamisawa et al. 1992). Spontaneous mutants which were likely to produce less IAA than the parent strain were selected on the microtiter plates, and then the putative mutants were grown in 5 ml of IAA-testing broth at 30°C with shaking for 6 days. The turbidity (A_{660}) and IAA content of each culture were then determined.

The culture supernatant of the parent and mutants was diluted ten times with a 0.1 M HCl solution, and its supernatant was directly analyzed by HPLC. Conditions for HPLC analysis were as follows: a column of Inertsil ODS-80A (150 mm × 4.6 mm i.d.) linked with three serial columns, a column temperature of 50°C, a mobile phase of 33% methanol–5% acetic acid, a flow rate of 0.7 ml min^{-1}, and a detector, a HITACHI F-1200 spectrofluorometer with an excitation wavelength of 280 nm and an emission wavelength of 350 nm.

Abbreviations: DAS, days after sowing; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; TLC, thin layer chromatography.

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Mutant T10 was further mutated with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) according to the method of Aird (1991) with some modifications. Cells were incubated on NTG-deposited plates at 30°C for 5 days. The selection and IAA determination were performed using the method described above.

B. elkanii USDA 31 and the IAA-deficient mutants were inoculated to *Glycine max* cv. Enrei. The inoculation procedure was carried out on described previously (Francisco and Akao 1993) except that the amount of inoculated bacteria was 10^8 cells/seed. Cells were washed twice with sterile saline and adjusted to the desired cell density with sterile deionized water. Sterile seeds were sown in sterile vermiculite contained in 500 ml glass jars. Plants were harvested 9 days after sowing (DAS), and the nodule number on the primary root was counted to explore the early responses of nodulation. Few or no nodules were observed on the lateral roots. For IAA application to soybean root systems, 20 ml of IAA solution (1 μM IAA, pH 6.0) or water (for control) was supplied on the vermiculite in each jar at 4 DAS (Francisco et al. 1991). Harvesting and counting were done at 9 DAS.

The Nod factor, a class of lipo-oligosaccharides that trigger the early steps of legume nodule formation, was analyzed by the modified method of Spaink et al. (1992). Cells were grown in a YM medium (Minamisawa et al. 1989) (pH 7.0) to A_{660} values of between 0.22 and 0.43 at 30°C with shaking, and subsequently diluted into an A_{660} value of 0.08 by the YM medium. Seven hundreds and forty kBq of [1-^{14}C]-labeled acetate (specific activity 2.0 GBq mmol^{-1}, CFA.13, obtained from Amersham Japan) was added to 0.5 ml of the diluted culture. Genistein was added at a final concentration of 2 μM as a flavonoid inducer. Cells were incubated for 18 h at 30°C with shaking at 120 rpm. Nod factors were extracted from the culture supernatant with water-saturated 1-butanol. The sample was taken to dryness and the residue was dissolved in 15 μl of water-saturated 1-butanol. Three microliters of each sample was applied to a reverse phase C18-coated silica (ODS; 100% octadecyl silanization) TLC plate and developed with acetonitrile-water (1 : 1 v/v). TLC plates were exposed to X-ray film (Kodak X-Omat AR) for three days.

Genomic DNAs isolated from the mutants and parent were digested with *Eco*R I or *Hin*d III, and then electrophoresed in 0.8% agarose gel (Minamisawa et al. 1990, 1992).

We isolated two spontaneous mutants (T10, T27) from approximately 1,000 colonies of *B. elkanii* USDA 31, which produced half the amount of IAA in the culture than the parent USDA 31 (Fig. 1A). To further reduce IAA production, T10 was mutated with NTG. As a result, one mutant which drastically reduced IAA production in the culture (Fig. 1A) was isolated, and designated TN3. The mutants and parent did not differ in growth kinetics (data not shown). To confirm whether the mutants were derived from *B. elkanii* USDA 31, the profiles of *Eco*R I or *Hin*d III digests of genomic DNAs were compared between the parent and mutants. The electrophoretic pattern of each mutant was identical to that of the parent (data not shown), indicating that all IAA-deficient mutants were derived from USDA 31.

(Brady)rhizobium cells inducing *nod* genes excrete a family of structurally related lipo-oligosaccharides, the Nod factors. These molecules trigger the early steps of

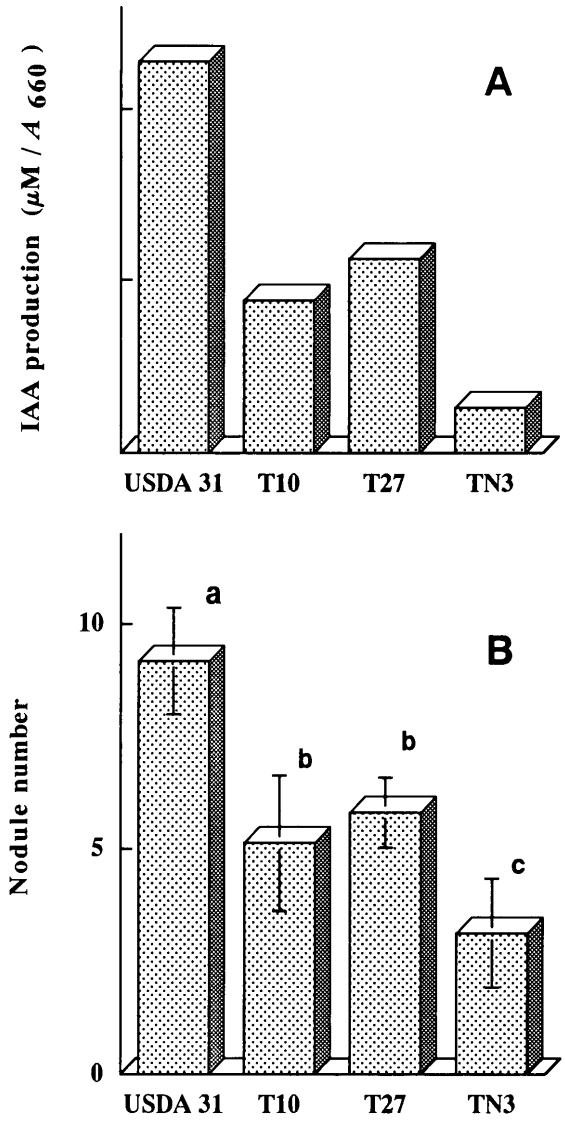

Fig. 1 IAA production of USDA 31 and IAA-deficient mutants (T10, T27 and TN3) in the culture (A), and nodule number on 9 day-old soybean roots inoculated with USDA 31 and the mutants (B). The value of the nodule number per plant is an average of ten replicates with a standard deviation (bar). Histograms followed by the same letter (a–c) do not differ significantly (p=0.01) by T-test.

legume nodule formation such as root hair deformation, cortical cell division, pre-infection thread formation and expression of early nodulin genes (Truchet et al. 1991, Dénarié et al. 1992, Carlson et al. 1993, Vijn et al. 1993). In association between *B. elkanii* and soybeans, the Nod factor application formed a nodule structure with vascular bundle tissues (Stokkermans and Peters 1994). If the mutagenesis takes place on the genes involved in Nod factor production, then the mutants should nullify or reduce the nodulation ability. Therefore, we examined the Nod factor production of the parent and IAA-deficient mutants (Fig. 2). Flavonoid-inducible bands were observed in all strains. In the presence of genistein, no differences could be detected in the patterns of TLC between the parent USDA 31 and three mutants, indicating that Nod factor production did not change in the mutants.

Inoculation with the mutants significantly reduced the nodule number on soybean roots as compared to that of the parent strain USDA 31 (Fig. 1B). The nodule number is likely to be correlated with IAA production in the culture (Fig. 1A, B). In particular, the nodule number of TN3 inoculation was reduced to 34%. To determine if the reduction in nodule number depends on the IAA production of *B. elkanii*, IAA solution was applied to the root system of soybean plants inoculated with mutant TN3 at 4DAS. This IAA application restored the nodule number of TN3-inoculated soybeans to the original level (Fig. 3). These results strongly suggest that IAA produced by *B. elkanii* is involved in soybean nodule formation.

In recent years, the localized plant hormones auxin and cytokinin have been shown to participate in the fundamental responses of nodule morphogenesis as well as Nod factors. The application of auxin transport inhibitors (Hirsch et al. 1989, Scheres et al. 1992) and cytokinin (Dehio and de Bruijin 1992, Cooper and Long 1994) to legume roots induced root cortical cell mitoses and/or nodule-like structures including the expression of early nodulin genes. One possible explanation is that such localized plant hormones are involved in the signal transduction via Nod factors to root cortical cell mitoses. Therefore, our results prompt us to speculate that local IAA production by invading *B. elkanii* may help to maintain meristem activity, which would enhance soybean nodulation. This speculation would also account for the higher competitive ability of wild-soybean and nodulation of rj_1 soybean plants by *B. elkanii*.

Martinez et al. (1993) have shown that *Rhizobium tropici* induces IAA excretion in response to the same flavonoids that elicit Nod factor production. A pSym mutant unable to induce IAA production has a diminished nodulation capacity (60%), which is consistent with our results although the IAA production of *B. elkanii* was unaffected by the addition of a flavonoid inducer, genistein (data not shown).

Up to now, we have not been able to isolate *B. elkanii*

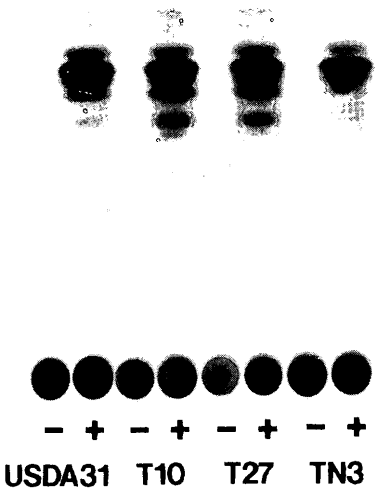

Fig. 2 Thin-layer chromatography (TLC) of Nod metabolites produced by wild-type strain USDA 31 and three IAA-deficient mutants (T10, T27 and TN3) in the absence (−) or presence (+) of genistein.

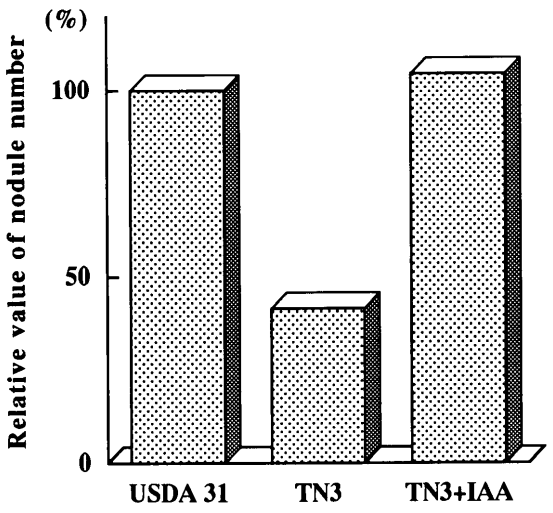

Fig. 3 The effect of IAA application on the nodule number of soybean plants inoculated with IAA-deficient mutant TN3. The average nodule number of soybean plants inoculated with USDA 31 was 4.5 nodules/plant. USDA 31, USDA 31 inoculation; TN3, TN3 inoculation; TN3+IAA, TN3 inoculation and IAA application (20 ml 1 μM IAA solution per jar).

mutants which are completely unable to produce IAA by spontaneous, NTG, and Tn5 mutageneses (Tn5 mutagenesis data is not shown). Thus, there is a possibility that *B. elkanii* may possess more than one copy of the genes involved in IAA biosynthesis and/or more than one IAA synthetic pathway. Similar situations have been reported for *Azospirillum* (Zimmer et al. 1991) and *Pseudomonas* (Oberhänsli et al. 1991). We are currently trying to elucidate the IAA biosynthetic pathways in *B. elkanii* and to construct a genetically characterized mutant completely lacking IAA production ability. This mutant would provide unequivocal evidence that *B. elkanii* IAA production involves soybean nodule formation and competitive ability.

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References

Aird, E.L.H., Brightwell, G., Jones, M.A. and Johnston, A.W.B. (1991) Identification of the exo loci required for exopolysaccharide synthesis in *Agrobacterium radiobactor* NCIB 11883. *J. Gen. Microbiol.* 137: 2287–2297.

Carlson, R.W., Sanjuan, J., Bhat, U.R., Glushka, J., Spaink, H.P., Wijfjess, A.H.M., van Brussel, A.A.N., Stokkermans, T.J.W., Peters, N.K. and Stacey, G. (1993) The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and type II strains of *Bradyrhizobium japonicum*. *J. Biol. Chem.* 268: 18372–18381.

Cooper, J.B. and Long, S.R. (1994) Morphogenetic rescue of *Rhizobium meliloti* nodulation mutants by *trans*-zeatin secretion. *Plant Cell* 6: 215–225.

Dehio, C. and de Bruijn, F.J. (1992) The early nodulin gene SrENOD2 from *Sesbania rostrata* is inducible by cytokinin. *Plant J.* 2: 117–128.

Dénarié, J., Debellé, F. and Rosenberg, C. (1992) Signaling and host range variation in nodulation. *Annu. Rev. Microbiol.* 46: 497–531.

Devine, T.E. and Weber, D.F. (1977) Genetic specificity of nodulation. *Euphytica* 26: 527–535.

Francisco, P.B., Akao, S. and Kokubun, M. (1991) The timing of exogenous 2,4-dichlorophenoxyacetate (2,4-D) application affects nodulation of soybeans, *Glycine max* (L.) Merr. *Soil Sci. Plant Nutr.* 37: 707–714.

Francisco, P.B. and Akao, S. (1993) Autoregulation and nitrate inhibition of nodule formation in soybean cv. Enrei and its nodulation mutants. *J. Exp. Bot.* 44: 547–553.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G. and Bisseling, T. (1989) Early noduline genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. *Proc. Natl. Acad. Sci. USA* 86: 1244–1248.

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.

Kuykendall, L.D., Saxena, B., Devine, T.E. and Udell, S.E. (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can. J. Microbiol.* 38: 501–505.

Martinez, E., Poupot, R., Prome, J.C., Pardo, M.A., Segovia, L., Truchet, G. and Denarie, J. (1993) Chemical signaling of *Rhizobium* nodulation bean. *In* New Horizons in Nitrogen Fixation. Edited by Palacios, R., More, J. and Newton, W.E. pp. 171–175. Kluwer Academic, Dordrecht.

Minamisawa, K. (1989) Comparison of extracellular polysaccharide composition, rhizobitoxine production, and hydrogenase phenotype among various strains of *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.

Minamisawa, K. and Fukai, K. (1991) Production of indole-3-acetic acid by *Bradyrhizobium japonicum*: a correlation with genotype grouping and rhizobitoxine production. *Plant Cell Physiol.* 32: 1–9.

Minamisawa, K., Iino, H., Saito, N., Onodera, S., Nakatsuka, Y., Nakajima, S., Kubota, M. and Asami, T. (1992) Determination of genotype group of *Bradyrhizobium japonicum* by indole-3-acetic acid production. (in Japanese) *J. Soil Sci. Plant Nutr.* 63: 459–462.

Minamisawa, K., Onodera, S., Kobayashi, N., Tanimura, Y., Yuhashi, K. and Akao, S. (1993) Competitiveness of different genotype groups of indigenous *Bradyrhizobium japonicum* for nodule occupancy. *In* New Horizons in Nitrogen Fixatin. Edited by Palacios, R., More, J. and Newton, W.E. pp. 632. Kluwer Academic, Dordrecht.

Oberhänsli, T., Défago, G. and Haas, D. (1991) Indole-3-acetic acid (IAA) synthesis in the biocontrol strain CHA0 of *Pseudomonas fluorescens*: role of tryptophan side chain oxidase. *J. Gen. Microbiol.* 137: 2273–2279.

Pueppke, S.G. and Payne, J.H. (1987) Responses of *Rj1* and *rj1* soybean isolines to inoculation with *Bradyrhizobium japonicum*. *Plant Physiol.* 84: 1291–1295.

Scheres, B., McKhann, H.I., Zalensky, A., Löbler, M., Bisseling, T. and Hirsch, A.M. (1992) The PsENOD12 gene is expressed at two different sites in Afghanistan pea pseudonodules induced by auxin transport inhibitors. *Plant Physiol.* 100: 1649–1655.

Spaink, H.P., Aarts, A., Stacey, G., Bloemberg, G.V., Lugtenberg, B.J.J. and Kennedy, E. (1992) Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. *Mol. Plant-Microbe Interact.* 5: 72–80.

Stokkermans, T.J.W. and Peters, N.K. (1994) *Bradyrhizobium elkanii* lipo-oligosaccharide signals induce complete nodule structures on *Glycine soja* Siebold et Zucc. *Planta* 193: 413–

420.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., De Billy, F., Promé, J.-C. and Dénarié, J. (1991) Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. *Nature* 351: 670–673.

Vijn, I., das Neves, L., van Kammen, A., Franssen, H. and Bisseling, T. (1993) Nod factors and nodulation in plants. *Science* 260: 1764–1765.

Zimmer, W., Aparicio, C. and Elmerich, C. (1991) Relationship between tryptophan biosynthesis and indole-3-acetic acid production in *Azospirillum*: identification and sequencing of a *trpGDC* cluster. *Mol. Gen. Genet.* 229: 41–51.

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