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Nodule formation and distribution of Rj_2Rj_3–genotype soybean infected with *Bradyrhizobium japonicum*

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Nodulation and nodule distribution of Rj_2Rj_3–genotype soybean cultivar CNS infected with *Bradyrhizobium japonicum* strain Is–34 which is a compatible strain with this genotype cultivar or *B. japonicum* Is–1 which is an incompatible strain were investigated. Nodule formation and distribution were quite different patterns between two type strains. On 28–day–old roots, the number of ineffective nodules of roots inoculated with Is-1 was more than five times of that of effective nodules of roots with Is–34. Although nodules on the single inoculation of Is–1 were almost ineffective, a small number of effective nodules were observed. However, this strange phenomenon was not observed on mixed inoculation of Is–34 and Is–1. Many root hairs curled on the epidermis of roots inoculated with either Is–34 or Is–1. Cortical cell division, however, initiated only in roots inoculated with Is–34, but did not in those with Is–1 at seven days after inoculation. These results show the first step of discrimination of ineffective nodule formation from effective nodule formation may be the initiation of induction of root cortical cell division on roots inoculated with Is–1.

INTRODUCTION

Soybean cultivars (*Glycine max* L. Merr.) are infected solely with *Bradyrhizobium japonicum* and nodulate on the root. The specific signal exchange between rhizobia and host plant during nodule formation is involved in the infection of rhizobia and nodule formation (Stacey *et al.*, 1995). It is, however, observed that some soybean cultivars can not form nodules or do ineffective nodules that can not fix nitrogen by infection of some strains of *B. japonicum* which exhibit certain physiological and genetical properties. It was reported that some soybean cultivars harbor nodulation–conditioning genes (*Rj*–genes) and nodulation takes place only with certain strains of *B. japonicum*. *Rj*–genes of these soybean cultivars were called rj_1, Rj_2, Rj_3, Rj_4 (Caldwell, 1966; Caldwell *et al.*, 1966; Caldwell and Vest, 1968; Vest, 1970; Vest and Caldwell, 1972). Soybean cultivars harboring *nts* (nitrate–tolelant symbiosis) gene are supernodulating mutants because autoregulatory responses are lacking in *nts*–cultivars and form a great number of nodules (Caetano–Anolles and Gressoff, 1993). Furthermore, the rhizobia can be classified into three nodulation–types, A, B, and C, based on the compatibility with *Rj*–soybean cultivars (Ishizuka *et al.*, 1991a,b). Nodulation type A can form effective nodules on soybean cultivars excepting rj_1–cultivars. Nodulation type B can not form effective nodules on Rj_4– and rj_1–cultivars. Nodulation type C can not form effective

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nodules on Rj_2Rj_3- and rj_1-cultivars. Therefore, there is a probable selective host specificity between two symbionts. Up to date, however, mechanisms involved in compatibility between *Rj*-soybean cultivars and *B. japonicum* strains were still unclear. In this study, we compared nodule formation and distribution in relation to host specificity between two symbionts, Rj_2Rj_3-soybean cultivar, CNS and *B. japonicum* strains of nodulation type C or nodulation type B.

MATERIALS AND METHODS

Plant and rhizobia

Soybean cultivar CNS was used as Rj_2Rj_3-genotype soybean. This cultivar can form effective nodules on roots by *B. japonicum* strains of nodulation type C, but cannot by strains of nodulation type B. *B. japonicum* strains Is-34 (nodulation type C) and Is-1 (nodulation type B) were used (Ishizuka *et al.*, 1991b). These strains were stored on YMA slants (Vincent, 1970) at 4°C.

Plant growth and nodulation

CNS seeds were sterilized with 70% ethanol and 5% sodium hypochlorite solution. Sterilized seeds were sown in 3 L sterilized vermiculite wetted to 50% water content with culture solution containing 1.6 mM K_2HPO_4, 2.0 mM $CaCl_2$, 2.5 mM $MgSO_4$ (Ishizuka *et al.*, 1991a) using a/5000 pots. After sowing, each seed was inoculated with 2 mL of a bacterial suspension diluted to 10^6 cells per mL with sterile 0.15 M NaCl. As uninoculated control, 2 mL of sterile 0.15 M NaCl was supplied per seed. Plant culture was done at 25°C and 70% relative humidity under natural light conditions in Biotron Institute Kyushu University. Plant roots were harvested on 7, 14, 21 and 28 days after sowing. Shoot growth was estimated by means of fresh weight of 4 to 12 plants. Nodule number on a primary root was counted in each 1 cm root section of the primary root, and these positions was presented as root length from the base of roots. Nodule number on lateral roots were counted on the lateral roots emerging in each 1 cm of a primary root up to 5 cm and in the primary root longer than 5 cm from the base of roots, and these positions were designated as Lateral root 0–1, Lateral root 1–2, Lateral root 2–3, Lateral root 3–4, Lateral root 4–5 and Lateral root 5–, respectively (Fig. 1). Nodules were classified to effective or ineffecitve based on the size and color of the interior of the nodules. Small nodules in which the interior was white and tumor-like structures were classified as ineffective.

Nodulation specificity by mixed inoculation

Nodulation specificity was examined by mixed inoculation and the serological identification method. Bacterial suspensions of Is-1 and Is-34 diluted to 10^6 cells per mL with sterile 0.15 M NaCl were mixed at a ratio of 1:1. CNS seeds were sown as discribed above and inoculated with the rhizobial strain mixture. Plant culture was done for 28 days as described above. The antisera against Is-1 or Is-34 were produced by injection to rabbits with cell suspensions of *B. japonicum* strains as somatic antigens according to the procedure of Vincent (1970). Serological identification of bacteroids was done as follows (Ishizuka *et al.*, 1993). The antisera were diluted to 1/500 with sterile 0.15 M NaCl containing 0.05% sodium azide before use. After 28 days of plant growth, the nodules

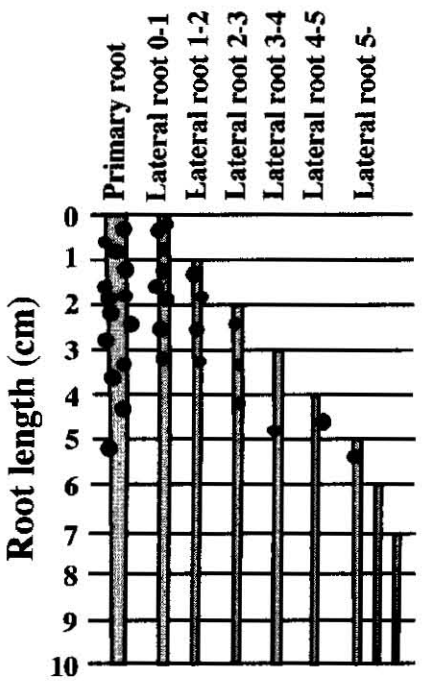

Fig. 1. Schematic presentation of distribution of nodules in different regions on the primary root and lateral roots.

were harvested, washed and put into a test tube containing 0.15 M NaCl. The test tube was heated in boiling water for 30 min. After cooling, each nodule was separately transferred to small test tubes and a small volume of sterile 0.15 M NaCl containing 0.05% sodium azide was added. The each nodule was crushed with a glass rod and dispersed using a vibrator. After precipitation of the nodule debris, each drop of the bacteroid suspension and the diluted antiserum were put into a well of a U-bottom microtiter assay plate (Becton Dickinson and Co., USA). The plate was covered with a thin polyethylene film and incubated at 37 °C for 1h followed by keeping at 4 °C overnight. The agglutination reaction was observed by comparison with the bacteroid-saline control.

Microscopic observation of root hair and root section

Root-hair curling with inoculation and root sections were observed on the primary root of 7-day-old seedlings. For root-hair observation, samples were prepared by barking a root epidermis and the epidermis was settled on a slide glass. To prepare the samples of root sections, the upper parts of tap root were cut into round slices as thin as possible using a razor blade. The sliced roots were settled on a slide glass for microscopic observation.

RESULTS

Plant growth

Plant growh was recorded by fresh wight of shoot (Fig. 2). Shoot growth was not different significantly until 21 days after sowing among plants inoculated with Is–34 or Is–1 and uninoculated plants. Since 21 days after sowing, however, the leaf color of plants uninoculated and inoculated with Is–1 was becoming pale. On 28 days, plants inoculated with Is–34 grew normally, while plants uninoculated and inoculated with Is–1 showed a typical nitrogen deficiency symptom. Futhermore, the 5th trifoliate leaf was emerging only on plants inoculated with Is–34 at 28 days after sowing.

Nodule formation and distribution

Time course of nodule formation was shown in Table 1. On the roots of control plants, effective or ineffective nodules were not observed even on 28–day–old roots. Nodule formation was observed at first at 14 days when Is–34 was inoculated. The nodules on roots inoculated with Is–34 were all effective. At 14 days in roots inoculated with Is–1, even ineffective nodules were not formed. Effective nodules were not formed but ineffective nodules were formed at 21 days when Is–1 was inoculated. The number of effective nodules formed by Is–34 inoculation was almost the same number as that of ineffective nodules formed by Is–1 inoculation. Effective nodules increased at 28 days on roots inoculated with Is–34. On the other hand, about two hundred ineffective nodules were formed by Is–1 inoculation but only a few effective nodules on the middle regions of 28–day–old roots (Table 1).

Nodule distribution was estimated on 14, 21, 28–day–old roots inoculated with Is–1

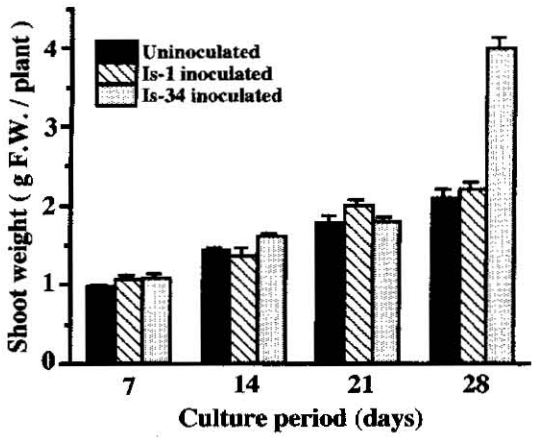

Fig. 2. Time course of shoot growth of CNS soybean with or without inoculation of *Bradyrhizobium japonicum*. Each value is a mean ± standard error.

Table 1. Changes in nodule number of CNS root inoculated with *Bradyrhizobium japonicum*.

Culture period (days)	Inoculant strain			
	Is-34		Is-1	
	Effective	Ineffective	Effective	Ineffective
7	N.D.	N.D.	N.D.	N.D.
14	25.8±1.6	N.D.	N.D.	N.D.
21	28.5±2.1	N.D.	N.D.	30.3±7.2
28	36.6±3.6	N.D.	1.4±0.5	205.8±44.4

Effective: nitrogen fixing nodule, Ineffective: non-nitrogen fixing nodule
Each value is a mean (per plant) ± standard error, N.D.: not detected

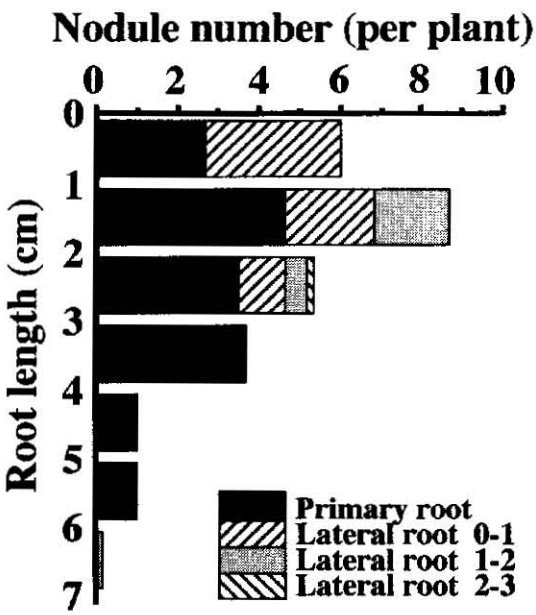

Fig. 3. Nodule distribution on 14-day-old CNS roots inoculated with *Bradyrhizobium japonicum* strain Is-34. Each value is a mean of 6 plants.

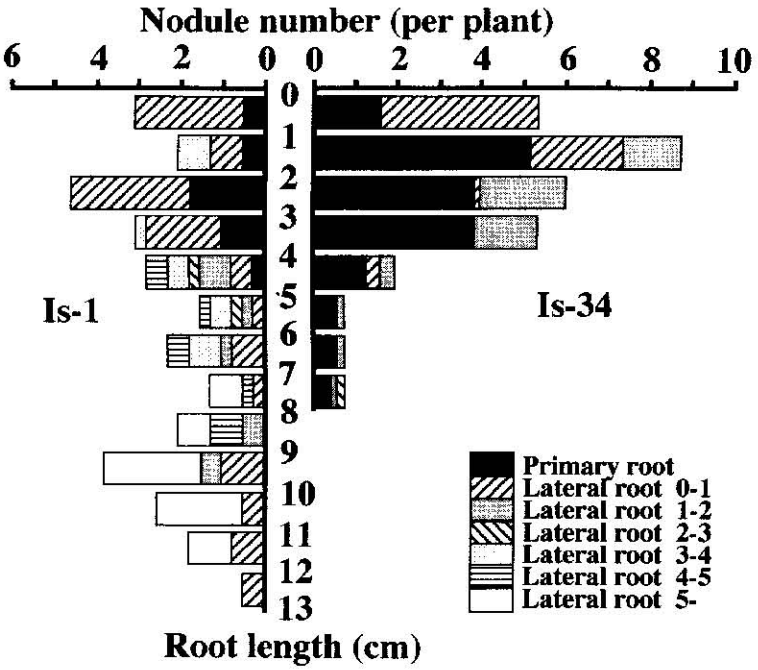

Fig. 4. Nodule distribution on 21-day-old CNS roots inoculated with *Bradyrhizobium japonicum* strains Is-34 and Is-1. Values of CNS roots inoculated with Is-34 are means of 6 plants, and those with Is-1 are means of 4 plants.

and Is-34. Sixty four % of effective nodules on 14-day-old roots inoculated with Is-34 were formed on a primary root and 90.5% of nodules were formed up to 4 cm from the base of roots (Fig. 3). On 21-day-old roots inoculated with Is-34 the nodulation pattern was similar to that of 14-day-old roots (Fig. 3 and 4). Fifty five % of nodules was observed on the primary root and 91% of nodules was formed up to 4 cm from the base of roots. On the other hand, unlike the nodule number mentioned above, the nodulation pattern was quite different between Is-1 and Is-34 inoculation (Fig. 4). In Is-1 inoculation, only 13.3% of ineffective nodules were present on the primary root and 47.6% of nodules were observed up to 4 cm from the base of roots. Most of ineffective nodules were formed the wide region on lateral roots. At 28 days, effective nodules were formed more on the upper regions of lateral roots inoculated with Is-34 (Fig. 5). Nodules of the primary root occupied 41.1% of total nodules and 69.3% of nodules were observed up to 4 cm from the base of root. In Is-1 inoculation, many ineffective nodules were formed mainly on lateral roots and the ineffective nodules formed on a primary root occupied only 4.3% of total nodules. Therefore, CNS inoculated with Is-34 nodulated both on the upper region of the primary root and on the base of lateral roots emerging from the upper region of the primary root. On the other hand, the region of ineffective nodulation on roots of CNS inoculated with Is-1 was mainly limited to lateral roots.

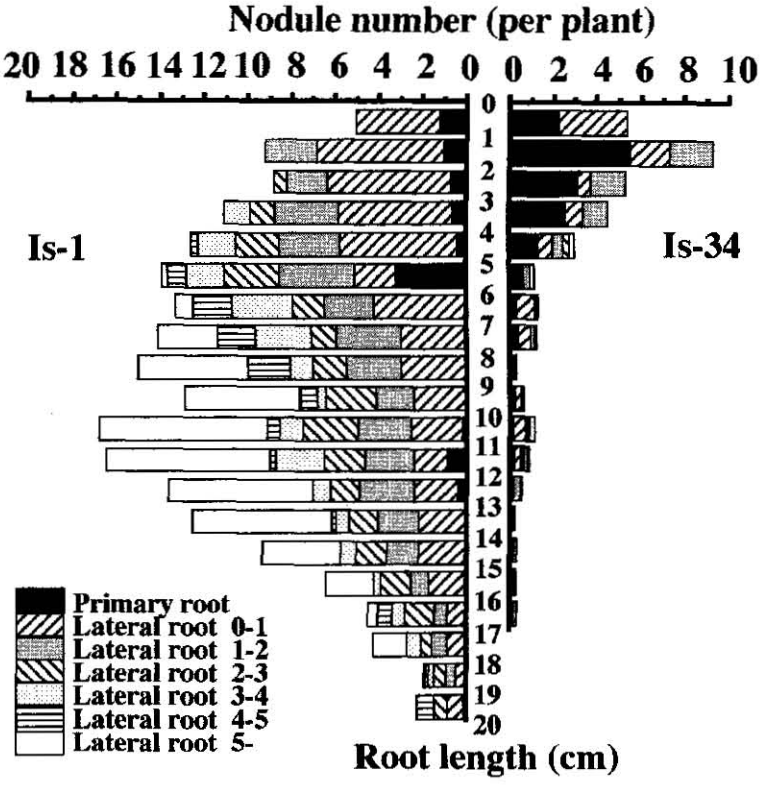

Fig. 5. Nodule distribution on 28-day-old CNS roots inoculated with *Bradyrhizobium japonicum* strains Is-34 and Is-1. Values of CNS roots inoculated with Is-34 are means of 12 plants, and those with Is-1 are means of 8 plants.

Nodulation specificity by mixed inoculation

A small number of effective nodule formation were observed on roots inoculated solely with incompatible strain Is-1 after 28 days plant culture. To confirm specificity between CNS and compatible strain Is-34 or incompatible strain Is-1, nodule occupancy of root inoculated with mixed bacterial suspensions was investigated by an immunological method using antisera against *B. japonicum* Is-1 and Is-34. All nodule bacteroid suspensions were crossreacted with the antiserum against *B. japonicum* Is-34. There was no nodule infected with incompatible strain Is-1 on roots inoculated with mixed bacterial suspension (data not shown).

Root-hair curling and cell division of cortex

Many root-hair curlings on root epidermis were found on roots inoculated with rhizobia regardless of their compatibility (Fig. 6A). Cortical cell division for formation of

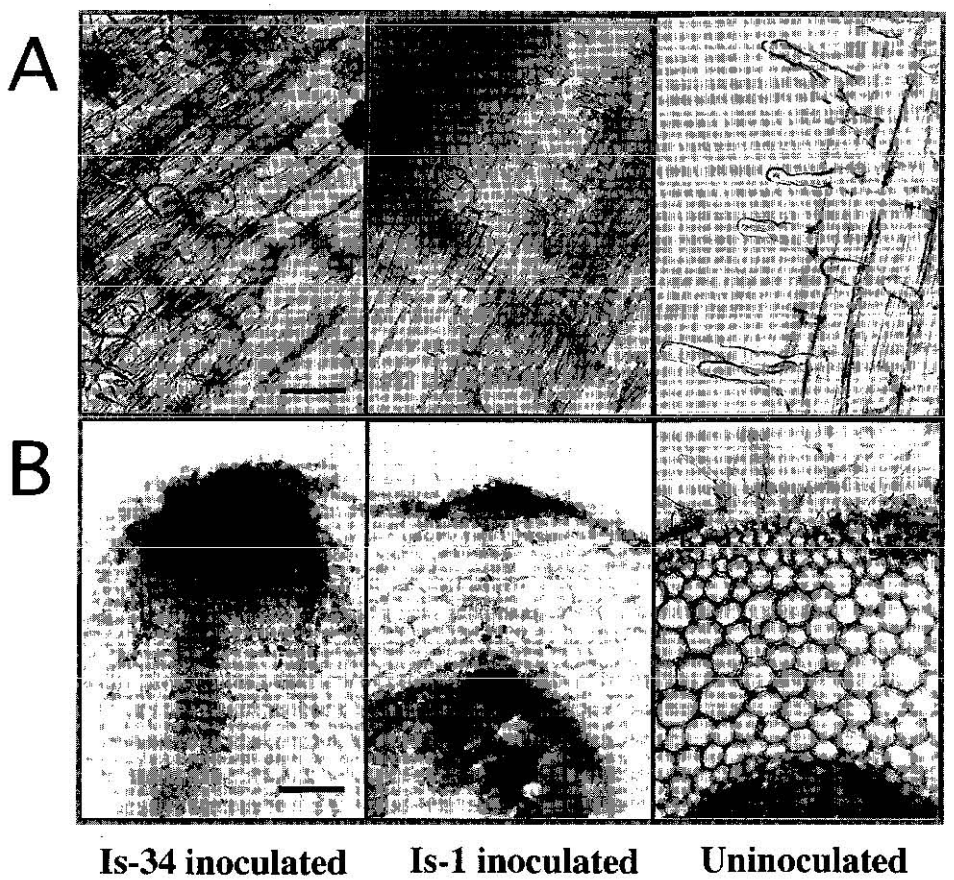

Fig. 6. Microscopic photograph of CNS root inoculated or uninoculated with *Bradyrhizobium japonicum* strains Is–34 and Is–1. A: Root epidermis of primary root of CNS inoculated or uninoculated with *B. japonicum* (Bar=50μm). B: Cross section of primary root of CNS inoculated or uninoculated with *B. japonicum* (Bar=100μm).

vascular tissue vigorously took place in roots inoculated with Is–34, while it was weak in roots inoculated with Is–1 (Fig. 6B).

DISCUSSION

In this study, we compared nodule formation, distribution and specificity to make clear host specificity between two symbionts, Rj_sRj_s–genotype soybean cultivar CNS and *B. japonicum* strains Is–34, nodulation type C or Is–1, nodulation type B. At 14 days when effecitive nodules were formed on roots inoculated with Is–34, any ineffective nodule was not yet observed on roots inoculated with Is–1. Furthermore, though a small number of

effective nodules were formed on the roots inoculated solely with incompatible strain Is–1, compatibility between CNS and compatible strain Is–34 was enhanced by inoculation of a mixture of compatible and incompatible strains. These results suggest some selective function of a host plant might work against Is–1 which is an incompatible strain during nodule development. Indeed, microscopic observation revealed brisk cortical cell division in roots inoculated with Is–34 and weak cortical cell division in those inoculated with Is–1 for nodule development at seven days after inoculation. On the other hand, root–hair curlings were invariably observed on roots inoculated with two different rhizobium strains regardless of the difference in their compatibility. Therefore, it is considered that the first selective action of CNS in the determination of effective or ineffective nodule formation against incompatible strain Is–1 may be the induction of cortical cell division. Generally, Nod factors that rhizobia produce induce root–hair curling and cortical cell division (Mylona *et al.*, 1995). Why the cortical cell division was retarded and weakened on the root inoculated with Is–1 regardless of normal root–hair curling? We suppose two possibilities. First, Nod factors may have different active sites for induction of root–hair curling and for cortical cell division, then Nod factors of these rhizobia have a common active site for root–hair curling but different active sites for cortical cell division. The second possibility may be that the host receptor of CNS has a different sensitivity against a range of concentrations of Nod factors produced by these compatible or incompatible strains. Furthermore, nodule number and distribution on roots were quite different patterns between them. Differences of nodule number and distribution between CNS inoculated with Is–34 and CNS inoculated with Is–1 are similar to those between Bragg and it's supernodulating mutant *nts* 382 lacking autoregulation response (Caetano–Anolles and Gresshoff, 1993). Because autoregulation of host plant is related with control of nodule number, the present results on ineffective nodule number and it's distribution suggest that autoregulation may not work on the CNS roots inoculated with incompatible strain Is–1.

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