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Incompatible Nodulation of *Bradyrhizobium elkanii* Strains BLY3-8 and BLY6-1 with Rj_3 Gene-Harboring Soybean Cultivars

Aung Zaw Htwe[1,2], Takeo Yamakawa[1]

[1]Laboratory of Plant Nutrition, Graduate School of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka, Japan
[2]Department of Agronomy, Yezin Agricultural University, Yezin, Naypyitaw, Myanmar
Email: aungzawhtwe333@gmail.com

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Abstract

Bradyrhizobia are known symbiotic partners of soybean. However, some soybean cultivars restrict nodulation by some *Bradyrhizobium* bacterial strains. These restrictions are related to compatibility between the *Rj* genes of soybean cultivars and nodulation types of inoculated bacteria. The objective of this study was to determine nodulation incompatibility of Type B strains with Rj_3 soybean cultivars. Newly isolated *B. elkanii* strains BLY3-8 and BLY6-1 from Myanmar and specific strain *Bradyrhizobium elkanii* USDA33, which are incompatible with Rj_3 soybean cultivars, and *B. japonicum* USDA110 were used as inoculants to check compatibility or incompatibility with Rj_3 soybean cultivars. Nitrogen fixation activity was measured by the acetylene reduction method. Ethylene concentration (reduction of acetylene) was determined by flame ionization gas chromatography. According to the inoculation test results, USDA110 was compatible with all soybean cultivars because it formed effective nodules (**Figure S1** in Appendix) and possessed nitrogenase activity. Similarly, *B. elkanii* strains BLY3-8, BLY6-1, and USDA33 were highly compatible with non-*Rj* and Rj_4-gene harboring soybean cultivars because they had the ability to form functional nodules and possessed nitrogenase activity. Inversely, BLY3-8, BLY6-1, and USDA33 were incompatible with Rj_3 soybean cultivars because they produced ineffective nodules. Consequently, the ratio of ineffective nodule number to total nodule number was >0.5. Therefore, nodule formation by the newly isolated *B. elkanii* strains BLY3-8 and BLY6-1 was restricted by the Rj_3 soybean cultivars potentially making them useful as specific strains to detect the Rj_3 gene in soybean cultivars.

Keywords

Bradyrhizobium elkanii, Nodulation, Incompatibility, Rj_3, Soybean

1. Introduction

Nodulation and symbiotic nitrogen fixation are important for soybean cultivation. Symbiotic nitrogen fixation provides 40% - 70% of the total nitrogen requirement of soybean (65 to >160 kg·N·ha^{-1}) [1]. Symbiotic nitrogen fixation is highly specific, as a particular species or strain of *Rhizobia* can perform the symbiotic association with only a specific leguminous species or cultivar [2]. This specificity involves molecular recognition of host plants and bacteria, through the exchange of signaling compounds that induce nodule formation and nitrogen fixation [3] [4].

Saeki *et al.* [5] reported that *Rj* gene soybean cultivars affect compatibility and preference for nodule formation between the host cultivar and rhizobial bacteria. *Bradyrhizobium* strains can be divided into nodulation Types A, B, and C based on compatibility of the bradyrhizobia with *Rj* gene soybean cultivars [6] [7]. Type A strains induce nodulation on all *Rj* genotype cultivars. However, Types B and C strains have restricted nodule formation on *Rj*$_2$*Rj*$_3$ and *Rj*$_4$ genotype cultivars, respectively. Htwe *et al.* [8] reported that strain Types A, B, and C account for 74%, 22%, and 4% of Myanmar *Bradyrhizobium* strains, respectively.

Although *Bradyrhizobium* bacteria are known symbiotic partners with soybean, some soybean cultivars restrict nodulation by some strains of *Bradyrhizobium*. These restrictions are due to the *Rj* (or *rj*) gene in soybean [9]. The *Rj* genotype may affect the efficiency of nodulation and nitrogen fixation in fields. Some nodulation *Rj* genes exist naturally or are induced artificially by mutations [9] and crosses of soybean cultivars [10]. A non-nodulating soybean line, called the *rj*$_1$-gene harboring cultivar, resulted from a cross between the "Lincoln" and "Richard" cultivars [11]. Soybean cultivars in Myanmar harboring non-*Rj*-, *Rj*$_2$*Rj*$_3$-, *Rj*$_3$-, or *Rj*$_4$-genes were determined in a previous study [12]. Among them, *Rj*$_4$-gene harboring soybean cultivars are widely grown in Myanmar and account for 60% of all strains [12]. Devine *et al.* [13] reported that > 60% of soybean in Southeast Asia have the *Rj*$_4$ gene.

The *Rj*-genotypes are mainly determined according to the inoculation method of Ishizuka *et al.* [10]. Strains Is-1, USDA 33, and Is-34 are used as inoculants because these strains are incompatible with *Rj*$_2$, *Rj*$_3$, *Rj*$_3$, and *Rj*$_4$ soybean cultivars, respectively [10] [14]. The *Rj*$_2$ and *Rj*$_4$ genes have also been identified by multiplex polymerase chain reaction (PCR) analysis [12] using primers designated by Yang *et al.* [15], Tang *et al.* [16], and Hayashi *et al.* [17]. However, this multiplex PCR analysis is incapable of detecting the *Rj*$_3$ gene in soybean cultivars. Therefore, detecting *Rj*$_3$ is based on an inoculation method using the specific *B. elkanii* strain USDA33. However, the nodulation phenotype of *B. elkanii* strain USDA33 is unstable [18]. *Bradyrhizobium* Type B strains, which have restricted nodule formation in *Rj*$_2$*Rj*$_3$-gene harboring cultivars [6] [7], can replace USDA33 and have the highest possibility of being effective for identifying the *Rj*$_3$ gene. This nodulation restriction is of interest for studying incompatibility of nodule formation in *Rj*$_3$ soybean cultivars. Therefore, we conducted this study to identify strains incompatible with the *Rj*$_3$ gene that can be used to identify the *Rj*$_3$ gene in soybean cultivars worldwide.

2. Materials and Methods

2.1. *Bradyrhizobium* Strains

Bradyrhizobium japonicum strains USDA110 (Type A), Is-1 (Type B), and Is-34 (Type C), as well as *B. elkanii* strain USDA33 (Type B) were obtained from the Plant Nutrition Laboratory, Kyushu University, Japan. The nodulation types in parentheses were reported by Ishizuka *et al.* [7]. Indigenous bradyrhizobia, such as *Bradyrhizobium* spp. strains SHY3-1 (Type B) and SHY6-1 (Type B), *B. japonicum* strains SHY3-10 (Type B) and SAY6-1 (Type B), and *B. elkanii* strains BLY3-8 (Type B) and BLY6-1 (Type B), were isolated from Myanmar strains and their nodulation types were reported previously [8].

2.2. Soybean Cultivars

Myanmar soybean cultivars [Yezin-3 (Rj_4), Yezin-6 (*non-Rj*), Yezin-9 (Rj_3), and Yezin-10 (Rj_2Rj_3)] were collected from the Food Legume Section, Department of Agricultural Research, Yezin, Myanmar. These cultivars were cultured in a Kyushu University greenhouse to produce seeds. The *Rj* genes (in parentheses) were identified by Htwe *et al.* [12] and Soe *et al.* [19]. Other cultivars [Bragg (*non-Rj*), T201 (rj_1), Fukuyutaka (Rj_4), D51 (Rj_3), IAC-2 (Rj_2Rj_3), A250 ($Rj_2Rj_3Rj_4$), B340 ($Rj_2Rj_3Rj_4$), C244 ($Rj_2Rj_3Rj_4$), and Orihime (*non-Rj*)] were obtained from the Plant Nutrition Laboratory, Department of Bioresources and Bioenvironmental Sciences, Kyushu University. The *Rj* genes (in parentheses) were described in Ishizuka *et al.* [6], Hayashi *et al.* [9], and Yamakawa *et al.* [20].

2.3. Incompatibility of the *B. elkanii* Strains BLY3-8 and BLY6-1 in Various Soybean Cultivars

Seeds were surface sterilized in 1% sodium hypochlorite solution for 5 min, rinsed five times with 10 mL of 99.5% ethanol, and washed five times with sterilized half-strength modified nitrogen-free Hoagland Nutrient (MHN) solution [21]. Seven seeds were sown in prepared culture pots filled with 1 L of vermiculite and 0.6 L of MHN solution. The *Bradyrhizobium* strains were cultured in A1E liquid medium [22] and incubated on a rotary shaker (100 rpm) at 30˚C for 7 days. The inoculant was prepared by diluting 1 mL of liquid bacterial culture with 99 mL of sterilized MHN solution to obtain a bacterial suspension of about 10^7 cells·mL^{-1}. Seeds were inoculated with the bacterial suspension at a rate of 5 mL/seed. Inoculation was done just after seed sowing. Then, the inoculated plants were cultivated under controlled conditions (25˚C and 75% relative humidity) and natural light for 4 weeks. Control pots were used to check for contamination. The plants were watered weekly with autoclaved deionized water. After 4 weeks, the plants were checked to determine whether effective or ineffective nodules had formed to detect nodulation incompatibility with the Rj_3 soybean cultivars. This experiment was conducted from January to November 2016.

2.4. Acetylene Reduction Assay to Measure Nitrogenase Activity

The acetylene reduction assay (ARA) was performed according to Haider *et al.*

[23] to measure nitrogenase activity. The soybean plants were cut at the cotyledonary nodes, and the root with intact nodules was placed in a 100-mL conical flask and sealed with a serum stopper. Then, 12 mL of acetylene gas was injected into the flask to replace the air. The flasks containing roots with intact nodules were incubated at room temperature (24˚C - 26˚C). Then, 1.0 mL of subsample was analyzed after 5 and 65 min. The ARA value, in terms of ethylene concentration per plant, was measured using a flame ionization gas chromatograph (GC-14A; Shimadzu, Kyoto, Japan) equipped with a stainless steel column (3 mm diameter, 0.5 m length). The column was filled with 60 - 80 mesh Porapak R (Nacalai Tesque, Inc., Kyoto Japan). Column, injection, and detection temperatures were 35˚C, 45˚C, and 170˚C, respectively. Nitrogen was the carrier gas.

3. Results

Compatibility or incompatibility for nodulation of *Bradyrhizobium* spp. strains SHY3-1 and SHY6-1, *B. japonicum* strains SHY3-10 and SAY6-1, and *B. elkanii* strains BLY3-8 and BLY6-1 on different *Rj* gene-harboring cultivars is shown in Table 1. The results showed that these strains were highly compatible with Yezin-6 (non-*Rj*) and Yezin-9 (Rj_3). Interestingly, of these strains, *B. elkanii* strains BLY3-8 and BLY6-1 were incompatible with D51 (Rj_3), although they nodulated on Yezin-9 (Rj_3). However the selected strains were incompatible with the Yezin-10 (Rj_2Rj_3) soybean cultivar. These results show that the Rj_2Rj_3 and Rj_3 soybean cultivars restricted nodule formation by *B. elkanii* strains BLY3-8 and BLY6-1.

As a continuing study based on the initial findings, *B. elkanii* strains BLY3-8 and BLY6-1 were tested for nodule formation on various soybean cultivars harboring different *Rj* genes. The results of *B. elkanii* strains BLY3-8 and BLY6-1 are shown in Table 2 and Table 3, respectively. The inoculation test results revealed that the BLY3-8 and BLY6-1 strains produced effective nodules in the range of 9.43 - 14.11/plant in the *non-Rj*-gene harboring cultivars Yezin-6 and Bragg. Similarly, they produced effective nodules in the range of 8.93 - 14.65/plant in the Rj_4 soybean cultivars Yezin-3 and Fukuyutaka. These results highlight that *B. elkanii* strains BLY3-8 and BLY6-1 were more compatible with the

Table 1. Preliminary testing of nodule restriction of different isolates by different soybean cultivars.

Cultivar (*Rj* gene)	Nodule no. plant^{-1} on inoculated strains					
	SHY3-1	SHY6-1	SHY3-10	SAY6-1	BLY3-8	BLY6-1
Yezin-6 (*non-Rj*)	High	High	High	High	High	High
Yezin-10 (Rj_2Rj_3)	None	None	Low	Low	None	None
Yezin-9 (Rj_3)	Medium	Medium	Medium	Medium	Medium	Medium
D51 (Rj_3)	Medium	Medium	Medium	Medium	None	None

High = 10 - 15 nodules plant^{-1}, Medium = 4 - 9 nodules plant^{-1}, Low = 1 - 3 nodules plant^{-1}, None = No nodulation. This division was based on Htwe *et al.* [8]. This experiment was conducted from January 2016 to February 2016.

Table 2. Detection for incompatibility of *B. elkanii* BLY3-8 with various soybean cultivars.

Cultivar (*Rj* gene)	Nodule number per plant					I/T	Incompatibility for Rj_3 gene
	Effective			Ineffective (I)	Total (T)		
	TR	LR	WR				
Yezin-6 (*non-Rj*)	3.30	6.20	9.50	0.00	9.50	0.00	-
Bragg (*non-Rj*)	3.36	6.67	10.03	0.00	10.03	0.00	-
Orihime (*non-Rj*)	0.00	0.07	0.07	0.31	0.38	0.82	+
Yezin-3 (Rj_4)	5.45	8.88	14.33	0.00	14.33	0.00	-
Fukuyutaka (Rj_4)	2.95	6.75	9.70	0.00	9.70	0.00	-
Yezin-9 (Rj_3)	7.86	2.50	10.36	0.00	10.36	0.00	-
D51 (Rj_3)	0.00	0.15	0.15	19.67	19.82	0.99	+
Yezin-10 (Rj_2Rj_3)	0.00	0.00	0.00	9.86	9.86	1.00	+
IAC-2 (Rj_2Rj_3)	0.00	0.00	0.00	15.48	15.48	1.00	+
A250 ($Rj_2Rj_3Rj_4$)	0.00	0.17	0.17	3.80	3.97	0.96	+
B340 ($Rj_2Rj_3Rj_4$)	0.20	0.00	0.00	18.67	18.87	0.99	+
C244 ($Rj_2Rj_3Rj_4$)	0.00	0.00	0.00	21.71	21.71	1.00	+

TR, LR, WR: tap root, lateral root and whole root, respectively. The number indicating in table is the mean of 7 plants for A20, B340 and C244 cultivars and 14 plants for other cultivars. + or − show the plants have or do not have the restriction ability for Rj_3 genes due to inoculation of incompatible strain BLY3-8 for Rj_3 gene-harbouring soybean cultivars. This experiment was conducted during April 2016.

Table 3. Detection for incompatibility of *B. elkanii* BLY6-1 with various soybean cultivars.

Cultivar (*Rj* gene)	Nodule number per plant					I/T	Incompatibility for Rj_3 gene
	Effective			Ineffective (I)	Total (T)		
	TR	LR	WR				
Yezin-6 (*non-Rj*)	4.29	9.82	14.11	0.00	14.11	0.00	−
Bragg (*non-Rj*)	3.43	6.00	9.43	0.00	9.43	0.00	−
Orihime (*non-Rj*)	0.00	0.07	0.07	0.15	0.22	0.68	+
Yezin-3 (Rj_4)	6.29	8.36	14.65	0.00	14.65	0.00	−
Fukuyutaka (Rj_4)	2.43	6.50	8.93	0.00	8.93	0.00	−
Yezin-9 (Rj_3)	7.36	2.79	10.15	0.00	10.15	0.00	−
D51 (Rj_3)	0.00	0.00	0.00	27.75	27.75	1.00	+
Yezin-10 (Rj_2Rj_3)	0.12	0.12	0.24	4.77	5.01	0.95	+
IAC-2 (Rj_2Rj_3)	0.00	0.10	0.10	9.21	9.31	0.99	+
A250 ($Rj_2Rj_3Rj_4$)	0.00	0.17	0.17	8.48	8.65	0.98	+
B340 ($Rj_2Rj_3Rj_4$)	0.20	0.00	0.20	25.60	25.80	0.99	+
C244 ($Rj_2Rj_3Rj_4$)	0.00	0.00	0.00	37.29	37.29	1.00	+

TR, LR, WR: tap root, lateral root and whole root, respectively. The number indicating in table is the mean of 7 plants for A20, B340 and C244 cultivars and 14 plants for other cultivars. + or − show the plants have or do not have the restriction ability for Rj_3 genes due to inoculation of incompatible strain BLY6-1 for Rj_3 gene-harbouring soybean cultivars. This experiment was conducted during April 2016.

non-*Rj* and *Rj*$_4$ cultivars. However, these two isolates were incompatible for nodule formation on *Rj*$_3$-harboring soybean cultivars, such as D51 (*Rj*$_3$), Yezin-10 (*Rj*$_2$*Rj*$_3$), IAC-2 (*Rj*$_2$*Rj*$_3$), A250 (*Rj*$_2$*Rj*$_3$*Rj*$_4$), B340 (*Rj*$_2$*Rj*$_3$*Rj*$_4$), and C244 (*Rj*$_2$*Rj*$_3$*Rj*$_4$). Although no effective nodules were formed, ineffective nodules (small and white colored) were produced on these *Rj*$_3$-harboring soybean cultivars. The ratio of ineffective nodules (I) to total number of nodules (T) (I/T ratio) was 0.95 - 1.00. Notably, the Orihime (*non-Rj*) soybean cultivar showed restricted effective nodule formation by BLY3-8 and BLY6-1, whereas Yezin-9 (*Rj*$_3$) did not, suggesting that the Orihime soybean cultivar might harbor the *Rj*$_3$ gene and Yezin-9 might not.

Thus, we performed another experiment to confirm these results (Table 4 and Table 5). The same results were obtained in which ineffective nodules formed on *Rj*$_3$ cultivars and effective nodules formed on non-*Rj* and *Rj*$_4$. The I/T ratio of the Orihime (*non-Rj*) soybean cultivar was 1.00, indicating that this cultivar is strongly restricted for forming effective nodules with BLY3-8 and BLY6-1. These results confirm that the Orihime soybean cultivar harbored *Rj*$_3$ genes. In our study, Yezin-9 (*Rj*$_3$) formed functional nodules with BLY3-8 and BLY6-1. These results need to be confirm by inoculating the *B. elkanii* strains BLY3-8, BLY6-1, and USDA33 to compare nodulation and nitrogenase activities of Yezin-9 (*Rj*$_3$) and D51 (*Rj*$_3$).

We performed inoculation tests using *B. elkanii* strains BLY3-8, BLY6-1, USDA33, and USDA110 to confirm whether Orihime and Yezin-9 harbor the *Rj*$_3$ gene allele. The results of nodulation and nitrogenase activity are shown in Tables 6-9. According to inoculation results of BLY3-8, BLY6-1, and USDA33, effective nodules formed on roots of Yezin-6 (non-*Rj*) and Fukuyutaka (*Rj*$_4$).

Table 4. Detection for incompatibility of *B. elkanii* BLY3-8 with various soybean cultivars.

Cultivar (*Rj* gene)	Nodule number per plant					I/T	Incompatibility for *Rj*$_3$ gene
	Effective			Ineffective (I)	Total (T)		
	TR	LR	WR				
Yezin-6 (*non-Rj*)	4.50	4.10	8.60	0.00	8.60	0.00	–
Orihime (*non-Rj*)	0.00	0.00	0.00	10.36	10.36	1.00	+
Yezin-9 (*Rj*$_3$)	7.08	2.17	9.25	0.00	9.25	0.00	–
D51 (*Rj*$_3$)	0.00	0.08	0.08	14.00	14.08	0.99	+
Yezin-10 (*Rj*$_2$*Rj*$_3$)	0.00	0.83	0.83	6.50	7.33	0.89	+
IAC-2 (*Rj*$_2$*Rj*$_3$)	0.00	0.00	0.00	1.33	1.33	1.00	+
A250 (*Rj*$_2$*Rj*$_3$*Rj*$_4$)	0.00	0.08	0.08	0.25	0.33	0.75	+
C244 (*Rj*$_2$*Rj*$_3$*Rj*$_4$)	0.00	0.00	0.00	9.42	9.42	1.00	+

Completely randomized design was used with three replications in this experiment. The same *Rj* gene harboring cultivars were grown in a 1-L pot by dividing into two halves. TR, LR, WR: tap root, lateral root and whole root, respectively. The number indicating in table is the mean of 12 plants for all cultivars. + or – show the plants have or do not have the restriction ability for *Rj*$_3$ genes due to inoculation of incompatible strain BLY3-8 for *Rj*$_3$ gene-harbouring soybean cultivars. This experiment was conducted during May 2016.

Table 5. Detection for incompatibility of *B. elkanii* BLY6-1 with various soybean cultivars.

Cultivar (*Rj* gene)	Nodule number per plant					I/T	Incompatibility for Rj_3 gene
	Effective			Ineffective (I)	Total (T)		
	TR	LR	WR				
Yezin-6 (*non-Rj*)	2.88	4.63	7.50	0.88	8.38	0.10	–
Orihime (*non-Rj*)	0.00	0.00	0.00	7.80	7.80	1.00	+
Yezin-9 (Rj_3)	6.58	2.25	8.83	0.00	8.83	0.00	–
D51 (Rj_3)	0.00	0.00	0.00	13.45	13.45	1.00	+
Yezin-10 (Rj_2Rj_3)	0.00	0.18	0.18	3.18	3.36	0.95	+
IAC-2 (Rj_2Rj_3)	0.00	0.08	0.08	0.50	0.58	0.86	+
A250 ($Rj_2Rj_3Rj_4$)	0.00	0.00	0.00	1.42	1.42	1.00	+
C244 ($Rj_2Rj_3Rj_4$)	0.00	0.08	0.08	5.25	5.33	0.98	+

Completely randomized design was used with three replications in this experiment. The same *Rj* gene harboring cultivars were grown in a 1-L pot by dividing into two halves. TR, LR, WR: tap root, lateral root and whole root, respectively. The number indicating in table is the mean of 12 plants for all cultivars. + or – show the plants have or do not have the restriction ability for Rj_3 genes due to inoculation of incompatible strain BLY6-1 for Rj_3 gene-harbouring soybean cultivars. This experiment was conducted during May 2016.

Table 6. Detection for incompatibility of *B. elkanii* BLY3-8 with various soybean cultivars.

Cultivar (*Rj* gene)	Nodule number per plant					I/T	ARA (μmol C_2H_4 h^{-1}·$plant^{-1}$)	Incompatibility for Rj_3 gene
	Effective			Ineffective (I)	Total (T)			
	TR	LR	WR					
T201 (*rj*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NNC
Yezin-6 (*non-Rj*)	7.25	4.75	12.00	0.00	12.00	0.00	0.34	-
Orihime (*non-Rj*)	0.00	0.00	0.00	4.33	4.33	1.00	0.00	+
Fukuyutaka (Rj_4)	2.57	5.29	7.86	0.00	7.86	0.00	0.18	-
Yezin-9 (Rj_3)	7.00	2.67	9.67	0.00	9.67	0.00	0.36	-
D51 (Rj_3)	0.17	0.00	0.17	1.50	1.67	0.90	0.00	+
IAC-2 (Rj_2Rj_3)	0.29	0.00	0.29	0.86	1.14	0.75	0.04	+
A250 ($Rj_2Rj_3Rj_4$)	0.43	0.00	0.43	1.00	1.42	0.70	0.02	+

TR, LR, WR: tap root, lateral root and whole root, respectively. NNC indicated in this table is non-nodulating cultivar. The number indicating in table is the mean of 7 plants for nodulation and 3 plants for ARA value. + or – show the plants have or do not have the restriction ability for Rj_3 genes due to inoculation of incompatible strain BLY3-8 for Rj_3 gene-harbouring soybean cultivars. This experiment was conducted from October 2016 to November 2016.

Table 7. Detection for incompatibility of *B. elkanii* BLY6-1 with various soybean cultivars.

Cultivar (*Rj* gene)	Nodule number per plant					I/T	ARA (μmol C_2H_4 h^{-1}·$plant^{-1}$)	Incompatibility for Rj_3 gene
	Effective			Ineffective (I)	Total (T)			
	TR	LR	WR					
T201 (*rj*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NNC
Yezin-6 (*non-Rj*)	9.00	3.50	12.50	0.00	12.50	0.00	0.44	−
Orihime (*non-Rj*)	0.14	0.00	0.14	5.50	5.64	0.97	0.00	+
Fukuyutaka (Rj_4)	3.50	6.33	9.83	0.00	9.83	0.00	0.28	−
Yezin-9 (Rj_3)	8.29	2.14	10.43	0.00	10.43	0.00	0.14	−
D51 (Rj_3)	0.00	0.00	0.00	3.57	3.57	1.00	0.00	+
IAC-2 (Rj_2Rj_3)	0.14	0.29	0.43	1.00	1.43	0.70	0.00	+
A250 ($Rj_2Rj_3Rj_4$)	0.00	0.29	0.29	1.00	1.29	0.78	0.02	+

TR, LR, WR: tap root, lateral root and whole root, respectively. NNC indicated in this table is non-nodulating cultivar. The number indicating in table is the mean of 7 plants for nodulation and 3 plants for ARA value. + or − show the plants have or do not have the restriction ability for Rj_3 genes due to inoculation of incompatible strain BLY3-8 for Rj_3 gene-harbouring soybean cultivars. This experiment was conducted from October 2016 to November 2016.

Table 8. Detection for incompatibility of *B. elkanii* USDA33 with various soybean cultivars.

Cultivar (Rj gene)	Nodule number per plant					I/T	ARA (μmol C_2H_4 h^{-1}·$plant^{-1}$)	Incompatibility for Rj_3 gene
	Effective			Ineffective (I)	Total (T)			
	TR	LR	WR					
T201 (*rj*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NNC
Yezin-6 (*non-Rj*)	2.67	5.00	7.67	0.00	7.67	0.00	0.09	−
Orihime (*non-Rj*)	0.00	0.00	0.00	7.67	7.67	1.00	0.00	+
Fukuyutaka (Rj_4)	1.00	7.50	8.50	0.00	8.50	0.00	0.06	−
Yezin-9 (Rj_3)	1.71	0.86	2.57	0.00	2.57	0.00	0.12	−
D51 (Rj_3)	0.00	0.14	0.14	3.86	4.00	0.96	0.05	+
IAC-2 (Rj_2Rj_3)	0.14	0.29	0.43	0.71	1.14	0.63	0.00	+
A250 ($Rj_2Rj_3Rj_4$)	0.00	0.00	0.00	0.86	0.86	1.00	0.00	+

TR, LR, WR: tap root, lateral root and whole root, respectively. NNC indicated in this table is non-nodulating cultivar. The number indicating in table is the mean of 7 plants for nodulation and 3 plants for ARA value. + or − show the plants have or do not have the restriction ability for Rj_3 genes due to inoculation of incompatible strain BLY3-8 for Rj_3 gene-harbouring soybean cultivars. This experiment was conducted from October 2016 to November 2016.

Table 9. Detection for incompatibility of *B. elkanii* USDA110 with various soybean cultivars.

Cultivar (Rj gene)	Nodule number per plant					I/T	ARA (μmol C_2H_4 h^{-1} $plant^{-1}$)	Incompatibility for Rj_3 gene
	Effective			Ineffective (I)	Total (T)			
	TR	LR	WR					
T201 (*rj*)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	NNC
Yezin-6 (*non-Rj*)	8.57	6.57	15.14	0.00	15.14	0.00	0.19	-
Orihime (*non-Rj*)	10.14	3.29	13.43	0.00	13.43	0.00	0.15	-
Fukuyutaka (Rj_4)	12.67	5.50	18.17	0.00	18.17	0.00	0.30	-
Yezin-9 (Rj_3)	4.71	8.00	12.71	0.00	12.71	0.00	0.18	-
D51 (Rj_3)	7.00	10.29	17.29	0.00	17.29	0.00	0.34	-
IAC-2 (Rj_2Rj_3)	4.86	11.86	16.71	0.00	16.71	0.00	0.16	-
A250 ($Rj_2Rj_3Rj_4$)	9.00	5.43	14.43	0.00	14.43	0.00	0.21	-

TR, LR, WR: tap root, lateral root and whole root, respectively. NNC indicated in this table is non-nodulating cultivar. The number indicating in table is the mean of 7 plants for nodulation and 3 plants for ARA value. + or − show the plants have or do not have the restriction ability for Rj_3 genes due to inoculation of incompatible strain BLY3-8 for Rj_3 gene-harbouring soybean cultivars. This experiment was conducted from October 2016 to November 2016.

Remarkably, Yezin-9 (Rj_3) formed effective nodules but Orihime (non-*Rj*) did not. Nitrogenase activities were measured to confirm the results. Yezin-6 (non-*Rj*), Fukuyutaka (Rj_4), and Yezin-9 (Rj_3) formed effective nodules and induced nitrogenase activity of 0.18 - 0.36 μmol C_2H_4/hour/plant by BLY3-8, 0.14 - 0.44 μmol C_2H_4/hour/plant by BLY6-1, and 0.06 - 0.12 μmol C_2H_4/hour/plant by USDA33. Nitrogenase activities of the other cultivars harboring the Rj_3 gene were relatively low or absent in some cultivars. When inoculated with USDA110, all cultivars except T201 (*rj*1) formed effective nodules and had nitrogenase activity (Table 9). T201 (*rj*1) was a non-nodulating cultivar. It is clear that Yezin-9 did not harbor the Rj_3 gene, whereas Orihime harbored the Rj_3 gene.

4. Discussion

The *Rj*(s) and *rj*(s) soybean cultivars depend on their compatibility with *Bradyrhizobium* and *Ensifer*/*Sinorhizobium* species [9]. In our study, selected *Bradyrhizobium* strains, such as *Bradyrhizobium* spp. strains SHY3-1 and SHY6-1, *B. japonicum* strains SHY3-10 and SAY6-1, and *B. elkanii* strains BLY3-8 and BLY6-1 on different *Rj* gene-harboring cultivars were highly compatible with Yezin-6 (non-*Rj*) and Yezin-9 (Rj_3). However, these strains did not form nodules on the Yezin-10 (Rj_2Rj_3) cultivar, which is the same finding reported previously [8] in which these strains did not nodulate on roots of the CNS (Rj_2Rj_3) cultivar. Exceptionally, *B. elkanii* strains BLY3-8 and BLY6-1 were incompatible with D51 (Rj_3). This result is in line with the findings of others in which formation of functional nodules by specific *Bradyrhizobium* strains was inhibited by the *Rj* genes, such as Rj_2, Rj_3, Rj_4, and *Rfg*1 [14], [24] [25] [26] [27].

Depending on the compatibility and incompatibility between host plant and

inoculated bacteria, the host plant produce effective or ineffective nodules. Effective nodules are generally large and yellow colored nodules with red pigmentation when cross-section through nodules, and have the ability to perform nitrogenase activity and nitrogen fixation. Ineffective nodules are generally small and white colored nodules with no red pigmentation when cross-section through nodules and cannot perform nitrogen fixation. *Bradyrhizobium elkanii* strains BLY3-8 and BLY6-1 were tested twice for nodule formation on different *Rj* gene soybean cultivars. According to the inoculation results, the BLY3-8 and BLY6-1 strains were compatible with *non-Rj* and Rj_4 soybean cultivars, except Orihime (*non-Rj*), although they were incompatible with Rj_3-harboring soybean cultivars, except Yezin-9 (Rj_3). The Rj_3-harboring soybean cultivars formed ineffective nodules. Consequently, the I/T ratio was > 0.5. As an exceptional case, the I/T ratio of Orihime (*non-Rj*) soybean cultivar was 1.00. Functional nodule formation of this cultivar was strongly restricted by BLY3-8 and BLY6-1. In contrast to Orihime (non-*Rj*), Yezin-9 (Rj_3) formed functional nodules with BLY3-8 and BLY6-1. An I/T ratio > 0.5 for Rj_3-gene harboring cultivars is a criterion for detecting incompatibility with Rj_3 gene soybean cultivars [20]. Thus, *B. elkanii* strains BLY3-8 and BLY6-1 were incompatible with Rj_3-genotye soybean cultivars.

BLY3-8, BLY6-1, and USDA33 formed effective nodules and induced nitrogen fixation in Yezin-6 (non-*Rj*) and Fukuyutaka (Rj_4). However, they did not form effective nodules on Rj_3 soybean cultivars and nitrogenase activity was relatively very low or absent in some Rj_3 cultivars. All USDA110 cultivars formed effective nodules and had nitrogenase activity, except that Yezin-9 (Rj_3) formed effective nodules and Orihime (non-*Rj*) did not when inoculated with BLY3-8, BLY6-1, or USDA33. In a previous experiment [12], Yezin-9 was assumed to harbor the Rj_3 gene because only 1 - 3 nodules formed per plant when inoculated with USDA33, whereas the mean nodule number per plant was 2.57 when inoculated with USDA33. These results indicate that Yezin-9 did not harbor the Rj_3 gene, whereas Orihime did.

We clarified the BLY3-8 and BLY6-1 inoculation results by identifying Rj_3 soybean cultivars compared with USDA33 because the ability of USDA33 to form nodules and fix nitrogen was very low in all cultivars compared with those of BLY3-8 and BLY6-1. Keyser *et al.* [28] reported that USDA33 nodulates poorly and is less effective for nitrogen fixation, which is why identifying whether an Rj_3 gene soybean cultivar is Rj_3 or non-*Rj* is a problem. This study demonstrated that Yezin-9 did not harbor the Rj_3 gene as reported previously [12], whereas Orihime harbored the Rj_3 gene but not the non-*Rj* gene, as described in Ishizuka *et al.* [6].

New molecular methods have been developed to identify R_j genes, such as Rj_2, Rfg_1, and Rj_4, using cloning [15] [16] [17]. However, molecular identification of the Rj_3 gene has not been developed. Therefore, its identification is only based on inoculation testing. The finding of new strains that are incompatible with Rj_3 soybean cultivars is the first step for developing a new identification method for

Rj$_3$ at the molecular level. The draft genomes of *B. elkanii* strains BLY3-8 and BLY6-1 were reported by Htwe *et al.* [29] to identify the causal incompatibility gene with *Rj*$_3$ genotype soybeans.

5. Conclusion

This study showed that the I/T ratios of *Rj*$_3$ genotype soybeans were >0.5 and the nitrogenase activity of *Rj*$_3$ genotype soybeans was relatively low or absent due to inoculation with *B. elkanii* strains BLY3-8 and BLY6-1. We confirmed that *Bradyrhizobium elkanii* strains BLY3-8 and BLY6-1 were incompatible with *Rj*$_3$ genotype soybeans. The inoculation test results show that nodule formation by *B. elkanii* strains BLY3-8 and BLY6-1 was restricted by *Rj*$_3$ soybean cultivars. These two strains could be useful for checking the presence or absence of the *Rj*$_3$ gene in soybean cultivars in the future.

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Supplementary

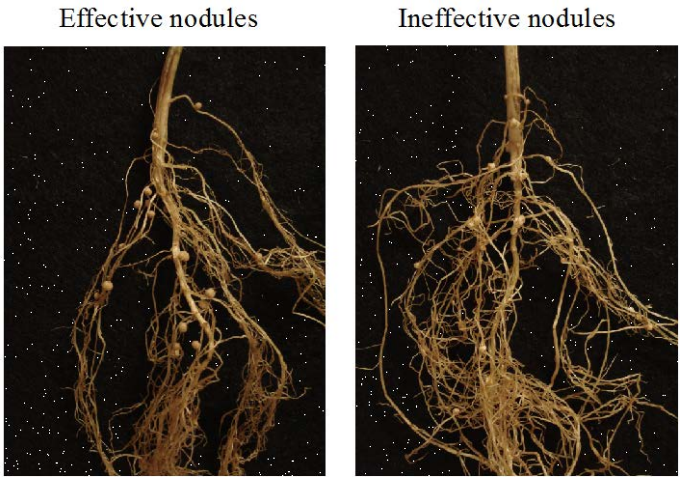

Figure S1. Effective nodules and ineffective nodules.