Identification of *Bradyrhizobium elkanii* Genes Involved in Incompatibility with Soybean Plants Carrying the *Rj4* Allele

Omar M. Faruque,^a Hiroki Miwa,^a Michiko Yasuda,^a Yoshiharu Fujii,^a Takakazu Kaneko,^b Shusei Sato,^c Shin Okazaki^a

Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan^a; Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan^b; Graduate School of Life Sciences, Tohoku University, Sendai, Japan^c

Symbioses between leguminous plants and soil bacteria known as rhizobia are of great importance to agricultural production and nitrogen cycling. While these mutualistic symbioses can involve a wide range of rhizobia, some legumes exhibit incompatibility with specific strains, resulting in ineffective nodulation. The formation of nodules in soybean plants (*Glycine max*) is controlled by several host genes, which are referred to as *Rj* genes. The soybean cultivar BARC2 carries the *Rj4* gene, which restricts nodulation by specific strains, including *Bradyrhizobium elkanii* USDA61. Here we employed transposon mutagenesis to identify the genetic locus in USDA61 that determines incompatibility with soybean varieties carrying the *Rj4* allele. Introduction of the Tn5 transposon into USDA61 resulted in the formation of nitrogen fixation nodules on the roots of soybean cultivar BARC2 (*Rj4 Rj4*). Sequencing analysis of the sequence flanking the Tn5 insertion revealed that six genes encoding a putative histidine kinase, transcriptional regulator, DNA-binding transcriptional activator, helix-turn-helix-type transcriptional regulator, phage shock protein, and cysteine protease were disrupted. The cysteine protease mutant had a high degree of similarity with the type 3 effector protein XopD of *Xanthomonas campestris*. Our findings shed light on the diverse and complicated mechanisms that underlie these highly host-specific interactions and indicate the involvement of a type 3 effector in *Rj4* nodulation restriction, suggesting that *Rj4* incompatibility is partly mediated by effector-triggered immunity.

Plants of the legume family can effectively make their own fertilizers by forming symbioses with a diverse group of nitrogen-fixing soil bacteria known as rhizobia. This cross-kingdom collaboration is characterized by the formation of the root nodule, a specialized plant organ that provides an optimized environment in which the bacteria convert atmospheric nitrogen into ammonia. The symbiotic interactions between leguminous plants and *Rhizobium* bacteria show high species specificity, which is determined by the exchange of signal molecules. The best-known determinants of host specificity derived from rhizobia are the so-called Nod factors (NFs), which comprise a family of lipochitooligosaccharides with various strain-specific chemical decorations (1). Legume roots excrete flavonoid compounds that can interact specifically with the rhizobial transcriptional factor NodD. Activation of NodD induces the expression of nodulation genes in rhizobia, which are required for the synthesis of NFs (2, 3). NFs are received by host NF receptors (NFRs) and activate host signal transduction pathways that lead to root hair infection and nodule organogenesis (2, 3). A recent study showed that introduction of the putative NFRs NFR1 and NFR5 of *Lotus japonicus* into *Medicago truncatula* initiated nodulation of the transgenic roots by the *L. japonicus* symbiont *Mesorhizobium loti* (4, 5). Thus, NFs and their receptor proteins contribute significantly to the determination of host specificity in root nodule symbiosis.

Recently, the type 3 secretion systems (T3SSs) of rhizobia have been implicated in the fine-tuning of the host range in a genotype-specific manner (6). T3SSs have been identified in a wide range of rhizobia, including *Rhizobium* sp. strain NGR234 (7), *Sinorhizobium fredii* USDA257 (8), *S. fredii* HH103 (9), *M. loti* MAFF303099 (10), *Bradyrhizobium diazoefficiens* (formerly *Bradyrhizobium japonicum*) USDA110 (11), and *Bradyrhizobium elkanii* USDA61 (12). Mutational analyses of these rhizobia have shown that the T3SS affects nodulation either positively or negatively, depending on the host plant. For example, in *B. diazoeffi-*

cians USDA110, deletion of *tts* genes led to a reduced nodule number with *Macroptilium atropurpureum* cv. Siratro and delayed nodulation with soybean (11). In *S. fredii* USDA257, the T3SS prevented rhizobial infection in the soybean cultivar McCall (13, 14). Inactivation of the T3SS affected the ability of *Rhizobium* sp. NGR234 to nodulate a range of tropical legumes (15).

Natural variation in symbiotic specificity has been well documented in soybean plants (*Glycine max*) (16). The formation of symbiotic root nodules in soybean plants is controlled by several loci, referred to as the *rj* or *Rj* loci (17). The recessive alleles *rj1* and *rj5* were identified to be putative NF receptors and are referred to as *nfr1* and *nfr5*, respectively (17–19), and *rj7* was identified to be *nts1*, which is involved in the systemic regulation of nodulation (20, 21). In contrast, the dominant alleles *Rj2*, *Rj3*, *Rj4*, and *Rfg1* have unique features that restrict nodulation with specific strains. The dominant nature of these genes is in contrast to the loss-of-function recessive alleles (*rj1*) in NFRs (18, 22, 23). *Rj2/Rfg1* restricts nodulation with *B. japonicum* USDA122 and *S. fredii* USDA257 (24, 25). Soybean varieties containing the *Rj4* allele are incapable of nodulation with *B. elkanii* USDA61 and *B. japonicum* Is-34 but compatible with *B. japonicum* USDA110 (26). USDA61

Received 10 June 2015 Accepted 13 July 2015

Accepted manuscript posted online 17 July 2015

Citation Faruque OM, Miwa H, Yasuda M, Fujii Y, Kaneko T, Sato S, Okazaki S. 2015. Identification of *Bradyrhizobium elkanii* genes involved in incompatibility with soybean plants carrying the *Rj4* allele. Appl Environ Microbiol 81:6710–6717. doi:10.1128/AEM.01942-15.

Editor: C. R. Lovell

Address correspondence to Shin Okazaki, sokazaki@cc.tuat.ac.jp.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.01942-15

TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Characteristics or sequence ^a	Reference or source
Bacterial strains		
<i>Bradyrhizobium elkanii</i>		
USDA61	Wild-type strain, Pol ^r	H. H. Keyser ^b
BErhcl	USDA61 derivative harboring an insertion in the <i>rhcJ</i> region, Pol ^r Km ^r Tc ^r	12
BE4-3	Tn5 mutant of USDA61, Pol ^r Km ^r	This study
BE6-3	Tn5 mutant of USDA61, Pol ^r Km ^r	This study
BE3-4	Tn5 mutant of USDA61, Pol ^r Km ^r	This study
BE2-1	Tn5 mutant of USDA61, Pol ^r Km ^r	This study
BE2-5	Tn5 mutant of USDA61, Pol ^r Km ^r	This study
BE7	Tn5 mutant of USDA61, Pol ^r Km ^r	This study
BE3-4S	USDA61 derivative containing a plasmid insertion, Pol ^r Km ^r Tc ^r	This study
BE2-5S	USDA61 derivative containing a plasmid insertion, Pol ^r Km ^r Tc ^r	This study
<i>Escherichia coli</i>		
DH10B	Cloning strain	Invitrogen
HB101	<i>recA hsdR hsdM pro</i> Sm ^r	Invitrogen
S17-1	<i>hsdR pro thi</i> (RP4-2 <i>km::Tn7 Tc::Mu</i> integrated into the chromosome) Sm ^r Sp ^r	52
Plasmids		
pUTKm	Transposon delivery vector, Ap ^r Km ^r	32
pRK2013	ColE1 replicon carrying RK2 transfer genes, Km ^r <i>tra</i>	53
pSUPSCAKm	Suicide vector, <i>oriT</i> of RP4, Tc ^r Km ^r	12
Oligonucleotides		
Linker 1	5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG-3'	34
Linker 2	5'-TGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT-3'	34
Y-linker primer	5'-CTGCTCGAATTCAAGCTTCT-3'	34
Tn5 primer	5'-GGCCAGATCTGATCAAGAGA-3'	34
3-4int_F	5'-AACAGCAGGTGCGAAGGTGAC-3'	This study
3-4int_R	5'-GAACTGAACGTGACGCAGAC-3'	This study
2-5int_F	5'-CGGGCGTACAGAATTAGCAT-3'	This study
2-5int_R	5'-GAAGTTACGACCGGGATCT-3'	This study

^a Pol^r, polymyxin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Ap^r, ampicillin resistant.

^b United States Department of Agriculture, Beltsville, MD.

is incompatible with carriers of the *Rj4* allele, such as soybean plants of the BARC2 cultivar, but compatible with carriers of the *rj4* allele, such as soybean plants of the BARC3 cultivar, which is a nearly isogenic line of BARC2 (27). These studies showed that *Rj4* incompatibility is strain specific and cultivar specific and appears to resemble gene-for-gene resistance in plant-pathogen interactions. This idea is supported by the finding that the *Rj2/Rfg1* gene encodes a member of the Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of proteins, which is typically involved in resistance to microbial pathogens (28).

B. elkanii fixes atmospheric nitrogen efficiently via symbiosis with soybean plants and is widely used as an inoculant for soybean plants in Brazil (29). However, *B. elkanii* USDA61 is incompatible with soybean varieties carrying the *Rj4* allele, which are found at high frequencies (>60%) in Southeast Asian countries, such as Myanmar, Indonesia, Malaysia, Thailand, and Vietnam, and at lower frequencies in northern Asia. Recently, it was reported that soybean plants carrying *Rj4* inoculated with a T3SS mutant of *B. elkanii* exhibited nodules (12). The specific genes and functional properties of *B. elkanii* that are involved in the nodulation restriction of soybean varieties carrying *Rj4* have not yet been elucidated. Here we identified the genetic loci in *B. elkanii* that regulate incompatibility with soybean varieties carrying *Rj4* through trans-

poson mutagenesis and subsequent screening of the mutants that overcame the nodulation restriction associated with the *Rj4* allele.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *B. elkanii* cultures were grown at 28°C in arabinose-gluconate (AG) medium (30) supplemented with appropriate antibiotics (50 µg/ml polymyxin and 200 µg/ml kanamycin). *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (31) supplemented with 50 µg/ml kanamycin.

Transposon mutagenesis and screening. The transposon mini-Tn5 was introduced into *B. elkanii* strain USDA61 by conjugation using *E. coli* strain S17-1 carrying the plasmid pUTKm (32) and *E. coli* HB101(pRK2013), as described by Krause et al. (11). Strains with transposon insertions were selected on AG agar plates containing 50 µg ml⁻¹ polymyxin and 200 µg ml⁻¹ kanamycin.

G. max cv. BARC2 seeds were surface sterilized with 70% ethanol for 1 min and then with 1% sodium hypochlorite for 30 s. The seeds were washed 10 times with sterilized distilled water, placed in a petri dish on wet sterile paper, and incubated at 25°C for 3 days under dark conditions. After 3 days of incubation, the germinated seedlings were sown in a plant box (CUL-JAR300; Iwaki, Tokyo, Japan) containing sterile vermiculite and watered with Broughton and Dilworth nitrogen-free plant nutrient solution (33) under a 16-h light and 8-h dark cycle in a plant growth cabinet (LPH-410SP; NK Systems Co. Ltd., Osaka, Japan). Two days after

FIG 1 *G. max* cv. BARC2 (*Rj4 Rj4*) plants inoculated with *B. elkanii* USDA61, Tn5 mutants, and T3SS mutant BERhcgj. Plants were photographed 30 days after inoculation. —, uninoculated control. Bars, 10 cm.

the transfer, the BARC2 (*Rj4 Rj4*) plants were separately inoculated with the Tn5 mutants.

After 28 days of cultivation, the nodules were sampled and immersed in 70% ethanol for 1 min, sterilized with a 1% NaOCl solution for 1 min, and rinsed with distilled water five times. The sterilized nodules were crushed in 0.5 ml of 0.9% NaCl using a sterilized toothpick. An aliquot of the crushed nodule suspension was streaked on AG medium with 200 $\mu\text{g ml}^{-1}$ kanamycin and 50 $\mu\text{g ml}^{-1}$ polymyxin and incubated for 7 days at 28°C.

Cloning and nucleotide sequence analysis of the region flanking Tn5. Genomic DNA was extracted from the Tn5 mutant strains using a Wizard genomic DNA purification kit. The sequences flanking the transposon were amplified using the Y-linker method (34). The oligonucleotides used in this experiment are shown in Table 1. The Y linker was designed to have a 3' overhang complementary to the sticky end generated by the *Nla*III restriction enzyme. The genomic DNA was digested with *Nla*III and ligated with the Y linker. The PCR was carried out using a Kapa *Taq* Extra HotStart system (Nippon Genetics Co., Tokyo, Japan). The thermal cycling conditions were as follows: initial denaturation was carried out for 2 min at 95°C, followed by 35 cycles of denaturation, annealing for 30 s at 58°C, and extension for 90 s at 68°C. After the final cycle, an extension step of 5 min at 70°C was added. The PCR products were analyzed on a 2% agarose gel and stained with ethidium bromide. The PCR fragments were sequenced directly using the Tn5-specific primer. Homology searches were carried out using the BLASTN program in RhizoBase, a genome database for rhizobia; the National Center for Biotechnology Information (NCBI) website; and an in-house database for the *B. elkanii* USDA61 genome (T. Kaneko, unpublished data).

In order to confirm that the observed phenotypes of the mutants were caused by transposon insertion and not by a secondary mutation independent of the transposon, genes encoding a putative DNA-binding transcriptional activator and cysteine protease, which were identified from BE3-4 and BE2-5, respectively, were mutated via single-crossover recombination. For this, internal regions of genes encoding a putative DNA-binding transcriptional activator and cysteine protease were amplified by PCR. The primers 3-4int_F and 3-4int_R were used to amplify the internal region of the gene encoding a putative DNA-binding transcriptional activator; the primers 2-5int_F and 2-5int_R were used to amplify the internal region of the gene encoding a putative cysteine protease. The PCR products were cloned into the vector pSUPSCAKm and were mobilized into *B. elkanii* USDA61 via conjugation as described previously (12). The mutants generated by integration of the plasmids were selected on PSY medium containing polymyxin and kanamycin. Integration of the plasmids was confirmed by PCR.

For microscopic analysis, nodules were excised from roots, immediately fixed with 0.1 M cacodylate buffer containing 2.5% glutaraldehyde, and incubated overnight at 4°C. Sections were obtained by hand using a razor blade. The nodule sections were observed using a stereoscopic microscope (SZX9; Olympus, Tokyo, Japan).

Nucleotide sequence accession numbers. The sequences of mutants BE4-3, BE6-3, BE3-4, BE2-1, BE2-5, and BE7 have been submitted to

GenBank and may be found under accession numbers [KT267565](#), [KT267566](#), [KT267564](#), [KT267562](#), [KT267563](#), and [KT267567](#), respectively.

RESULTS

Random mutagenesis using the Tn5 transposon followed by screening for nodulating mutants carrying the *Rj4* allele was performed to investigate the molecular mechanisms underlying the incompatibility of *Rj4*-carrying varieties with USDA61. About 12,000 Tn5 mutants of USDA61 were inoculated into 30 seedlings of the BARC2 cultivar, which were not nodulated by parental strain USDA61. Through a comparison with the symbiotic phenotype of wild-type strain USDA61, Tn5 mutants were isolated from the nodules formed on soybeans of BARC2. Kanamycin-resistant strains were isolated from the nodules and reinoculated into BARC2 plants to confirm the nodulation ability. Six mutants (BE4-3, BE6-3, BE3-4, BE2-1, BE2-5, and BE7) were confirmed to nodulate soybean varieties carrying the *Rj4* allele: plants inoculated with these mutant strains clearly exhibited higher nodule numbers (7 to 81 nodules per plant) than wild-type strain USDA61 (0.67 nodule per plant).

To characterize the symbiotic phenotype of these mutants, we performed inoculation tests on BARC2 and measured the nodule numbers, nodule weights, and plant fresh weights 30 days after the inoculation of Tn5 mutants (Fig. 1 and 2). Wild-type strain USDA61 formed only a small number of nodules on BARC2 plants. Symptoms of nitrogen deficiency were observed, and plant growth was severely impaired (Fig. 1). With the exception of the weak nodulation phenotype of BE6-3, all of the mutants tested were able to form mature nodules and had a plant growth-promoting effect on BARC2, suggesting efficient nitrogen-fixing activity (Fig. 1). We then performed inoculation tests with the BERhcgj mutant, which has a mutation in the essential component of the T3SS, the *rhcgj* gene, and therefore cannot secrete effector proteins. BERhcgj mutants formed nodules on BARC2 plants and promoted plant growth: the fresh weight of BERhcgj-inoculated plants was almost twice that of noninoculated control plants or USDA61-inoculated plants. This showed that the T3SS of USDA61 has a strong negative effect on the nodulation of soybean varieties carrying the *Rj4* allele.

Although all of the mutants formed nodules, they each exhibited a slightly different symbiotic phenotype. The nodules were classified into two types by size (<2 mm and ≥ 2 mm), and the number of each was scored. The BE7 mutant formed about 80 nodules per plant, which was the highest number among the Tn5 mutants (Fig. 2a). However, about 70% of the nodules were smaller than 2 mm, and the nodule weight per plant was only 0.4 g

(a) Nodule number

(b) Plant weight

(c) Nodule weight

FIG 2 Symbiotic properties of *G. max* cv. BARC2 inoculated with *B. elkanii* USDA61, Tn5 mutants, and T3SS mutant BErhcj. Nodule number (a), plant weight (b), and nodule weight (c) were measured 30 days after inoculation. The open bars and closed bars in panel a show the numbers of small nodules (<2 mm) and large nodules (≥2 mm), respectively. N.D., not determined. The values are means for triplicate samples, and the error bars indicate standard deviations. Statistical analysis (Student's *t* test) was performed by comparison of each mutant to the wild type. *, $P < 0.05$; **, $P < 0.01$.

(Fig. 2a and c). The BE3-4 mutant exhibited the strongest plant growth-promoting effect, with the plant fresh weight being 10 g (Fig. 2a to c). The BE3-4, BE2-1, and BE2-5 mutants had a greater number of large nodules than the other mutants and a higher nodule weight per plant (Fig. 2a and c). The BE6-3 mutant formed only a few nodules on BARC2 plants and had a nodule weight of only 0.3 g (Fig. 2a and b). Thus, BE6-3 had only a weak plant growth-promoting effect compared with that of the control plants (Fig. 2b).

Microscopic analysis of the nodules showed that those formed by Tn5 mutants were similar to the typical soybean nodules

formed by *B. elkanii* strains (Fig. 3). There was red coloration inside the nodules, indicative of leghemoglobin expression, which suggested efficient nitrogen-fixing activity and successful infection of Tn5 mutants (Fig. 3). The size and shape of the nodules formed by the Tn5 mutants were comparable to those of the BErhcj mutants.

To identify and characterize the genes disrupted by the Tn5 insertion, Y-linker PCR was performed using the genomic DNA of these mutants. The results showed that all of the mutants contained the Tn5 fragment and that the PCR fragment size differed for each. A BLAST search of the sequences flanking Tn5 revealed that they were all present in the complete genome of *B. elkanii* USDA61, which has been completely sequenced (Kaneko et al., unpublished). Among the mutants, the Tn5 insertions were located in different regions of the USDA61 genome. The BE4-3 mutant had a Tn5 insertion in the 2,049-bp open reading frame (ORF) encoding histidine kinase which showed similarity to the *Xanthomonas axonopodis* pv. citri cvgSY gene (GenBank accession no. AAM36539.1) (Table 2). BE6-3 had a Tn5 insertion in the 1,098-bp ORF encoding a transcriptional regulator which had 38% identity with the *nwsB* gene of *Agrobacterium radiobacter* (GenBank accession no. ACM30865.1). BE3-4 contained a Tn5 insertion in the 900-bp ORF encoding a DNA-binding transcriptional regulator which had 58% identity with the *gcvA* gene of *Pseudomonas fluorescens* (GenBank accession no. AEV63066.1). BE2-1 carried a mutation in the 1,131-bp ORF encoding a phage shock protein, and BE7 carried a mutation in the 171-bp ORF encoding a transcriptional regulatory protein (Table 2). In the BE2-5 mutant, a Tn5 insertion was found in the 3,987-bp ORF encoding cysteine protease (Table 2); notably, this gene had 26% identity with *Xanthomonas campestris* pv. vesicatoria *xopD* (GenBank accession no. CAJ22068.1), which is known to be a type 3 effector protein. Unfortunately, we could not isolate any mutants with mutations in genes related to the T3SS machinery.

In order to confirm that the observed phenotypes of the mutants were caused by transposon insertion and not by a secondary mutation independent of the transposon, we reintroduced the mutations into the wild-type background. Genes encoding a putative DNA-binding transcriptional activator and cysteine protease, which were identified from BE3-4 and BE2-5, respectively, were mutated via single-crossover recombination. The newly constructed mutants, designated BE3-4S and BE2-5S, respectively, were inoculated on BARC2 plants, and their symbiotic phenotypes were compared with the symbiotic phenotype of wild-type strain USDA61. As a result, both BE3-4S and BE2-5S formed numerous mature nodules like the transposon mutants did, while wild-type strain USDA61 formed only a small number of nodules (Fig. 2). These results confirm that the genes with transposon insertions were responsible for the altered nodulation phenotypes.

DISCUSSION

This study used Tn5 mutagenesis and screening for mutants compatible with soybean plants carrying the *Rj4* allele to investigate the molecular mechanism of incompatibility with soybean plants carrying *Rj4*. *Rj4* is a dominant allele that discriminates groups of rhizobia and determines host specificity in a strain-specific (or serogroup-specific) manner (16). This is in contrast to the nodulation restriction mechanism of the recessive *rj1* allele, in which rhizobia cannot activate the host signaling pathway due to a lack of

FIG 3 Roots and nodules of *G. max* cv. BARC2 inoculated with *B. elkanii* USDA61, Tn5 mutants, and T3SS mutant BErhcj. Roots were photographed 30 days after inoculation. Bars, 1 cm (root) and 1 mm (nodule and section).

NFRs. The incompatibility with soybean plants carrying the *Rj4* allele appears to be more similar to gene-for-gene resistance in plant-pathogen interactions. We speculated that rhizobia might be able to activate NF signaling in host plants but are rejected due to a strong defense response induced by factors acting as negative regulators of infection. The successful isolation of mutants compatible with soybean plants carrying *Rj4* in the current study supports our speculation that inactivation by a Tn5 insertion abolishes the production or secretion of such negative factors in *Rj4* incompatibility.

T3SSs play important roles in plant-pathogen interactions. Effector proteins secreted by T3SSs can interfere with host signaling pathways and suppress host defense responses. However, once

effector proteins are recognized by host plants, a defense response called effector-triggered immunity is induced and eventually eradicates the bacterial infection. In the current study, the T3SS mutant BErhcj was able to form nodules on soybean plants of the BARC2 cultivar. This suggests that an as-yet-unidentified rhizobial effector protein secreted by the T3SS induces effector-triggered immunity in BARC2 and prevents wild-type strain USDA61 from infecting the plants and forming nodules. Similarly T3SS-mediated incompatibility has been found in the interaction between soybean plants carrying the *Rj2* allele and *B. japonicum* USDA122 (35).

Although there were phenotypic variations among the mutants compatible with soybean plants carrying *Rj4*, they all formed ma-

TABLE 2 Results of BLAST searches of Tn5 flanking sequences

Strain	GenBank accession no.	Gene with Tn5 insertion		Strain and locus tag (GenBank accession no., % identity by BLASTP analysis)
		Length (bp)	Deduced gene product	
BE4-3	KT267565	2,049	Histidine kinase	<i>Bradyrhizobium diazoefficiens</i> USDA110 blr0516 (BAC45781.1 , 66%), <i>B. japonicum</i> E109 RN69_02365 (AJA59390.1 , 65%), <i>Bradyrhizobium</i> sp. strain ORS278 BRADO0236 (CAL74199.1 , 65%), <i>B. japonicum</i> USDA6 BJ6T_04860 (BAL05784.1 , 65%), <i>X. axonopodis</i> pv. citri 306 XAC1672 (AAM36539.1 , 40%) <i>R. etli</i> CFN42 RHF_CHO1536 (ABC90339.1 , 39%)
BE6-3	KT267566	1,098	Transcriptional regulator	<i>Methylobacterium nodulans</i> Mnod_6522 (ACL61291.1 , 42%), <i>B. diazoefficiens</i> USDA110 blr2746 (BAC48011.1 , 45%), <i>Sinorhizobium meliloti</i> AK83 Sinme_5303 (AEG56916.1 , 34%), <i>Methylobacterium oryzae</i> MOC_6064 (AIQ93819.1 , 35%), <i>Agrobacterium radiobacter</i> SSDB Arad_9929 (ACM30865.1 , 38%)
BE3-4	KT267564	900	DNA-binding transcriptional activator	<i>B. diazoefficiens</i> USDA110 blr7904 (BAC53169.1 , 87%), <i>Bradyrhizobium</i> sp. strain S23321 S23_08810 (BAL74101.1 , 86%), <i>B. japonicum</i> USDA6 BJ6T_85940 (BAL13836.1 , 86%), <i>Pseudomonas fluorescens</i> F113 PSF113_3064 (AEV63066.1 , 58%), <i>Pantoea vagans</i> Pvag_2480 (ADO10644.1 , 42%)
BE2-1	KT267562	1,131	Phage shock protein	<i>Rhizobium leguminosarum</i> bv. trifolii CB782 RLEG12_25645 (AHG46413.1 , 56%), <i>R. leguminosarum</i> bv. trifolii WSM2304 Rleg2_2880 (ACI56148.1 , 55%), <i>B. japonicum</i> E109 RN69_25795 (AJA63358.1 , 52%), <i>B. diazoefficiens</i> USDA110 blr4468 (BAC49733.1 , 50%), <i>Salmonella enterica</i> SeD_A1642 (ACH74367.1 , 27%)
BE2-5	KT267563	3,987	Cysteine protease	<i>B. japonicum</i> USDA6 BJ6T_88360 (BAL14078.1 , 51%), <i>B. diazoefficiens</i> USDA110 blr8244 (BAC53509.1 , 51%), <i>Xanthomonas campestris</i> pv. vesicatoria XCV0437 (CAJ22068.1 , 26%), <i>S. fredii</i> HH103 SFHH103_04358 (CCE98838.1 , 54%), <i>X. campestris</i> pv. campestris ATCC 33913 XCC2896 (AAM42168.1 , 29%)
BE7	KT267567	171	Helix-turn-helix-type transcriptional regulator	<i>Rhodopseudomonas palustris</i> TIE-1 Rpal_3493 (ACF01994.1 , 54%), <i>R. palustris</i> CGA009 RPA3083 (CAE28524.1 , 55%), <i>S. fredii</i> USDA 257 USDA257_c56150 (AFL54130.1 , 76%), <i>R. palustris</i> BisB5 RPD_2990 (ABE40216.1 , 39%)

ture red nodules similar to those produced by *B. japonicum* strains compatible with soybean plants carrying *Rj4*, and they all promoted plant growth. These observations suggest that the mutants were capable of successful infection and that efficient nitrogen fixation was taking place within the nodules. It is therefore likely that a single-layer genetic barrier exists in the incompatibility between soybean plants carrying *Rj4* and USDA61: the inactivation of genes associated with the rhizobial negative factor allowed the mutants to overcome the nodulation restriction conferred by *Rj4* and successfully achieve infection and nodule development. A similar mechanism was reported previously for incompatibility with soybean plants carrying *Rj2*, wherein the inactivation of genes by a Tn5 insertion allowed *B. japonicum* Is-1 to form fully developed nitrogen-fixing nodules on soybean plants carrying *Rj2* (36).

Sequencing analysis revealed that genes with various functions were involved in incompatibility with soybean plants carrying *Rj2* (Table 2). Notably, these clearly differed from the *B. japonicum* Is-1 genes involved in incompatibility with soybean plants carrying *Rj2* (36). This suggests that the molecular mechanisms underlying incompatibility with soybean plants carrying *Rj2* and *Rj4* might differ, although T3SSs have been suggested to determine both types of nodulation restriction (12, 35).

The BE4-3 mutant carried a Tn5 insertion in the gene encoding histidine kinase, which shared similarity with *fixL* of *Rhizobium etli* CFN42 (39% identity). *R. etli* CFN42 FixL acts as an oxygen sensor and provides the regulatory network for *fix* and *nif* gene expression under symbiotic and free-living microaerobic conditions (37). FixL regulates the expression of hundreds of diverse genes under symbiotic conditions (38). It might also affect the expression of the negative factor in incompatibility with soybean plants carrying *Rj4*.

BE6-3 had a mutation in the gene encoding a transcriptional regulator, which shared similarity with the *nwsB* gene of *Agrobacterium radiobacter* (38% identity). The role of rhizobial NwsB appears to extend to either the activation or repression of the nodulation genes (39). *B. japonicum* *nwsB* is a functional homolog of *nodW*, which is essential for the nodulation of cowpea, siratro, and mung bean, but not soybean (39). The NodVW two-component system provides an alternative pathway for *nod* gene activation in NodD1 mutants, which are still able to nodulate host plants (40–42). The redundancy of *nodW* and its functional homolog, *nwsB*, in *B. japonicum* might explain the weak nodulation phenotype of BE6-3, implying that the lack of *nwsB* might be partially complemented by *nodW*.

The BE3-4 mutant contained a Tn5 insertion in a DNA-binding transcriptional activator which shared similarity with *gcvA* of *Pseudomonas fluorescens* (58% identity). *gcvA* is the transcriptional regulator of the glycine cleavage system (GCV) (43). Glycine or functioning C₁ metabolism is essential for the effective nodulation of soybean by *B. japonicum* (44). Notably, *S. fredii* USDA257 failed to nodulate soybean cultivar McCall, while the *gcv* mutant of USDA257 was able to form nitrogen-fixing nodules on host plants (45). This suggests that the GCV might have various effects on C₁ metabolism and alter some essential cell functions, resulting in the modification or synthesis of signal molecules that might regulate soybean cultivar specificity (45).

In the BE2-5 mutant strain, Tn5 was inserted into the gene encoding cysteine protease, which shared similarity with *xopD* of *Xanthomonas campestris* (29% identity). XopD acts as a T3S effec-

tor and has weak ubiquitin-like modifier (SUMO) isopeptidase activity (46). Overexpression of *xopD* from *Xanthomonas* in *Ara-bidopsis* leaves induces a host defense response that is dependent on salicylic acid (47). This suggests that the cysteine protease of *B. elkanii* USDA61 identified in the current study might be a type 3 effector and stimulate the plant immune response, eventually leading to nodule restriction in soybean varieties carrying the *Rj4* allele.

Recent genetic studies in soybean plants revealed that *Rj4* encodes thaumatin-like proteins (17). These proteins have been reported to have various biological functions, including antifungal activity; β -1,3-glucan binding and digestion activity; inhibition of xylanase, α -amylase, and trypsin; and protection from abiotic stresses, including cold, salinity, and drought (48). In addition, several thaumatin-like proteins are capable of binding proteins, such as *Saccharomyces cerevisiae* glycoproteins and G-protein-coupled receptors, or binding hormones, like cytokinins (48). It remains unclear how the *Rj4* protein restricts nodulation with strains incompatible with soybean plants carrying the *Rj4* allele and how *Rj4* interferes with the rhizobial genetic components identified in the current study. Host specificity is largely determined by NF, but rhizobial surface polysaccharides are also involved in the modulation of the host range (49–51). Rhizobial surface polysaccharides, such as exopolysaccharides, lipopolysaccharides, and cyclic glucans, have been implicated in facilitating infection thread formation and nodule development (51). It has been proposed that surface polysaccharides play a role in the evasion or suppression of host defense responses, a feature that is shared by pathogenic and symbiotic bacteria (49–51). Thus, one can speculate that *Rj4* might recognize rhizobial cell surface components, such as extracellular polysaccharides, or recognize effector proteins directly when coming into contact on the root surface with a strain incompatible with soybean plants carrying *Rj4*.

Our mutant screening and sequencing analysis identified several genetic components of USDA61 involved in incompatibility with soybean plants carrying the *Rj4* allele and suggests that diverse and complicated mechanisms might underlie these highly host-specific interactions. We also revealed that T3SS mutants and putative type 3 effector mutants could overcome nodulation restriction in soybean plants carrying *Rj4*, implying that incompatibility with soybean plants carrying *Rj4* is partly mediated by effector-triggered immunity. Further analysis of mutants compatible with soybean plants carrying *Rj4* and T3SSs will improve our understanding of the intraspecies host specificity in legume-rhizobium interactions.

ACKNOWLEDGMENTS

We thank T. Devine (Agricultural Research Service, United States Department of Agriculture, Beltsville, MD, USA) for kindly providing soybean seeds. We thank K. Suzuki for her kind help.

This work was supported in part by a grant-in-aid (number 25450082) to S.O.

REFERENCES

1. Lerouge P, Roche P, Faucher C, Maillat F, Truchet G, Promé JC, Dénarié J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* 344:781–784. <http://dx.doi.org/10.1038/344781a0>.
2. Cullimore JV, Ranjeva R, Bono JJ. 2001. Perception of lipo-chitooligosaccharidic Nod factors in legumes. *Trends Plant Sci* 6:24–30. [http://dx.doi.org/10.1016/S1360-1385\(00\)01810-0](http://dx.doi.org/10.1016/S1360-1385(00)01810-0).

3. Radutoiu S, Madsen LH, Madsen EB, Jurkiewicz A, Fukai E, Quistgaard EMH, Albrechtsen AS, James EK, Thirup S, Stougaard J. 2007. LysM domains mediate lipochitin-oligosaccharide recognition and *Nfr* genes extend the symbiotic host range. *EMBO J* 26:3923–3935. <http://dx.doi.org/10.1038/sj.emboj.7601826>.
4. Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R. 2003. LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302:630–633. <http://dx.doi.org/10.1126/science.1090074>.
5. Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Grønlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425:585–592. <http://dx.doi.org/10.1038/nature02039>.
6. Deakin WJ, Broughton WJ. 2009. Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. *Nat Rev Microbiol* 7:312–320. <http://dx.doi.org/10.1038/nrmicro2091>.
7. Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. *Nature* 387:394–401. <http://dx.doi.org/10.1038/387394a0>.
8. Krishnan HB, Lorio J, Kim WS, Jiang G, Kim KY, DeBoer M, Pueppke SG. 2003. Extracellular proteins involved in soybean cultivar-specific nodulation are associated with pilus-like surface appendages and exported by a type III protein secretion system in *Sinorhizobium fredii* USDA257. *Mol Plant Microbe Interact* 16:617–625. <http://dx.doi.org/10.1094/MPMI.2003.16.7.617>.
9. De Lyra MDCCP, López-Baena FJ, Madinabeitia N, Vinardell JM, Espuny MDR, Cubo MT, Bellogin RA, Ruiz-Sainz JE, Ollero FJ. 2006. Inactivation of the *Sinorhizobium fredii* HH103 *rhcJ* gene abolishes nodulation outer proteins (Nops) secretion and decreases the symbiotic capacity with soybean. *Int Microbiol* 9:125–133.
10. Okazaki S, Okabe S, Higashi M, Shimoda Y, Sato S, Tabata S, Hashiguchi M, Akashi R, Göttfert M, Saeki K. 2010. Identification and functional analysis of type III effector proteins in *Mesorhizobium loti*. *Mol Plant Microbe Interact* 23:223–234. <http://dx.doi.org/10.1094/MPMI-23-2-0223>.
11. Krause A, Doerfel A, Göttfert M. 2002. Mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. *Mol Plant Microbe Interact* 15:1228–1235. <http://dx.doi.org/10.1094/MPMI.2002.15.12.1228>.
12. Okazaki S, Zehner S, Hempel J, Lang K, Göttfert M. 2009. Genetic organization and functional analysis of the type III secretion system of *Bradyrhizobium elkanii*. *FEMS Microbiol Lett* 295:88–95. <http://dx.doi.org/10.1111/j.1574-6968.2009.01593.x>.
13. Meinhardt L, Krishnan H, Balatti P, Pueppke S. 1993. Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. *Mol Microbiol* 9:17–29. <http://dx.doi.org/10.1111/j.1365-2958.1993.tb01665.x>.
14. Bellato C, Krishnan H, Cubo T, Temprano F, Pueppke S. 1997. The soybean cultivar specificity gene *noIX* is present, expressed in a *nodD*-dependent manner, and of symbiotic significance in cultivar-nonspecific strains of *Rhizobium* (*Sinorhizobium*) *fredii*. *Microbiology* 143:1381–1388. <http://dx.doi.org/10.1099/00221287-143-4-1381>.
15. Viprey V, Del Greco A, Golinowski W, Broughton WJ, Perret X. 1998. Symbiotic implications of type III protein secretion machinery in *Rhizobium*. *Mol Microbiol* 28:1381–1389. <http://dx.doi.org/10.1046/j.1365-2958.1998.00920.x>.
16. Devine TE, Kuykendall LD. 1996. Host genetic control of symbiosis in soybean (*Glycine max* L.). *Plant Soil* 186:173–187. <http://dx.doi.org/10.1007/BF00035072>.
17. Hayashi M, Saeki Y, Haga M, Harada K, Kouchi H, Umehara Y. 2012. *Rj* (*rj*) genes involved in nitrogen-fixing root nodule formation in soybean. *Breed Sci* 61:544–553. <http://dx.doi.org/10.1270/jsbbs.61.544>.
18. Indrasumunar A, Kereszt A, Searle I, Miyagi M, Li D, Nguyen CDT, Men A, Carroll BJ, Gresshoff PM. 2010. Inactivation of duplicated Nod factor receptor 5 (NFR5) genes in recessive loss-of-function non-nodulation mutants of allotetraploid soybean (*Glycine max* L. Merr.). *Plant Cell Physiol* 51:201–214. <http://dx.doi.org/10.1093/pcp/pcp178>.
19. Indrasumunar A, Searle I, Lin MH, Kereszt A, Men A, Carroll BJ, Gresshoff PM. 2011. Nodulation factor receptor kinase *lxa* controls nodule organ number in soybean (*Glycine max* L. Merr.). *Plant J* 65:39–50. <http://dx.doi.org/10.1111/j.1365-3113.2010.04398.x>.
20. Searle IR, Men AE, Laniya TS, Buzas DM, Iturbe-Ormaetxe I, Carroll BJ, Gresshoff PM. 2003. Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. *Science* 299:109–112. <http://dx.doi.org/10.1126/science.1077937>.
21. Nishimura R, Hayashi M, Wu G-J, Kouchi H, Imaizumi-Anraku H, Murakami Y, Kawasaki S, Akao S, Ohmori M, Nagasawa M, Harada K, Kawaguchi M. 2002. HAR1 mediates systemic regulation of symbiotic organ development. *Nature* 420:426–429. <http://dx.doi.org/10.1038/nature01231>.
22. Zhukov V, Radutoiu S, Madsen LH, Rychagova T, Ovchinnikova E, Borisov A, Tikhonovich I, Stougaard J. 2008. The pea *Sym37* receptor kinase gene controls infection-thread initiation and nodule development. *Mol Plant Microbe Interact* 21:1600–1608. <http://dx.doi.org/10.1094/MPMI-21-12-1600>.
23. Stacey G. 1995. *Bradyrhizobium japonicum* nodulation genetics. *FEMS Microbiol Lett* 127:1–9. <http://dx.doi.org/10.1111/j.1574-6968.1995.tb07441.x>.
24. Trese AT. 1995. A single dominant gene in McCall soybean prevents effective nodulation with *Rhizobium fredii* USDA257. *Euphytica* 81:279–282. <http://dx.doi.org/10.1007/BF00025618>.
25. Caldwell B. 1966. Inheritance of a strain-specific ineffective nodulation in soybeans. *Crop Sci* 6:427–428. <http://dx.doi.org/10.2135/cropsci1966.0011183X000600050010x>.
26. Sadowsky MJ, Cregan PB. 1992. The soybean *Rj4* allele restricts nodulation by *Bradyrhizobium japonicum* serogroup 123 strains. *Appl Environ Microbiol* 58:720–723.
27. Devine TE, O'Neill JJ, Kuykendall LD. 1993. Near isogenic lines of soybeans as tools to identify nodulation specific mutants of *Bradyrhizobium elkanii*. *Plant Soil* 149:205–209. <http://dx.doi.org/10.1007/BF00016610>.
28. Yang S, Tang F, Gao M, Krishnan HB, Zhu H. 2010. R gene-controlled host specificity in the legume-rhizobia symbiosis. *Proc Natl Acad Sci U S A* 107:18735–18740. <http://dx.doi.org/10.1073/pnas.1011957107>.
29. Rumjanek NG, Dobert RC, Van Berkum P, Triplett EW. 1993. Common soybean inoculant strains in Brazil are members of *Bradyrhizobium elkanii*. *Appl Environ Microbiol* 59:4371–4373.
30. Sadowsky MJ, Tully RE, Cregan PB, Keyser HH. 1987. Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybean. *Appl Environ Microbiol* 53:2624–2630.
31. Green MR, Sambrook J. 2012. Molecular cloning: a laboratory manual, 4th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. Herrero M, De Lorenzo V, Timmis KN. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* 172:6557–6567.
33. Broughton WJ, Dilworth MJ. 1971. Control of leghaemoglobin synthesis in snake beans. *Biochem J* 125:1075–1080.
34. Kwon YM, Rieke SC. 2000. Efficient amplification of multiple transposon-flanking sequences. *J Microbiol Methods* 41:195–199. [http://dx.doi.org/10.1016/S0167-7012\(00\)00159-7](http://dx.doi.org/10.1016/S0167-7012(00)00159-7).
35. Tsukui T, Eda S, Kaneko T, Sato S, Okazaki S, Kakizaki-Chiba K, Itakura M, Mitsui H, Yamashita A, Terasawa K, Minamisawa K. 2013. The type III secretion system of *Bradyrhizobium japonicum* USDA122 mediates symbiotic incompatibility with *Rj2* soybean plants. *Appl Environ Microbiol* 79:1048–1051. <http://dx.doi.org/10.1128/AEM.03297-12>.
36. Tsurumaru H, Yamakawa T, Tanaka M, Sakai M. 2008. Tn5 mutants of *Bradyrhizobium japonicum* Is-1 with altered compatibility with *Rj2*-soybean cultivars. *Soil Sci Plant Nutr* 54:197–203. <http://dx.doi.org/10.1111/j.1747-0765.2007.00225.x>.
37. David M, Davaan M-L, Batut J, Dedieu A, Domergue O, Ghai J, Hertig C, Boistard P, Kahn D. 1988. Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. *Cell* 54:671–683. [http://dx.doi.org/10.1016/S0092-8674\(88\)80012-6](http://dx.doi.org/10.1016/S0092-8674(88)80012-6).
38. Dixon R, Kahn D. 2004. Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol* 2:621–631. <http://dx.doi.org/10.1038/nrmicro954>.
39. Loh J, Stacey G. 2003. Nodulation gene regulation in *Bradyrhizobium japonicum*: a unique integration of global regulatory circuits. *Appl Environ Microbiol* 69:10–17. <http://dx.doi.org/10.1128/AEM.69.1.10-17.2003>.
40. Grob P, Hennecke H, Göttfert M. 1994. Cross-talk between the two-component regulatory systems NodVW and NwsAB of *Bradyrhizobium japonicum*. *FEMS Microbiol Lett* 120:349–353. <http://dx.doi.org/10.1111/j.1574-6968.1994.tb07057.x>.
41. Göttfert M, Holzhäuser D, Bani D, Hennecke H. 1992. Structural and functional analysis of two different *nodD* genes in *Bradyrhizobium japonicum* USDA110. *Mol Plant Microbe Interact* 5:257–265. <http://dx.doi.org/10.1094/MPMI-5-257>.

42. Göttfert M, Lamb JW, Gasser R, Semenza J, Hennecke H. 1989. Mutational analysis of the *Bradyrhizobium japonicum* common *nod* genes and further *nod* box-linked genomic DNA regions. *Mol Gen Genet* 215:407–415. <http://dx.doi.org/10.1007/BF00427037>.
43. Stauffer LT, Stauffer GV. 2005. GcvA interacts with both the α and σ subunits of RNA polymerase to activate the *Escherichia coli* *gcvB* gene and the *gcvTHP* operon. *FEMS Microbiol Lett* 242:333–338. <http://dx.doi.org/10.1016/j.femsle.2004.11.027>.
44. Rossbach S, Hennecke H. 1991. Identification of *glyA* as a symbiotically essential gene in *Bradyrhizobium japonicum*. *Mol Microbiol* 5:39–47. <http://dx.doi.org/10.1111/j.1365-2958.1991.tb01824.x>.
45. Lorio JC, Kim WS, Krishnan AH, Knshnan HB. 2010. Disruption of the glycine cleavage system enables *Sinorhizobium fredii* USDA257 to form nitrogen-fixing nodules on agronomically improved North American soybean cultivars. *Appl Environ Microbiol* 76:4185–4193. <http://dx.doi.org/10.1128/AEM.00437-10>.
46. Kim JG, Taylor KW, Mudgett MB. 2011. Comparative analysis of the XopD type III secretion (T3S) effector family in plant pathogenic bacteria. *Mol Plant Pathol* 12:715–730. <http://dx.doi.org/10.1111/j.1364-3703.2011.00706.x>.
47. Tan CM, Li M-Y, Yang P-Y, Chang SH, Ho Y-P, Lin H, Deng W-L, Yang J-Y. 2015. *Arabidopsis* HFR1 is a potential nuclear substrate regulated by the *Xanthomonas* type III effector XopD_{Xcc8004}. *PLoS One* 10: e0117067. <http://dx.doi.org/10.1371/journal.pone.0117067>.
48. Liu JJ, Sturrock R, Ekramoddoullah AKM. 2010. The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. *Plant Cell Rep* 29:419–436. <http://dx.doi.org/10.1007/s00299-010-0826-8>.
49. D’Haeze W, Holsters M. 2004. Surface polysaccharides enable bacteria to evade plant immunity. *Trends Microbiol* 12:555–561. <http://dx.doi.org/10.1016/j.tim.2004.10.009>.
50. Soto MJ, Domínguez-Ferreras A, Pérez-Mendoza D, Sanjuán J, Olivares J. 2009. Mutualism versus pathogenesis: the give-and-take in plant-bacteria interactions. *Cell Microbiol* 11:381–388. <http://dx.doi.org/10.1111/j.1462-5822.2009.01282.x>.
51. Jones KM, Sharopova N, Lohar DP, Zhang JQ, VandenBosch KA, Walker GC. 2008. Differential response of the plant *Medicago truncatula* to its symbiont *Sinorhizobium meliloti* or an exopolysaccharide-deficient mutant. *Proc Natl Acad Sci U S A* 105:704–709. <http://dx.doi.org/10.1073/pnas.0709338105>.
52. Simon R, Priefer U, Puhler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Nat Biotechnol* 1:784–791. <http://dx.doi.org/10.1038/nbt1183-784>.
53. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA* 76:1648–1652. <http://dx.doi.org/10.1073/pnas.76.4.1648>.