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The Efficient Strategy of Plasmid Rescue from Tn*5* Mutants Derived from *Bradyrhizobium japonicum* Is–1, Based on Whole Genome Sequence Information of Strain USDA110

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We previously reported that Tn*5* mutants derived from *Bradyrhizobium japonicum* Is–1 were isolated. In this report, Tn*5*–encoded kanamycin resistance cassette and its flanking sequence were concurrently cloned into cosmid vector from Tn*5* mutants. In general, the genomic southern blot analysis is often performed to select appropriate restriction enzyme before performing the plasmid rescue from cosmid clone. Therefore plasmid rescue experiment consumes the time, especially when more than one Tn*5* mutant was obtained. To improve this situation, tentative restriction map of strain Is–1 was constructed based on whole genome sequence information of strain USDA110. Plasmid rescue was performed with restriction enzymes selected according to this map and these enzymes were appropriate for plasmid rescue. This strategy is thought to be effective for saving the time of plasmid rescue in *B. japonicum* strains other than USDA110.

INTRODUCTION

Transposon mutagenesis is a powerful strategy to identify the novel gene. In rhizobia, Tn*5* mutagenesis has been used for the identifications of *nod*, *fix* and *nif* genes (Deshmane and Stacey 1989; Rossen *et al*., 1984; Corbin *et al*., 1982). Once Tn*5* mutant is isolated, it is necessary to determine the Tn*5*–falanking sequence to deduce the gene product disrupted by Tn*5* insertion. When both a kanamycine resistant gene and its flanking sequence were cloned from Tn*5* mutant, "rescue" method (Guilhabert *et al*., 2001) is often used. The rescue method consists of a four–step procedure: 1) the genomic cosmid library of Tn*5* mutant is constructed; 2) a cosmid clone containing the Tn*5* is selected with Kanamycin–containing medium; 3) a cosmid is digested with appropriate restriction enzyme and digested–DNA fragment containing the Tn*5* is cloned into plasmid vector; 4) constructed plasmid is sequenced to determine the Tn*5*–flanking sequence. The plasmid rescue experiment consumes time, especially when more than one mutant was obtained. Actually we obtained more than one Tn*5* mutant of *Bradyrhizobium japonicum* Is–1 with altered compatibility with Rj_2–soybean cultivars (Tsurumaru *et al*., 2008). Though the change of host range was common to these Tn*5* mutants, Tn*5* insertion sites were different from each other and all homologous sequences to Tn*5*–flanking sequence were found in the complete genome sequence of *B. japonicum* USDA 110. In cases like this, PCR–based method to identify the gene disrupted by Tn*5* is efficient (Kwon and Ricke, 2000). However PCR–based method has sometimes disadvantage such as that there is amplification error (Cline *et al*., 1996). Because the complete genome sequences of *B. japonicum* strains other than USDA110 are not determined, it is not able to confirm the accuracy of sequence of PCR products in these strains. In Tn*5* mutants of strain Is–1, it is preferable to use conventional approach such as plasmid rescue, which is not based on PCR method, for determining the complete sequence of the gene disrupted by Tn*5*.

In this report, we constructed the tentative restriction map that contains Tn*5* insertion site of strain Is–1, based on whole genome sequence of *B. japonicum* strain USDA110 (Kaneko *et al*., 2002). According to this map, the appropriate restriction enzyme was selected for digesting the cosmid containing the Tn*5* and recloning this DNA fragment into plasmid vector. This strategy saves the time in the third–step procedure (described above) of the rescue method.

MATERIALS AND METHOD

Bacterial strains and plasmids

Bacterial strains, cosmids and plasmids used in this study were described in Table 1. Tn*5* mutants of *B. japonicum* strain Is–1 were grown on HM salt medium (Cole and Elkan, 1973) supplemented with 0.1% arabinose at 30 °C. *E. coli* strains were grown on Luria–Bertani medium (Sambrook *et al*., 1989) at 37 °C. Antibiotics were added to the media at the following final concentrations: kanamycin at 50 μg mL^{-1} for Tn*5* mutant and ampicillin at 100 μg mL^{-1} and kanamycin at 50 μg mL^{-1} for *E. coli* harboring Tn*5*–containing cosmid or plasmid clone (Table 1).

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Table 1. Bacterial strains, cosmids and plasmids used in this study

Strain and plasmid	Relevant characteristics*1	Reference or source
B. japonicum		
Is–1	incompatible with Rj_3Rj_3 soybean and compatible Rj_4 one	Ishizuka *et al.* (1991)
1C1	Is–1::Tn*5*, Km^r	Tsurumaru *et al.* (2008)
1C2	Is–1::Tn*5*, Km^r	Tsurumaru *et al.* (2008)
5C1	Is–1::Tn*5*, Km^r	Tsurumaru *et al.* (2008)
6C1	Is–1::Tn*5*, Km^r	Tsurumaru *et al.* (2008)
7C1	Is–1::Tn*5*, Km^r	Tsurumaru *et al.* (2008)
7C2	Is–1::Tn*5*, Km^r	Tsurumaru *et al.* (2008)
10C1	Tn*5*–induced spontaneous mutant, Km^r	Tsurumaru *et al.* (2008)
10C2	Is–1::Tn*5*, Km^r	Tsurumaru *et al.* (2008)
E. coli		
JM109	*rec*A1 ; cloning strain	Takara Bio Inc., Shiga, Japan
Cosmid		
SuperCos I	constructing SuperCos I–Neo, Ap^r, Km^r	Stratagene, CA, USA
SuperCos I–Neo	cloning of partially digested genomic DNAs, Ap^r	This study
pSUP1C1	genomic DNA of 1C1 is cloned, Ap^r, Km^r	This study
pSUP1C2	genomic DNA of 1C2 is cloned, Ap^r, Km^r	This study
pSUP5C1	genomic DNA of 5C1 is cloned, Ap^r, Km^r	This study
pSUP6C1	genomic DNA of 6C1 is cloned, Ap^r, Km^r	This study
pSUP7C1	genomic DNA of 7C1 is cloned, Ap^r, Km^r	This study
pSUP7C2	genomic DNA of 7C2 is cloned, Ap^r, Km^r	This study
pSUP10C2	genomic DNA of 10C2 is cloned, Ap^r, Km^r	This study
Plasmid		
pGEM–3Zf(−)	Subcloning vector, Ap^r	Promega, MA, USA
pGEM–5Zf(+)	Subcloning vector, Ap^r	Promega, MA, USA
pGApa1C1	6.0–kbp *Apa* I fragment of pSUP1C1–1 is subcloned, Ap^r, Km^r	This study
pGH1C2	4.5–kbp *Hinc* II fragment of pSUP1C2–5 is subcloned, Ap^r, Km^r	This study
pGAcc5C1	5.0–kbp *Acc* I fragment of pSUP5C1–1 is subcloned, Ap^r, Km^r	This study
pGA6C1	4.6–kbp *Aat* II fragment of pSUP6C1–3 is subcloned, Ap^r, Km^r	This study
pGSphIII7C1–1	4.4–kbp *Sph* I fragment of pSUP7C1–9 is subcloned, Ap^r, Km^r	This study
pGA7C2	5.5–kbp *Aat* II fragment of pSUP7C2–4 is subcloned, Ap^r, Km^r	This study
pGApa10C2	9.3–kbp *Apa* I fragment of pSUP10C2–1 is subcloned, Ap^r, Km^r	This study

*1 Km^r: kanamycin resistance, Ap^r: ampicillin resistance.

Tn*5*–flanking sequence

Kwon and Ricke (2000) developed efficient method to amplify specifically the transposon–flanking sequences with unique Y–shape linkers. We previously isolated Tn*5* mutants derived from strain Is–1 and showed Tn*5*–flanking sequences according to their method (Tsurumaru *et al.*, 2008). All homologous sequences to them were found in the genome of *B. japonicum* USDA110. The open reading frames (ORFs) containing homologous sequence inside or upstream of itself and the sequence positions of these ORFs are shown in Table 2. The complete genome sequence information of *B. japonicum* USDA110 was obtained from RhizoBase (http://bacteria.kazusa.or.jp/rhizobase/Bradyrhizobium/index.html). This information was used for constructing tentative restriction map of strain Is–1.

Table 2. Open reading frame (ORF), containing homologous sequence to Tn*5*–flanking sequence of Tn*5* mutants derived from strain Is–1, in RhizoBase

Tn*5* mutant	ORF*1	Location*2	Length
1C1	*bll4521*	5009041 – 5010171 b	1130 bp
1C2*3	*bsr7468*	8203674 – 8203889 b	215 bp
5C1	*blr1414*	1540332 – 1540634 b	302 bp
6C1	*bll1193*	1307182 – 1309080 b	1898 bp
7C1	*bll5593*	6149267 – 6150277 b	1010 bp
7C2	*blr5786*	6353093 – 6353491 b	398 bp
10C2	*bll5123*	5682342 – 5683715 b	1373 bp

*1 ORF of USDA110 which contains the homologous sequence to Tn*5*–flanking sequence inside or upstream of itself is showed.
*2 Location of ORF in the genome of USDA110 is showed.
*3 Homologous sequence to Tn*5*–flanking sequence of 1C2 is located in the upstream of *bsr7468*.

Construction of a cosmid vector

SuperCos I–Neo was constructed as described below. First, SuperCos I cosmid vector (Stratagene, CA, USA) was digested by *Bal* I (Roche Diagnostics, Basel, Switzerland) and *Sma* I (Takara Bio Inc., Shiga, Japan) to eliminate the neomycin resistant gene. Finally the *Bal* I/*Sma* I–digested SuperCos I was self–ligated using T4 DNA ligase (Stratagene, CA, USA).

Cloning of kanamycin resistance region into cosmid vector

Genomic DNA was isolated by the method of

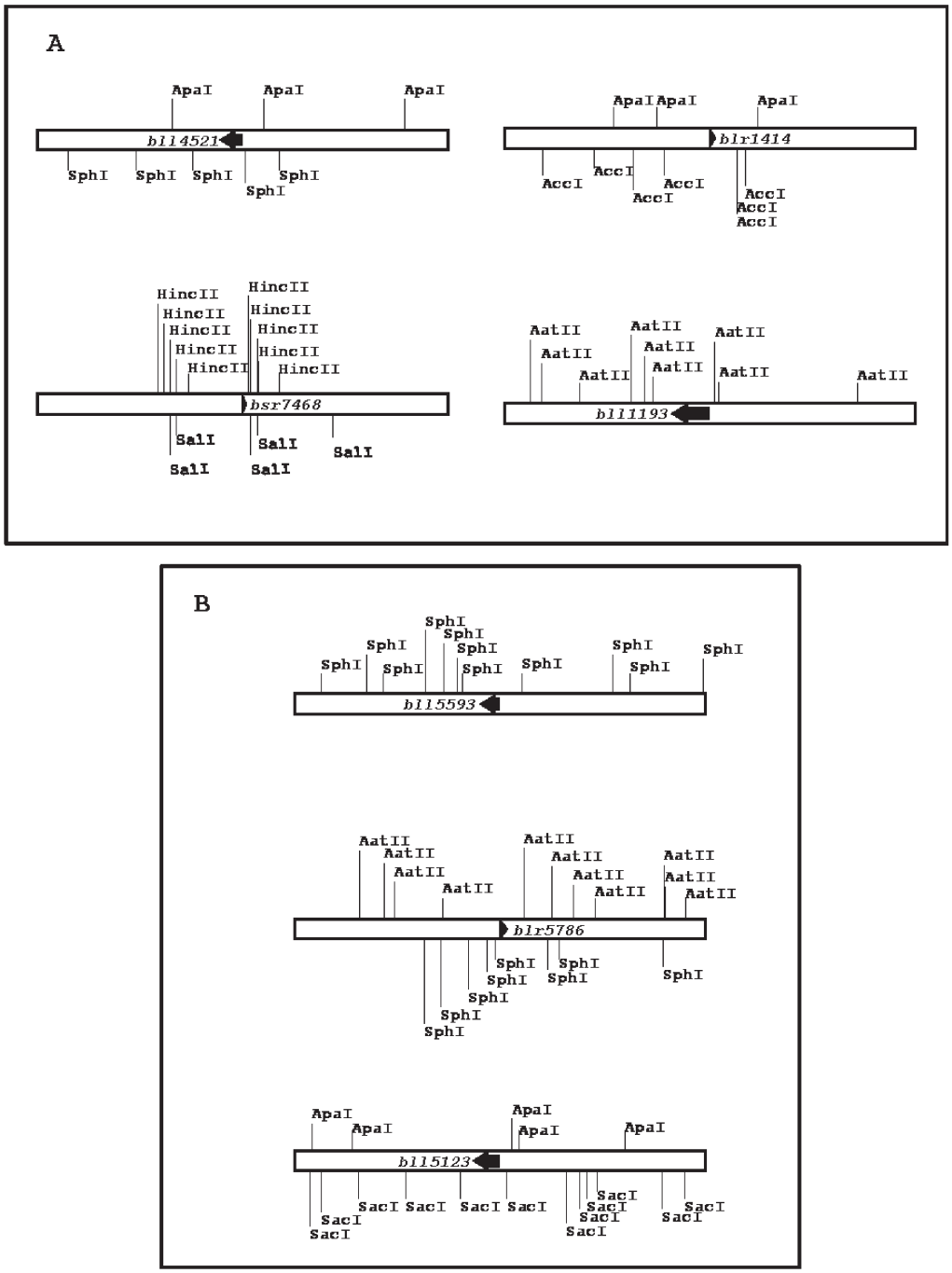

Fig. 1. The restriction maps in the vicinities of the genes, which have high similarities with Tn*5*–flanking sequences, in *B. japonicum* strain USDA110.
The *bll4521*, *bsr7468*, *blr1414* and *bll1193* genes are showed in panel A. The *bll5593*, *blr5786* and *bll5123* genes are showed in panel B. These genes have high similarities with Tn*5*–flanking sequences of 1C1, 1C2, 5C1, 6C1, 7C1, 7C2 and 10C2, respectively. The sequences in the vicinities of these genes were obtained from the RhizoBase. The restriction maps were constructed by Genetyx software (Genetyx, Tokyo, Japan) with these sequences. Because we assumed that the vicinities of Tn*5*–inserted genes in Tn*5* mutants of *B. japonicum* strain Is–1 have also high similarities with ones of above–described genes in *B. japonicum* strain USDA110, these maps were served as the tentative restriction maps in Tn*5* mutants for plasmid rescue. In this study, we confirmed that the strategy based on this assumption was viable (as described in results).

Pitcher *et al*. (1989). The kanamycin resistance cassette and its flanking sequence (Kanamycin resistance region) were cloned into SuperCos I–Neo following instructions of SuperCos I cosmid vector kit and Gigapack Ⅲ Gold–4 packaging Extract (Stratagene, CA, USA).

Construction of the tentative restriction map based on the RhizoBase and plasmid rescue according to this map

A 2000–bp sequence containing ORF which has high similarity to the Tn*5*–flanking sequence was obtained from the RhizoBase. This sequence extends from 1000 bp upstream of to 1000 bp downstream of the translation start site of this ORF. Restriction map of this sequence was constructed with Genetyx software (Genetyx, Tokyo, Japan). These maps were used as the tentative restriction map for plasmid rescue experiment (Fig. 1). According to these tentative restriction maps, restriction enzymes were selected. These restriction enzymes do not cut both Tn*5* (accession number of DNA data bank of Japan, AB158755) and the ORF that have high similarity to the Tn*5*–flanking sequence (Tsurumaru *et al*., 2008). The pGEM–3Zf(−) or pGEM–5Zf(+) were used as cloning vector.

RESULTS

The rescue of the kanamycin resistance region

Partially digested genomic DNAs from all Tn*5* mutants were ligated into the SuperCos I–Neo cosmid vector. The cosmid clones containing transposon–encoded kanamycin resistance gene were obtained from all mutants except for mutant 10C1 (Table 1). No cosmid clone showing the kanamycin resistance was obtained from 10C1. According to the tentative restriction map (Fig. 1), *Apa* I, *Hinc* Ⅱ, *Acc* I, *Aat* Ⅱ, *Sph* I, *Aat* Ⅱ and *Apa* I were used for plasmid rescue from cosmid clones of 1C1, 1C2, 5C1, 6C1, 7C1, 7C2 and 10C2 respectively. Constructed plasmids were showed in Table 1 and sequenced commercially by Macrogen Inc. (Seoul, Korea).

Deposition of the sequences determined in this study

All restriction enzymes selected according to the tentative restriction map were worked for plasmid rescue. The sequences of these plasmids determined in this study were deposited to DNA Data Bank of Japan (DDBJ) database (sequential accession numbers AB243409 through AB243415). Sequence analysis showed that these plasmids contained Tn*5*–flanking sequences that were determined by using the method of Kwon and Ricke (2000). A 773 bp sequence extending from 1,110 b to 1,882 b in 1C1 (accession number AB243409), 756 bp sequence extending from 905 to 1,660 b in 5C1 (AB243411), 227 bp sequence extending from 1,625 to 1,851 b in 6C1 (AB243412), 1,896 bp sequence extending from 1,050 to 2,945 b in 7C1 (AB243413) and 234 bp sequence extending from 1,174 to 1,407 b in 10C2 (AB243415) correspond to the Tn*5*–flanking sequences, respectively. A 369 bp sequence extending from 2,573 to 2,941 b in 1C2 (AB243410) and 932 bp sequence extending from 501 to 1,432 b in 7C2 (AB243414) correspond to a part of the Tn*5*–flanking sequences.

DISCUSSION

The tentative restriction map of strain Is–1 was constructed with both information of Tn*5*–flanking sequence and RhizoBase. The restriction enzymes used for plasmid rescue were selected according to these maps. Because this strategy worked in case of strain Is–1, it may be viable in *B. japonicum* strains other than Is–1. However this strategy may have disadvantage because it does not work when a Tn*5*–flanking sequence from mutant has no similarity with any sequence in the RhizoBase. In addition, we can not tell whether a Tn*5*–flanking sequence in mutant derived from *B. elkanii* has high similarity with the sequence of some gene in RhizoBase, because *B. japonicum* is clearly a separate species from *B. elkanii* on the basis of 16S rDNA similarities and total DNA homology values (Willems *et al*., 2001).

Once the Tn*5*–disrupted gene is identified, it is necessary to confirm whether its gene is responsible for the phenotype change in Tn*5* mutant. Therefore the complementation experiment is usually performed after identification of Tn*5*–disrupted gene. A complementation experiment consists of a four–step procedure: 1) the genomic cosmid library of wild type strain is constructed; 2) a cosmid clone containing the Tn*5* insertion site is selected from it's library by colony hybridization with a DNA probe based on Tn*5*–flanking sequence in Tn*5* mutant; 3) a cosmid is digested with appropriate restriction enzyme and digested–DNA fragment is cloned into plasmid vector; 4) constructed plasmid is transformed into Tn*5* mutant to confirm whether the complemented mutant shows the wild type phenotype. In second–step in complement experiment, there are sometimes negative clones in candidate clones selected by colony hybridization. The strategy with tentative restriction map may be applied for selection of true positive clones from Is–1 genomic library. Basically, it is only necessary to confirm that there is Tn*5*–flanking sequence in tentative map based on end sequence of candidate clones. The rapid selection protocol of positive clones will be described in more detail in the next paper to confirm that Tn*5* insertion is responsible for the acquisition of ability to nodulate Rj_2 soybean.

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