PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2009, 36(5): 556~565
www.pibb.ac.cn

Development and Optimization of Method for Generating Unmarked *A. tumefaciens* Mutants*

GUO Min-Liang**, ZHU Qing, GAO Dian-Kun
(*College of Bioscience and Biotechnology, Yangzhou University, Yangzhou* 225009, China)

Abstract *Agrobacterium tumefaciens* possesses many advantages as a model bacterium for the study of a wide variety of biological processes. Gene disruption or inactivation is a powerful and direct tool for investigation of *in vivo* gene functions. The intensive study of *A. tumefaciens* has increased the need for simple and highly efficient procedures to manipulate its genome. The *sacB* gene was used as a counterselectable marker to develop a gene replacement procedure that allows precise insertion, deletion, and allele substitution of any gene sequence in *A. tumefaciens* without altering the genome in any other way. A kanamycin resistance (Km^R) cassette was constructed to the suicide vector as the positive selection marker. The suicide plasmid containing DNA fragments homologous to the flanking sequences of the target gene was integrated into the recipient cell genome at the target gene locus by intermolecular homologous recombination, generating the Km^R-single cross-over colonies. The effect of homologous sequence length on the intermolecular homologous recombination was analyzed. The second cross-over colonies generated by intramolecular homologous recombination occurring between two tandem repeats were simply screened out by counter-selection of *sacB*. Data showed that the intervening sequence length between two repeats significantly affected the intramolecular homologous recombination frequency in *A. tumefaciens*, indicating that *A. tumefaciens* adopted the homologous recombination mechanism similar to that in *E. coli*. All these results demonstrated that investigators could minimize the numbers of colonies to be analyzed and reduce the overall workload by optimizing the relative length of two homologous fragments and using the specific type of single cross-over transformants for screening the second cross-over event. This mutagenesis strategy had successfully been used to generate the double unmarked Δ*vbp2*Δ*vbp3* mutant in two *A. tumefaciens* strains.

Key words *Agrobacterium tumefaciens*, counterselectable marker gene, homologous recombination mechanism, unmarked mutant
DOI: 10.3724/SP.J.1206.2008.00618

Agrobacterium tumefaciens is a soilborne phytopathogenic bacterium. It causes the crown gall disease of dicotyledonous plants by transferring a segment of DNA (designated T-DNA) from its Ti-plasmid to plant cells [1, 2]. Because the wild-type T-DNA coding region can be replaced by any DNA sequence without any effect on its transfer from *A. tumefaciens* to the plant, *A. tumefaciens*-mediated T-DNA transfer to plant has become the most widely used method for the introduction of foreign genes into plant cells [1~3]. In addition, *A. tumefaciens*-mediated T-DNA transfer to plant is the only known natural example of DNA transport between kingdoms [2]. The T-DNA is transferred into eukaryotic cells in the form of nucleoprotein complex. Thus, *A. tumefaciens*-mediated T-DNA transfer system also provides a fascinating model system to study the molecular mechanism of a wide variety of biological processes, including bacterial detection of host signaling chemicals[4], intercellular transfer of macromolecules[5], importing of nucleoprotein into plant nuclei [1, 6], and interbacterial chemical signaling *via* autoinducer-type quorum sensing [7]. It has been proved that reverse genetics is a powerful approach for the molecular mechanism investigation of these biological processes, in which the gene of interest is mutated or inactivated to study the resulting effects on *A. tumefaciens*.

So far, most mutants in *A. tumefaciens* were

*This work was supported by a grant from The National Natural Science Foundation of China (30870054).
**Corresponding author.
Tel: 86-514-87979054, E-mail: guoml@yzu.edu.cn
Received: September 6, 2008 Accepted: October 13, 2008

constructed by transposon inactivation with an antibiotic resistance or other selection markers[8]. The insertion of an antibiotic resistance marker into the target gene provides direct selection of rare recombinants. While powerful, this method does have drawbacks. First, the insertion of an antibiotic resistance marker may exert polar effects on the upstream or downstream genes. For example, the polar effect can prevent the disruption of a nonessential gene if the target gene is located in an operon upstream of an essential gene. Secondly, in many cases it is common for investigators to introduce multiple mutations into a bacterial cell. The accumulation of antibiotic resistance markers in the mutated strains could become cumbersome and possibly compromise interpretations of experimental results. Also, there are a limited number of antibiotic resistance markers available for use in *A. tumefaciens*.

An ideal gene replacement system is one that can generate the defined mutants by unmarked in-frame deletions or the introduction of point mutations. The mutants generated by unmarked in-frame deletions do not carry antibiotic resistance genes and cannot revert, and thus the mutations do not exert the polar effects on the expression of the adjacent genes. By extension, this ideal gene replacement system could be used for allelic exchange of point mutations, which allow for a finer dissection of gene function. The combined utilization of selectable and counterselectable markers makes this ideal gene replacement methodology be successfully used in several bacteria[9~16]. We once used this gene replacement system to generate unmarked *A. tumefacins* mutants also[17, 18]. However, no detail about this method and its homologous recombination mechanism was reported though many investigators had pointed out that the length of the homologous sequences might affect the homologous recombination rate. Therefore, the homologous recombination rate and the frequency to generate the desirable mutants became uncontrollable and unpredictable when this method was used to generate unmarked mutants. We try to optimize this gene replacement method by means of the study of the homologous recombination mechanism in *A. tumefaciens* underlying this method and provide the detailed data about its mechanism to researchers so that they could minimize the numbers of colonies to be analyzed and reduce the overall workload for screening the desirable mutants when they use this method to construct the unmarked bacterial mutants.

This method uses the *sacB* gene of *Bacillus* spp as the counterselectable marker to construct a suicide vector, which lacks agrobacterial origin of replication and thus is not able to replicate in *A. tumefaciens*. When this suicide vector was used to deliver the recombination substrate to the agrobacterial recipient cell and integrated to the recipient cell genome, the *sacB* gene would confer the recipient cell sucrose sensitivity (Suc^S) and allow efficient counterselection of integrated vector in the present of sucrose[9, 19]. The genes chosen for this study are the Atu4860 gene and the Atu4856 gene (accession: NC_003305.1) in the U Wash version of *A. tumefaciens* genome[20], which are located on the linear chromosome. The proteins encoded by these two genes were identified to be homologous to a VirD2-binding protein and were involved in the tumorigenesis[17]. These two genes were designed as *vbp2* and *vbp3* respectively[17, 18]. Data in this work showed the *sacB*-based gene replacement system is reproducible for allelic exchange of unmarked deletion in the chromosome of *A. tumefaciens*. Results also told us how to optimize the gene replacement procedure and increase the frequency to generate desirable mutants.

1 Materials and methods

1.1 Bacterial strains, plasmids, primers and bacterial culture conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strain DH5α was used as the host for plasmid replication. Two *Agrobacterium tumefaciens* strains A348 and GMI9017 were used as parent strains to construct unmarked double deletion (*Δvbp2Δvbp3*) mutants. All chromosomal genome sequences in three *A. tumefaciens* strains (C58, A348, and GMI9017) are the same except the harboring plasmids pAtC58, pTiC58, and pTiA6NC.

The sequences, purposes and origins of the primers used in this study are described in Table 2. *E. coli* strains were cultured at 37℃ in Luria-Bertani (LB) liquid or agar medium[21]. *A. tumefaciens* strains were cultured at 28 ℃ in MG/L liquid or agar medium[22]. LB and MG/L media were supplemented with 5 mg/L tetracycline, 50 mg/L (for *E. coli*.) or 100 mg/L (for *A. tumefaciens*) kanamycin when necessary.

Table 1 Bacterial strains and plasmids used in this study

Bacterial strain and plasmid	Relevant characteristic (s) [1)]	Source or reference
Strains		
Escherichia coli DH5α	*EndA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* (*argF-lacZYA*) U169 φ*80dlacZ*. For DNA cloning	Bethesda Research Laboratories
Agrobacterium tumefaciens		
C58	Wild type, nopaline-type pTiC58 plasmid	Laboratory collection
A348	Wild type, A136 (pTiA6NC) (octopine-type)	Laboratory collection
GMI9017	Derivative of C58 cured of pAtC58, Sm^S, Sp^R, Rf^R	[23]
Plasmids		
pEX18Tc	Counterselectable plasmid carrying *sacB* marker, *oriT*, Tc^R	[19]
pCB301	A minim binary vector plasmid, Km^R	[24]
pEX18K	Derivative of pEX18Tc in which Tc^R was replaced by *npt*Ⅲ from pCB301, Km^R	This study
pEXKmVD	pEX18K carrying a 2 183 bp *Hin*dⅢ fragment from *vbp2* downstream, Km^R	This study
pEXKmVUD	pEXKmVD carrying a 1 395 bp *Bam*HI fragment from *vbp2* upstream, Km^R	This study
pEXKmV3	pEX18K carrying a 1 323 bp *Bam*HⅠ fragment, 375 bp of this fragment from *vbp3* upstream, 948 bp of this fragment from *vbp3* downstream, Km^R	This study

[1)] Km^R, Tc^R, Sp^R and Rf^R, resistant to kanamycin, tetracycline, spectinomycin, rifampicin respectively. Sm^S, sensitive to streptomycin.

Table 2 Primers used in this study

Primers	Sequence and purpose	Origin
PnptIIIF	5' gaagatctctcgagttggcagcatcacc 3' (Amplify *npt*Ⅲ from plasmid pCB301)	Plasmid pCB301
PnptIIIR	5' gaagatcttactaaaacaattcatccag 3' (Amplify *npt*Ⅲ from plasmid pCB301)	Plasmid pCB301
Pex18F	5' gaagatctgttgaatactcatactcttc 3' (Amplify backbone of pEX18Tc)	Plasmid pEX18Tc
Pex18R	5' gaagatcttgtcagaccaagtttactcat 3' (Amplify backbone of pEX18Tc)	Plasmid pEX18Tc
Pdow3	5' tccaagcttgatcatatcccgcacag 3' (Amplify downstream of *vbp2* gene)	Linear chromosome - 2045222
Pdow4	5' cccaagcttccagcgcgagtaccag 3' (Amplify downstream of *vbp2* gene)	Linear chromosome - 2047409
Pup10	5' acaggatcccttcctgccacgcc 3' (Amplify upstream of *vbp2* gene)	Linear chromosome - 2043756
Pup11	5' gtgggatccatgaactttatacgcttc 3' (Amplify upstream of *vbp2* gene)	Linear chromosome - 2045151
Pscreen1	5' ctcgagagaggagacgcatcg 3' (Screen the *vbp2*-deleting mutant)	Linear chromosome - 2044997
Pscreen2	5' cagcgcatcctcgaactcctc 3' (Screen the *vbp2*-deleting mutant)	Linear chromosome - 2045303
Pvbp3u	5' ggggatcctgaggacctgcggcag 3' (Amplify upstream of *vbp3* gene)	Linear chromosome - 2039858
Pvbp3l1	5' ctcgcgcatccgggagg-**tgc**-tccgcttatcgacggtgtcc 3' (Overlapped primer linking the upstream and downstream fragment of *vbp3* gene, amplify upstream of *vbp3* gene)	Linear chromosome - 2040233 Link to 2040735
Pvbp3l2	5' ggacaccgtcgataagcgga-**gca**-cctcccggatgcgcgag 3' (Overlapped primer linking the upstream and downstream fragment of *vbp3* gene, amplify downstream of *vbp3* gene)	Linear chromosome - 2040233 Link to 2040735
Pvbp3d	5' cttggatccgtcccgagaaagtcgc 3' (Amplify downstream of *vbp3* gene)	Linear chromosome - 2041683
Pex18tc1	5' ctcttcgctattacgccagctgg 3' (Differentiate the single cross-over colonies)	Plasmid pEX18Tc

1.2 DNA manipulations

DNA manipulations followed standard molecular protocols [21]. *E. coli* DH5α strain was routinely used as the host for DNA cloning experiments. *E. coli* competent cells were prepared according to the Inoue protocol [21] and DNA was transferred into *E. coli* cells by heat-shock [21]. Plasmids were introduced into *A. tumefaciens* by electroporation [22]. For accurately calculating the recombination frequency, the plasmid concentration and the number of recipient *A. tumefaciens* cells were calibrated to the same for different transformation experiments. Total DNA of *A. tumefaciens* C58 strain was prepared according to Charles and Nester[25].

1.3 Construction of suicide vector

To obtain a *sacB*-based suicide vector suitable for *A. tumefaciens* gene replacement, the tetracycline resistance (Tc^R) gene in plasmid pEX18Tc [19] was replaced by Km^R gene. Primers Pex18F and Pex18R were used to amplify a 5 kb non-Tc^R-resistance fragment from plasmid pEX18Tc. A 1.04 kb Km^R-encoding cassette fragment was amplified by primers PnptⅢF and PnptⅢR from plasmid pCB301[24]. These two fragments were ligated to generate the Km^R-plasmid pEX18K. This kanamycin-resistance suicide vector was used to construct gene replacement plasmid.

2 Results

2.1 Development of gene replacement system

2.1.1 Constructions of gene replacement plasmids. The *vbp2* gene replacement plasmid pEXKmVUD was constructed as follows. First, a 2.183 kb *vbp2* downstream fragment was amplified by primers Pdow3 and Pdow4 from *A. tumefaciens* C58 total DNA and inserted to the *Hin*d Ⅲ site of pEX18K to generate plasmid pEXKmVD. Then, primers Pup10 and Pup11 were used to amplify a 1.395 kb *vbp2* upstream fragment. Finally, the *vbp2* upstream fragment was inserted to the *Bam*H Ⅰ site of pEXKmVD to generate pEXKmVUD (Figure 1). In this plasmid, two *vbp2* flanking fragments were linked by a short vector MCS (multiple-cloning sites) fragment.

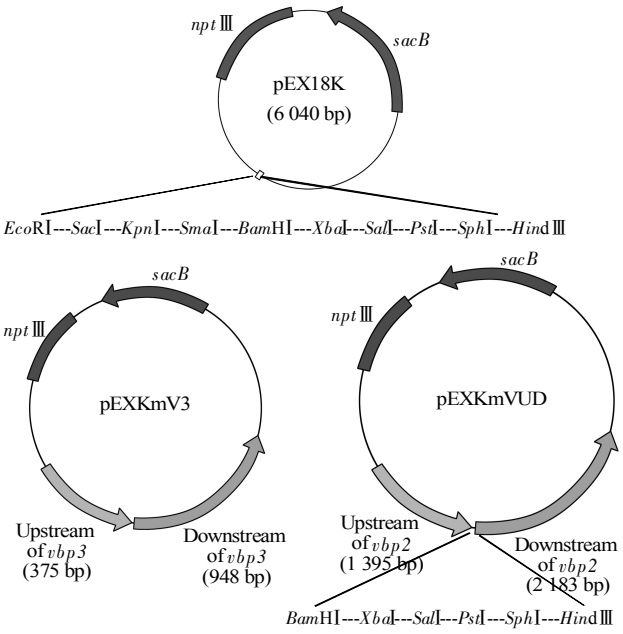

Fig. 1 Physical maps of plasmids for gene replacement

All three plasmids cannot replicate in *A. tumefaciens* but have the pMB1-based origin of replication for *E. coli*. The *npt* Ⅲ represents the kanamycin-resistance gene. The *sacB* represents the levansucrase-encoding gene that confers the host sucrose sensitivity (Suc^S).

To construct the *vbp3* gene replacement plasmid pEXKmV3, the upstream and downstream fragments of *vbp3* gene were linked by PCR *via* using overlapped primers. First, a 375 bp upstream fragment of *vbp3* gene was amplified by primer Pvbp3u and the overlapped primer Pvbp3l1 from *A. tumefaciens* C58 total DNA, a 948 bp downstream fragment of *vbp3* gene was amplified by primer Pvbp3d and the overlapped primer Pvbp3l2 from *A. tumefaciens* C58 total DNA. Then, these two PCR products were purified and mixed together to use as template for another PCR. By using primers Pvbp3u and Pvbp3d, a 1.323 kb DNA fragment, in which the upstream and downstream fragments of *vbp3* gene were linked together, could be amplified from this mixed template. This 1.323 kb fragment was inserted to the *Bam*H Ⅰ site of pEX18K to generate pEXKmV3 (Figure 1). In this plasmid, two *vbp3* flanking fragments were linked directly without any unwanted sequence.

2.1.2 Selection and identification of the single cross-over transformants. The purified gene replacement plasmid, which carries both upstream and downstream sequences of the target gene, was introduced into *A. tumefaciens* strain A348 and GMI9017 cells respectively by electroporation. DNA concentrations were calculated from A_{260}. The electroporated *A. tumefaciens* cells were plated on kanamycin-containing MG/L agar plate to select the single cross-over transformants. Since the gene replacement plasmid cannot replicate inside agrobacterial cells, the Km^R-transformant could only result from the integration of the plasmid into the agrobacterial genome. The integration of the gene replacement plasmid into the agrobacterial genome was achieved by the intermolecular homologous recombination. The gene replacement plasmid could be integrated into the agrobacterial genome on either the upstream (Figure 2a) or the downstream (Figure 2b) of the target gene in the single cross-over event and thus two types of single cross-over transformants could be generated. PCR was used to differentiate these two types of single cross-over transformants. Two primers that were designed for this PCR were shown in Figure 2. One primer is Pex18tc1, which anneals to the pEX18K vector sequence near the downstream of the target gene, and the other primer (Pup in Figure 2) is complementary to the upstream sequence of the target gene. We can differentiate these two types of single cross-over transformants depending on the size of the DNA fragment amplified by PCR.

2.1.3 Selection and identification of the second cross-over recombinants. The PCR-confirmed Km^R-transformants were used to screen for the second cross-over colonies. The single cross-over Km^R-transformants were plated on sucrose-containing MG/L plate to select the sucrose resistance (Suc^R) recombinants. The Suc^R-recombinants were generated

due to the second cross-over homologous recombination, which resulted in the loss of the integrated suicide plasmid. The second cross-over homologous recombination could also occur in two ways, either on the upstream homologous region (A2 and B1 in Figure 2) or on the downstream homologous region (A1 and B2 in Figure 2). So, two types of Suc^R-recombinants were identified: the wild type colonies (A2 and B2 in Figure 2) and the deletion (or gene replacement) mutants (A1 and B1 in Figure 2). The wild type and mutant colonies were also differentiated by PCR using two primers complementary to the upstream and downstream sequences of the target gene.

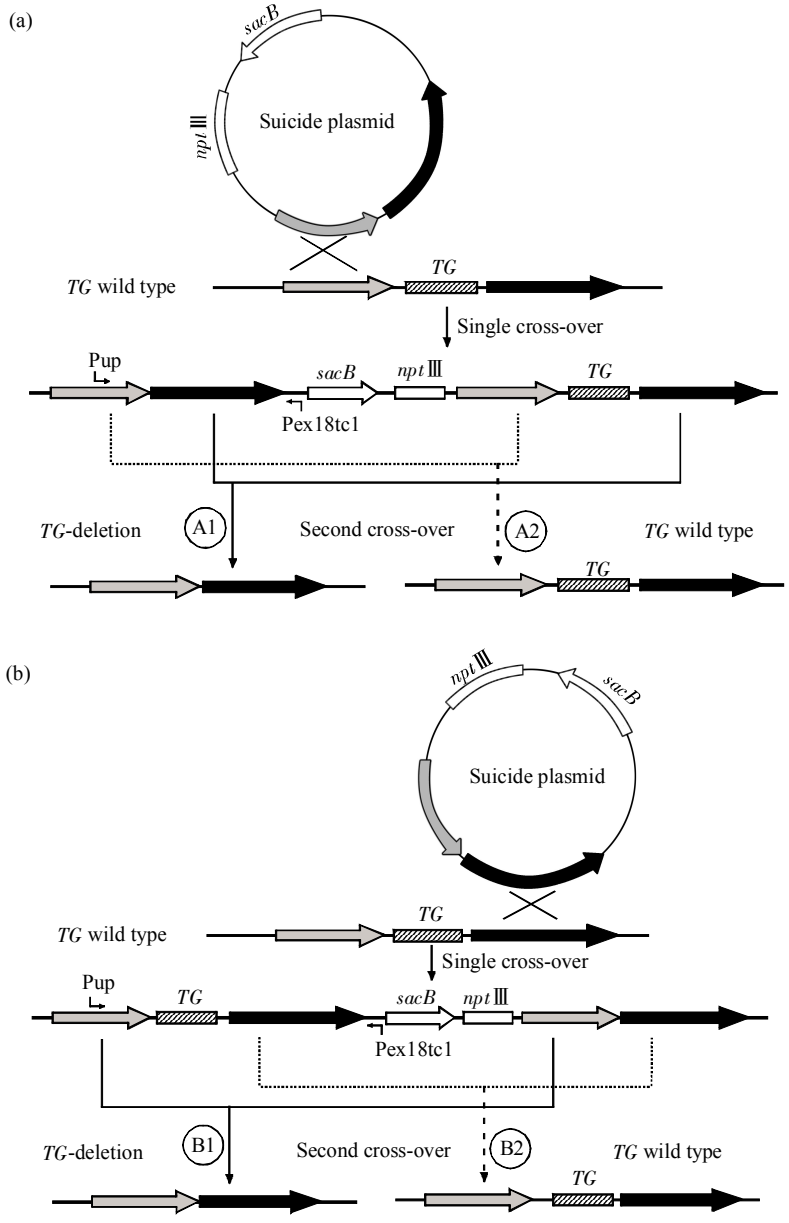

Fig. 2 Schematic diagram of gene replacement strategy

The suicide plasmid carrying the upstream (shadow arrows) and downstream (black arrows) sequences of the target gene (*TG*) was introduced to the recipient cell and then integrated into the recipient genome DNA at the locus of the target gene by homologous recombination . The *npt* Ⅲ (open rectangles) represents kanamycin-resistant gene. The *sacB* (open arrows) is a counterselectable marker gene that confers sucrose sensitivity. A fragment of target gene was shown in stippled rectangles. Colonies having undergone single cross-over homologous recombination (×) were screened as kanamycin resistance and sucrose sensitivity. Second cross-over homologous recombination event was selected by plating the single cross-over colonies on media containing 5% sucrose. The *TG*-deletion (or replacement) mutants were isolated from the kanamycin-sensitive and sucrose-resistant colonies. (a) The suicide plasmid was integrated into the *A. tumefacien* genome by homologous recombination at the upstream of the target gene. (b) The suicide plasmid was integrated into the *A. tumefacien* genome by homologous recombination at the downstream of the target gene. Second cross-over homologous recombination may occur in the two fragments homologous to the upstream of *TG* (A2 and B1, two shadowy arrows) or in the two fragments homologous to the downstream of TG (A1 and B2, two black arrows).

2.2 Optimization of the gene replacement procedure

2.2.1 The effects of homologous sequence length on the intermolecular homologous recombination. Two gene replacement plasmids, pEXKmVUD and pEXKmV3, were electroporated into *A. tumefaciens* strains A348 and GMI9017 respectively. These two plasmids carry different length of DNA sequences homologous to the flanking sequences of target genes. The plasmid pEXKmVUD carries a 2.183 kb *vbp2* downstream fragment and a 1.395 kb *vbp2* upstream fragment. The plasmid pEXKmV3 carries a 948 bp *vbp3* downstream fragment and a 375 bp *vbp3* upstream fragment. To demonstrate the effect of homologous DNA length on the frequency of the intermolecular homologous recombination, the Km^R-transformants resulting from the integration of the gene replacement plasmid into the host genome were analyzed by PCR and the frequencies of the single cross-over event were measured as the numbers of single cross-over colonies per μg of DNA used in the electroporation (Table 3). All Km^R-colonies resulted from the homologous recombination within the target loci, *i.e.* no spontaneous Km-resistant mutant or illegitimate integration of the gene replacement plasmid into the host genome other than the target loci was detected. Results in Table 3 showed that the frequency of the first homologous recombination event (*i.e.* the integration frequency of the gene replacement plasmid into the host's genome DNA or the frequency of intermolecular homologous recombination) is significantly dependent upon the length of homologous DNA. Statistic analysis showed that the R^2s in strains A348 and GMI9017 are 0.9739 and 0.9689 respectively.

Table 3 Frequency of the single cross-over event[1)]

Length of homologous sequence (Integration locus of gene replacement plasmid)	Frequency of the single cross-over event ± *s* (colonies/μg DNA) [2)]	
	A348	GMI9017
2 183 bp (*vbp2* downstream of pEXKmVUD)	120 ± 14	84 ± 7
1 395 bp (*vbp2* upstream of pEXKmVUD)	62 ± 9	48 ± 10
948 bp (*vbp3* downstream of pEXKmV3)	30 ± 7	16 ± 4
375 bp (*vbp3* upstream of pEXKmV3)	11 ± 3	4 ± 2

[1)]Plasmids carrying DNA fragments homologous to the flanking sequences of the target genes were electroporated into *A. tumefaciens* cells. The plasmid would integrate into the *A. tumefaciens* genome by intermolecular homologous recombination to generate Km^R-trasformants. The Km^R-trasformants were screened out by plating the electroporated agrobaterial cells on the kanamycin-containing MG/L agar plate and identified by PCR. [2)]The frequency of the single cross-over event (*i.e.* the integration frequency of the plasmid into the host's genome DNA) was measured as the Km^R-transformant colonies per μg of DNA used in the electroporation. The numbers of the Km^R-transformant colonies were the means of triplicates and the frequency of the single cross-over event was the means of three independent experiments. *s* represents the standard derivations.

2.2.2 Analysis of the frequency of the second cross-over event. Following the successful isolation and identification of single cross-over transformants, two types of the single cross-over transformants were used to select the second cross-over colonies respectively. The second cross-over can result in either the wild-type allele or the mutant allele remaining in the host genome, depending on where the second cross-over event occurs (Figure 2). Thus, the selected Km^S-Suc^R-colonies can carry either the wild-type or mutant allele and were differentiated by PCR. The frequencies of second cross-over event were measured as the numbers of second cross-over colonies per input single cross-over transformant and were showed in Table 4. The second cross-over event occurs between the tandem repeat regions located on both sides of the suicide vector (Figure 2). In contrast to the single cross-over, the second cross-over is an intramolecular homologous recombination, which results in the excision of the integrated suicide plasmid from host genome. Previous study from *E. coli* showed that for intramolecular homologous recombination the length of intervening sequence between tandem direct repeats had a greater influence over the deletion frequency than the length of the homologous sequence of the tandem direct repeats[26]. To demonstrate whether the intervening sequence length between tandem repeats affects the intramolecular recombination frequency in *A. tumefaciens*, the sequence length between two tandem repeats was calculated and also showed in

Table 4. These data showed that the length of intervening sequence greatly affected the frequency of second cross-over event, indicating that the mechanism of the intramolecular homologous recombination in *A. tumefaciens* may be similar to that in *E. coli*.

Results in Table 4 also showed that different percentage of mutant colonies could be obtained if different type of single cross-over transformant was used to screen the second cross-over colonies. During the isolation and identification of *vbp2* mutant, 78% (A348 strain) and 73% (GMI9017 strain) of the second cross-over colonies were mutant colonies when the single cross-over transformant resulting from the upstream integration was used to screen the second cross-over colonies, whereas, only 21% (A348 strain) and 16% (GMI9017 strain) of the second cross-over colonies were mutant colonies when the other type of single cross-over transformant was used to screen the second cross-over colonies.

Table 4 Frequency of second cross-over event occurred between the different tandem repeats[1)]

Loci of single cross-over	Loci of second cross-over	Genotype of the second cross-over colony[2)]	Length between tandem repeats/bp[3)]	Frequency of second cross-over event/10^{-7}($\bar{x} \pm s$)[4)]	
				A348	GMI9017
vbp2 gene					
Downstream	Upstream	Mutant	8 237	69 ± 6	44 ± 5
Upstream	Upstream	Wild type	8 187	71 ± 6	77 ± 9
Upstream	Downstream	Mutant	7 450	258 ± 21	212 ± 22
Downstream	Downstream	Wild type	7 400	266 ± 19	239 ± 21
vbp3 gene					
Downstream	Upstream	Mutant	7 454	86 ± 7	52 ± 7
Upstream	Upstream	Wild type	6 952	171 ± 9	139 ± 15
Upstream	Downstream	Mutant	6 881	184 ± 11	148 ± 17
Downstream	Downstream	Wild type	6 379	234 ± 24	198 ± 23

[1)]The single cross-over Km^R-transformant carries the tandem repeats of the target gene flanking sequences and the *sacB* gene because of the plasmid integration, and shows sucrose sensitivity. Therefore, the single cross-over Km^R-transformant was plated on sucrose-containing MG/L agar plate to select the second cross-over colonies. [2)]The genotype of second cross-over colonies were identified by PCR. [3)]The length between tandem repeats does not include the length of repeat sequence itself. [4)]Frequency of second cross-over event was measured as the numbers of second cross-over colonies per input single cross-over Km^R-transformant cell. The numbers of second cross-over colonies were the means of triplicates and the frequency of second cross-over event was the means of three independent experiments. *s* represents the standard derivations.

2.3 The isolation and identification of *A. tumefaciens* mutants

The DNA fragments amplified from the deletion region of the selected second cross-over colonies were further identified by restriction enzyme digestion and verified by DNA sequencing to examine whether the defined *A. tumefaciens* mutants were obtained. The *vbp2* replacement plasmid pEXKmVUD was constructed in two steps. Firstly, the downstream fragment was inserted to the *Hin*d Ⅲ site and then the upstream fragment was inserted to the *Bam*H Ⅰ site. Thus, a short MCS (multiple-cloning sites) fragment was left in the region between the upstream and downstream fragments (Figure 1). This implied that in the expected *vbp2* mutant, the *vbp2* gene fragment between the upstream and downstream fragments should be replaced by the short MCS fragment. Figure 3 showed the isolation and identification of *vbp2* mutant. Compared with the *vbp2* wild type, a shorter DNA fragment (lane *4* in Figure 3) was amplified from the *vbp2* mutant. Since the short MCS fragment that was left in the region between the upstream and downstream fragments carried the *Bam*H Ⅰ site (Figure 1), the DNA fragment amplified from the *vbp2* mutant could be digested by *Bam*H Ⅰ into two fragments (lane *1* in Figure 3). The PCR fragments amplified from both the *vbp2* wild type and *vbp2* mutant were also sequenced. Sequencing results showed that in the *vbp2* mutant, a *vbp2* gene fragment was deleted and replaced by a short DNA sequence from the multiple-cloning sites of the vector as expected (Figure 4). The *vbp3* mutant was also identified by PCR and verified by DNA sequencing (data not showed). Unlike *vbp2* mutant, no unwanted

DNA sequence was introduced into *vbp3* mutant because the upstream and downstream fragments of *vbp3* gene were linked directly by PCR with overlap extension.

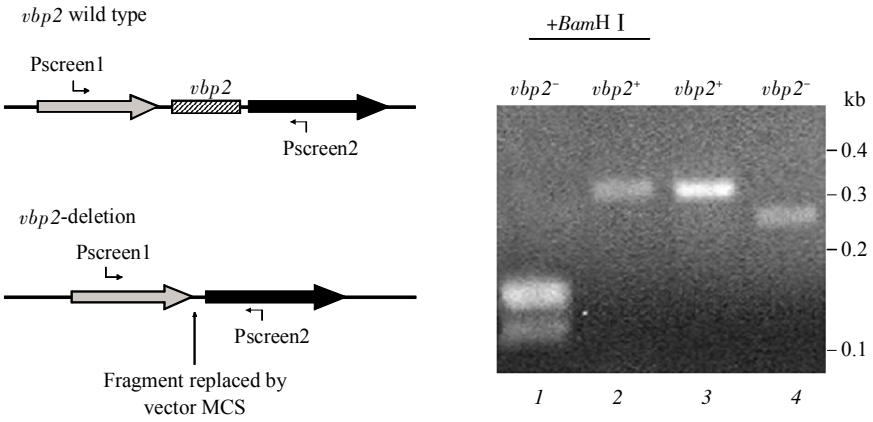

Fig. 3 Screening of *vbp2*-deletion mutants

The *vbp2*-deletion mutants were screened by PCR. Two oligonucleotides Pscreen1 (annealed to upstream of *vbp2*) and Pscreen2 (annealed to downstream of *vbp2*) were used as the primers. The PCR products from a *vbp2*-deletion mutant (lanes *1* and *4*) and from a wild type (lanes *2* and *3*) were treated by *Bam*H Ⅰ (lanes *1* and *2*) or without restriction digestion (lanes *3* and *4*). The PCR product from *vbp2*-deletion mutant was digested to two fragments (lane *1*) because the deleted *vbp2* gene fragment was replaced by a small fragment from vector′s multiple-cloning sites (MCS)(see plasmid pEXKmVUD in Figure 1). The PCR product from the wild type could not be digested to two fragments (lane *2*) because the wild type DNA fragment does not have the *Bam*H Ⅰ site. These two PCR fragments were sequenced to confirm that the *vbp2* fragment was deleted as expected (sequence data was shown in Figure 4). *vbp2*⁻ represents *vbp2*-deletion mutant. *vbp2*⁺ represents *vbp2* wild type.

vbp2 wild type: -----agcacttt-tggcatttga-tcaacgtgtc-gaagcgtata-aagttcatggat-accaccag-cgcgattgcg-tgggtgtcgc-aatcgcaagc-tagtcagata-cctcgttccc-

vbp2 mutant: -----agcacttt-tggcatttga-tcaacgtgtc-gaagcgtata-aagttcatgga-----tcc-tct-aga-gtc-gac-ctg-cag-gca-tgc-aag----------

atg-atg-aaa-tcc-tcc-ctt-gat-cat-atc-ccg-cac-aga-aag-cag-cga-gag-ctc-gcc-cgt-gcg----

----------ctt-gat-cat-atc-ccg-cac-aga-aag-cag-cga-gag-ctc-gcc-cgt-gcg----

Fig. 4 Sequence of the deleted *vbp2* fragment

The bold letters indicate the deleted *vbp2* fragment. The shadowy letters represent the small fragment replaced by vector′s multiple-cloning sites (MCS). The underlined letters highlight the start code.

3 Discussion

This study reports an efficient procedure for gene deletion and replacement in the *A. tumefaciens* chromosome by utilizing the counterselectable marker *sacB* gene. The replacement of tetracycline resistance gene in the suicide vector by kanamycin resistance gene abolished the problem of spontaneous tetracycline resistance in *A. tumefaciens* during the selection of single cross-over transformants. Using this gene deletion and replacement method, secondary mutations can be made without the problem of finding another resistance marker. In this study, we have isolated the unmarked double mutant (Δ*vbp2*Δ*vbp3*) from *A. tumefaciens* strains A348 and GMI9017 respectively, demonstrating the usefulness of this procedure for various gene deletion (or replacement) in different *A. tumefaciens* strains. Sequencing results demonstrated that the defined mutant alleles were obtained. Therefore, there is no longer a need to consider unknown mutations caused by random mutagenesis.

To optimize this gene deletion and replacement method, we investigated the effects of homologous sequence length on the recombination frequency and analyzed the intramolecular recombination mechanism. We concluded that the frequency to generate desirable mutants is controllable and able to greatly increase by optimizing the gene replacement procedure. The relative length of two homologous

fragments on each side of the target gene determines the relative frequency of the two types of single cross-over transformants. Different type of single cross-over transformants can result in different frequency of the desirable mutant colonies because of the difference of the intervening sequence length between two tandem repeats where second cross-over event occurs. Therefore, high frequency of the desirable mutant colonies can be obtained by designing the relative length of two homologous fragments and using the specific type of single cross-over transformants for screening the second cross-over event. This is particularly important when the mutant is not fitness or does not have a screenable phenotype. This strategy can minimize the numbers of colonies to be analyzed and thus significantly reduce the overall workload.

The *sacB* gene encodes levansucrase, which synthesizes lavens (high molecular-weight fructose polymers) in the presence of sucrose. The accumulation of lavens in the periplasm of gram-negative bacteria is toxic and thus causes the bacterial cells to die[27]. Although the effect of the *sacB* gene in the presence of sucrose has been demonstrated in most of the gram-negative bacteria [9], the high frequency of spontaneous sucrose resistance has been reported in many bacteria [28, 29]. Results in this study showed that the frequency of spontaneous sucrose resistance was very low in *A. tumefaciens*. More than 95% of Suc^R-colonies were second cross-over colonies when the single cross-over transformant was screened against 5% sucrose for the second cross-over colonies (data not shown). This indicates that the *sacB* gene can be used as a counterselectable marker in *A. tumefaciens*.

Acknowledgements This study was financially supported by startup fund for scientific research from Yangzhou University. We thank Dr Pan S Q, National University of Singapore, for providing bacterial strains and plasmids.

References

1 Tzfira T, Citovsky V. *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Curr Opin Biotechnol, 2006, **17**(2): 147～154

2 McCullen C A, Binns A N. *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annu Rev Cell Dev Biol, 2006, **22**: 101～127

3 陈 英, 黄敏仁, 王明庶. 植物遗传转化新技术和新方法. 中国生物工程杂志, 2005, **25**(9): 94～98
Chen Y, Huang M R, Wang M X. Chin Biotechnology, 2005, **25**(9): 94～98

4 Winans S C. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol Rev, 1992, **56**(1): 12～31

5 Cascales E, Christie P J. Definition of a bacterial type Ⅳ secretion pathway for a DNA substrate. Science, 2004, **304**(5674): 1170～1173

6 Ziemienowicz A, Merkle T, Schoumacher F, *et al*. Import of *Agrobacterium* T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins. The Plant Cell, 2001, **13**(2): 369～383

7 Newton J A, Fray R G. Integration of environmental and host-derived signals with quorum sensing during plant-microbe interactions. Cellular Microbiology, 2004, **6**(3): 213～224

8 Stachel S E, Nester E W. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J, 1986, **5**(7): 1445～1454

9 Reyrat J, Pelicic V, Gicquel B, *et al*. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect Immun, 1998, **66**(9): 4011～4017

10 Jr P M. Allelic exchange of unmarked mutations in *Mycobacterium tuberculosis*. Methods Mol Biol, 2008, **435**: 191～201

11 Bei W, He Q, Yan L, *et al*. Construction and characterization of a live, attenuated apxⅡCA inactivation mutant of *Actinobacillus pleuropneumoniae* lacking a drug resistance marker. FEMS Microbiol Lett, 2005, **243**(1): 21～27

12 Sato T, Fukui T, Atomi H, *et al*. Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*. Appl Environ Microbiol, 2005, **71**(7): 3889～3899

13 Roux F L, Binesse J, Saulnier D, *et al*. Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. Appl Environ Microbiol, 2007, **73**(3): 777～784

14 钱 锋, 潘卫庆, 杜景伶. Cre/l0xP 介导的伤寒杆菌染色体上插入基因的切除. 生物化学与生物物理进展, 2001, **28**(5): 732～735
Qian F, Pan W Q, Du J L. Prog Biochem Biophys, 2001, **28**(5):732～735

15 王玉飞, 陈泽良, 赵红庆, 等. 以克隆载体为自杀载体快速构建布鲁氏菌的无痕缺失突变株. 微生物学通报, 2007, **34**(4)：642～645
Wang Y F, Chen Z L, Zhao H Q, *et al*. Microbiology, 2007, **34**(4)：642～645

16 徐引弟, 郭爱珍, 刘维红, 等. 绿荧光蛋白基因在猪霍乱沙门氏菌 C500 株 asd- 缺失株平衡致死载体系统中的表达. 中国兽医学报，2007, **27**(4): 493～496
Xu Y D, Guo A Z, Liu W H, *et al*. Chin J Veter Sci, 2007, **27**(4): 493～496

17 Guo M L, Hou Q, Hew C L, *et al*. *Agrobacterium* VirD2-binding protein is involved in tumorigenesis and redundantly encoded in conjugative transfer gene clusters. Mol Plant-Microbe Interact,

2007, **20**(10): 1201～1212

18 Guo M L, Jin S, Sun D, *et al*. Recruitment of conjugative DNA transfer substrate to *Agrobacterium* type Ⅳ secretion apparatus. Proc Natl Acad Sci USA, 2007, **104**(50): 20019～20024

19 Hoang T T, Karkhoff-Schweizer R R, Kutchma A J, *et al*. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene, 1998, **212**(1): 77～86

20 Gooder B, Hinkle G, Gattung S, *et al*. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science, 2001, **294**(5550): 2323～2328

21 Sambrook J F, Russell T. Molecular Cloning: A Laboratory Manual. 3rd. New York: Cold Spring Harbor Laboratory Press, 2001. 1.31～1.138

22 Cangelosi G A, Best E A, Martinetti G, *et al*. Genetic analysis of *Agrobacterium*. Methods Enzymol, 1991, **204**: 384～397

23 Rosenberg C, Huguet T. The pAtC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumour induction. Mol Gen Genet, 1984, **196**(3): 533～536

24 Xiang C, Han P, Lutziger I, *et al*. A mini binary vector series for plant transformation. Plant Mol Biol, 1999, **40**(4): 711～717

25 Charles T C, Nester E W. A chromosomally encoded two-component sensory transduction system is required for virulence of *Agrobacterium tumefaciens*. J Bacteriol, 1993, **175**(20): 6614～6625

26 Bi X, Liu L F. A replicational model for DNA recombination between direct repeats. J Mol Biol, 1996, **256**(5): 849～858

27 Gay P, Lecoq D, Steinmetz M, *et al*. Cloning structural gene *sacB*, which codes for exoenzyme levansucrase of *Bacillus subtilis*: expression of the gene in *Escherichia coli*. J Bacteriol, 1983, **153**(3): 1424～1431

28 Parish T, Stoker N G. Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis tlyA plcABC* mutant by gene replacement. Microbiology, 2000, **146**(pt8): 1969～1975

29 Picardeau M, Brenot A, Girons I S. First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa flab* results in non-motile mutants deficient in endoflagella. Mol Microbiol, 2001, **40**(1): 189～199

产生无标记农杆菌突变体方法的建立及优化 *

郭敏亮 ** 诸 庆 高佃坤

(扬州大学生物科学与技术学院，扬州 225009)

摘要 农杆菌已经用作许多生物过程研究的模型细菌，为了解析这些生物过程的分子机理，对农杆菌的某些基因进行突变就显得非常重要．以自杀性基因 *sacB* 作为反向可选择性标记基因，利用同源重组的原理，建立了一种可对农杆菌基因进行准确插入、删除和位点置换的突变方法，所获突变体不带任何不需要的外源 DNA 序列．通过详细研究同源序列的长度对农杆菌同源重组效率和突变体产生概率的影响，以及对农杆菌中的同源重组机理的分析，提出了优化该突变体产生方法的方案，即通过设计不等长的上下游同源序列和选择其中一种类型的单交换重组体来筛选二次交换重组体的方法，可以显著地提高理想突变体的产生概率．研究结果对如何提高突变体的产生概率和减少突变体筛选的工作量具重要的参考价值．利用该方法成功地获得了两个基因被同时删除而且不含抗性标记的农杆菌突变株．

关键词 农杆菌，反向可选择性标记基因，同源重组机理，无标记突变体

学科分类号 Q933 **DOI:** 10.3724/SP.J.1206.2008.00618

* 国家自然科学基金资助项目(30870054).

** 通讯联系人．Tel: 0514-87979054, E-mail: guoml@yzu.edu.cn

收稿日期：2008-09-06，接受日期：2008-10-13