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ORIGINAL RESEARCH PAPER

Multiple genes deletion based on Cre-*loxP* marker-less gene deletion system for the strains from the genus of *Pectobacterium*

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Abstract
Objective To introduce the Cre-*loxP* system for constructing marker-less multiple-gene deletion mutants in *Pectobacterium*, overcoming limitations of antibiotic markers and enhancing the understanding of pathogenic mechanisms.
Results Firstly, a plasmid named pEX18-Cre, containing a *sacB* sucrose suicide gene, was constructed to express Cre recombinase in *Pectobacterium*. Secondly, a mutant in which the *loxP-Km* fragment replaced the target gene was obtained through homologous recombination double-crossover with the chromosome. Finally, pEX18-Cre was introduced into the mutant to excise the DNA between the *loxP* sites, thereby removing the markers and achieving multiple gene deletions. By utilizing the Cre-*loxP* system, we successfully constructed multiple marker-less gene deletion mutants in *Pectobacterium* strains.
Conclusions The Cre-*loxP* system efficiently creates marker-less multiple-gene deletion mutants, enhancing the study of *Pectobacterium* pathogenic mechanisms by overcoming antibiotic marker limitations.

Keywords Antibiotic marker · Cre-*loxP* multiple genes deletion · *Pectobacterium*

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Introduction

The strains from *Pectobacterium carotovorum* are devastating plant pathogens that affect a variety of crops, vegetables, and ornamentals, causing wilt, rot, and black-leg (Gijsegem et al. 2021). To understand the molecular pathogenic mechanism of *Pectobacterium*, genetic manipulation is often the initial step, as it is in many other pathogens, and there are usually sorts of genes linked with pathogenicity, but we lack effective techniques available for deleting multiple genes other than homologous recombination with different antibiotic markers in *P. carotovorum,* which can help us obtain single- or two-gene deletion

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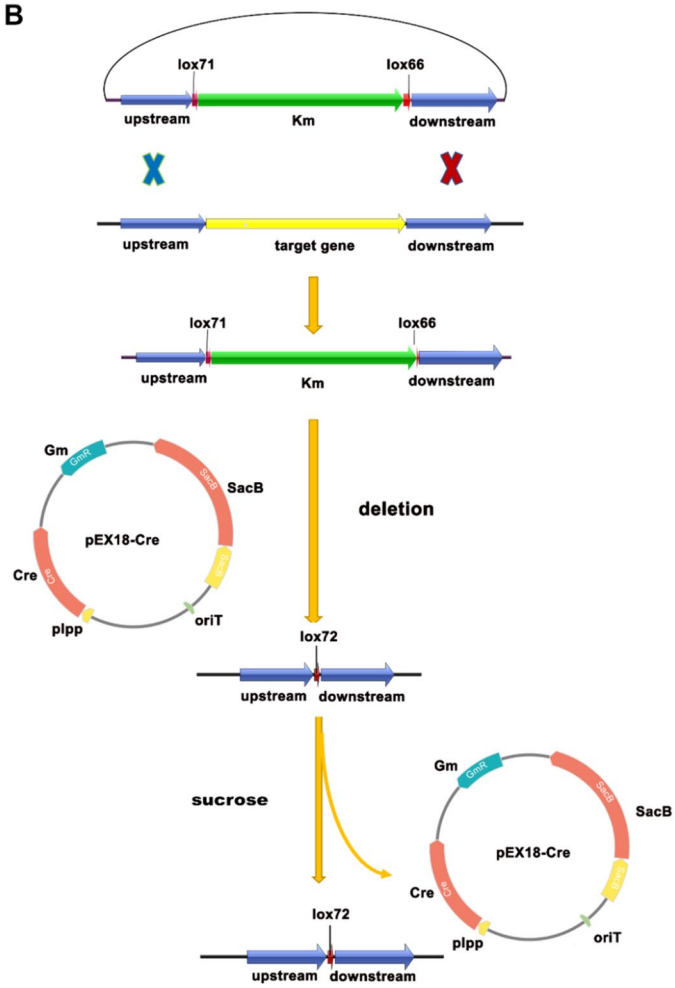
A

Name	Sequences (5'-3')
loxP	ATAACTTCGTATA GCATACAT TATACGAAGTTAT
lox66	ATAACTTCGTATA GCATACAT TATACGAACGGTA
lox71	TACCGTTCGTATA GCATACAT TATACGAAGTTAT
lox72	TACCGTTCGTATA GCATACAT TATACGAACGGTA

B



mutations. However, the antibiotic markers might result in some unexpected effects on our understanding of the pathogenicity. The main challenge is the inability to perform homologous double-crossover without resistance fragments.

Cre recombinase, discovered in phage P1 in 1981, belongs to the λInt supergene family (Sternberg et al. 1981; Hoess et al. 1984; Yarmolinsky & Hoess. 2015). This 38 kDa monomer protein is composed of 343 amino acids and possesses catalytic activity. It can specifically recognize 34 bp *loxP* sequences to facilitate the deletion or recombination of gene sequences between *loxP* sites (Yarmolinsky & Hoess. 2015). Cre recombinases can act on DNA substrates with various structures, including linear, circular, and superhelix DNA, without the need for cofactors. The *loxP* sequence (locus of X-over P1) is composed of two 13 bp inverted repeats and 8 bp spacers, with the spacers determining the direction of *loxP*. During catalytic DNA strand exchange, Cre covalently binds to DNA, with the 13 bp inverted repeat serving as the binding domain of the Cre enzyme.

◂**Fig. 1** Cre-*loxP* system application in *Pectobacterium*. **A** Sequences of the wild *loxP* and its mutants. The *loxP* is original sequence. *lox71* and *lox66* are *loxP* variants having 5 bp changed in the left and right elements, respectively. The mutated sequences are underlined. After modification by Cre recombination, the *lox71-Km-lox66* fragment will turn to be the *lox72* site that contains mutations in both repeats and exhibits reduced affinity for Cre recombinase. **B** Markerless multiple gene knock out method schematic in *Pectobacterium*. The recombinant plasmid will be chemically transformed into *E. coli* S17-1 competent cell using heat shock. After homologous gene fragments double crossover, we will obtain the mutant in which the *loxP-Km* fragment (*lox71-Km-lox66* cassette) has replaced the target genes. pEX18-Cre plasmid then transferred into the mutant strains containing *loxP-Km*. A positive colony will not be able to survive on the LA (Rif + Km) selective plate because the *loxP-Km* fragment was deleted by Cre recombination. The above-mentioned strains were inoculated into LB medium without sucrose and cultured for 20 h in a shaker at 28 °C. A 1:100 dilution of the overnight cultured bacteria solution was transferred to fresh LB medium containing 5% sucrose. A sterilized inoculation ring was dipped into the bacterial solution from the previous step and spread on LA (Rif + 10% sucrose) resistant selective medium to facilitate shedding of the vector. The obtained single colony was verified by Gm, and the resistant medium containing both Gm and Km was used for reverse screening. Finally, a mutant strain with the Km resistance fragment deleted and no Cre expression vector was obtained

When two *loxP* sites are present in the cell genome, the presence of Cre recombinase induces sequence recombination between these sites. The outcome of recombination is determined by the orientation of the two *loxP* sites. If the two *loxP* sites are located on the same DNA strand, the Cre recombinase can effectively delete the sequence between them, as depicted in Fig. 1. However, after recombination, two *loxP* sites would still remain that still exhibit high affinity for Cre recombinase. Therefore, in this study, we utilized two mutant *loxP* sites (*lox71* and *lox66*) to replace the original *loxP* site. The sequences of these mutant sites are provided in Fig. 1A.

Previous research has demonstrated the extensive utilization of the Cre-*loxP* system for multiple gene deletion in various organisms such as mice, plants, yeast, and pathogenic bacteria (Banerjee & Biswas. 2008; Guan et al. 2017; Sauer & Henderson. 1988; Kos. 2004; Chen et al. 2017; Liang et al. 2018). However, its application in *Pectobacterium* strains has not been reported yet. In this study, we firstly introduced the Cre-*loxP* system to construct marker-less multiple-gene deletion mutants in *Pectobacterium* strains.

Materials and methods

Bacterial strains and growth conditions

All the *Pectobacterium* strains and derivate strains were cultured in Luria–Bertani (LB) medium (Wang et al. 2016) with rifampicin (Rif) at 100 μg·mL^{-1} at 28 °C, and *Escherichia coli* containing pEX18 vectors in LB medium with gentamicin (Gm) at 50 μg·mL^{-1} at 37 °C.

Cre expression plasmid construction

The plasmids and synthetic oligonucleotides utilized in this study are documented in Table 1 and Table 2, respectively. The plasmid pEX18Gm is a reliable vector containing the *sacB* sucrose suicide gene, while *cre* is transcribed autonomously by the robust constitutive *E. coli* promoter P_{lpp}. Subsequently, we obtained the Cre expression plasmid, pEX18Gm-Cre (Fig. 1B), which exhibits potent Cre expression in *Pectobacterium* and can be eliminated through sucrose reverse selection.

Preparation of *Pectobacterium* strains genomic DNA

Bacterial DNA genome extraction using the Bacterial Genomic DNA Extraction Kit (TIANGEN, CN), detail steps as below (Wang et al. 2016).

Construction of plasmids for gene deletion containing a *lox71-Km-lox66* cassette

Based on homologous recombination, the upstream and downstream fragments of the target genes are supposed to double-crossover with chromosome. We constructed a plasmid that contained both the upstream and downstream fragments of the target genes, as well as a *lox71-Km-lox66* cassette. So, we can get a mutant that *lox71-Km-lox66* cassette replaced the target gene. The upstream and downstream fragments were amplified from *Pectobacterium* strains genomic DNA, while the *lox71-Km-lox66* cassette was amplified from pET30a. After restriction and ligation, the upstream and downstream

Table 1 strains and plasmids used in this study

Strains and plasmids	The complete genotype	Orgins
Bacterial strains		
Escherichia coli		
S17-1λpir	*λpir pro hsdR*, *recA*	TaKaRa
DH5α	Standard cloning host, carbapenem sensitivity	TaKaRa
Pectobacterium carotovorum subsp. *carotovorum*		
PccS1	Wild type (NCBI GenBank accession no. CP063773.1), Rif ^{R}	isolated from *Zantedeschia elliotiana* in Nanjing, China
Δ*0112*::*loxP-km*	*PccS1_0112* gene knockout mutant of strain PccS1 with *loxP-Km* fragment, Rif^{R}	This study
Δ*0112*::lox72	*PccS1_0112* gene knockout mutant of strain PccS1 with *lox72* site, Rif^{R}	This study
Δ*0112/0181::loxP-Km*	*PccS1_0112* and *PccS1_0181* gene knock-out mutant of strain PccS1 with *loxP-Km* fragment, Rif^{R}	This study
Δ*0112/0181*::lox72	*PccS1_0112* and *PccS1_0181* gene knock-out mutant of strain PccS1 with *lox72* site, Rif^{R}	This study
Δ*0112/0181/3542::loxP-Km*	*PccS1_0112*, *PccS1_0181* and *PccS1_3542*gene knock-out mutant of strain PccS1 with *loxP-Km* fragment, Rif^{R}	This study
Δ*0112/0181/3542*::lox72	*PccS1_0112*, *PccS1_0181* and *PccS1_3542* gene knock-out mutant of strain PccS1 with *lox72* site, Rif^{R}	This study
Δ*1260*::*loxP-km*	*Pcb_1260* gene knockout mutant of strain *Pcb* with *loxP-Km* fragment, Km^{R}	This study
Δ*1260*::lox72	*Pcb_1260* gene knockout mutant of strain *Pcb* with *lox72* site	This study
Δ*1260/0092(loxP-Km)*	*Pcb_1260* and *Pcb_0092* gene knock-out mutant of strain *Pcb* with *loxP-Km* fragment, Km^{R}	This study
Δ*1260/0092*::lox72	*Pcb_1260* and *Pcb_0092* gene knock-out mutant of strain *Pcb* with *lox72* site	This study
pEX18GM	Allelic exchange suicide vector, *sacB* oriT(RP4) *LacZ*, Cre (Causes recombination), Gm^{R}	(Kovach et al., 1995)
pEX18-Cre	Broad host range vector with a P_{lac} promoter, Gm^{R}	This study

fragments, along with the *lox71-Km-lox66* cassette, were successfully cloned into pEX18. The resulting ligated product was then chemically transformed into *E. coli* DH5α competent cells using heat shock. The primers used for this process can be found in Table 2.

lox71-Km-lox66 marker replace target gene

The recombinant plasmid will be chemically transformed into *E. coli* S17-1 competent cell using heat shock. The LB plates containing X-gal (100 μg·mL^{-1}), Gm (100 μg·mL^{-1}), and Km (100 μg·mL^{-1}) were utilized to select the positive colony. Subsequently, we mixed the wild type strain with *E. coli* S17-1, and performed conjugation transfer using *E. coli* S17-1 as the donor to introduce the plasmid into PccS1.

To identify strains with double exchange homologous recombination events, the trans conjugants were plated on LA medium containing 10% sucrose and Km (100 μg·mL^{-1}). In this manner, we obtained the mutant in which the *lox71-Km-lox66* cassette has replaced the target genes.

Removal of antibiotic marker

Escherichia coli S17-1 containing pEX18-Cre and the mutant strains were concentrated and mixed at a 1:1 ratio. The mixture was then dropped onto the filter membrane of an LB (Rif + Gm) resistant plate and incubated at 28 °C for 20 h. Next, the concentrated, mixed culture strains were eluted onto an LA (Rif + Gm) medium. Individual colonies on the plate were selected for PCR verification. Using a

Table 2 Primers for this study

Primers	Sequence (5′–3′)	Purpose
Cre-F	ATGTCCAATTTACTGACCGT	To amplify the Cre
Cre-R	CCCAAGCTTCTAATCGCCATCTTCCAGCA	
plpp-F	CGATAACCAGAAGCAATAAAAAATCAAATC	To amplify the plpp
plpp-R	TATTAATACCCTCTAGATTGAGTTAATCTC	
lox71–Km -F	TACCGTTCGTATAGCATACATTATACGAAGTTATGAAGCTCCCTCGTGC	To amplify the lox71–Km–lox66 cassette from pET30a
lox66–Km -R	TACCGTTCGTATAATGTATGCTATACGAAGTTATCAGGTGGCACTTTTCG	
0112-1F	CGGGGTACCCAGCGTCGCTGCCTCGGTCGCCAAC	To construct mutant strain Δ0112
0112-1R	AGGAACTATGCCAGGAGC	
0112-2F	GCTCTAGAGAAATCCACTACCGCAAAAGTGCCT	
0112-2R	CCCAAGCTTCCTGGCGTCTGGCGAAAT	
0181-1F	CGGGGTACCCGGCCTGCTCCGCTTTTCTCAGCGC	To construct mutant strain Δ0181
0181-1R	TCTGTATTCCCTGTCTGC	
0181-2F	GCTCTAGAGCCTGAAATCCACTACTGCAAAAGT	
0181-2R	CCCAAGCTTAGGTAAGTGATTCGGGTG	
3542-1F	CGGGGTACCTAAAGACACCCTTTTTACCTTCGAC	To construct mutant strain Δ3542
3542-1R	CGGAATTCCCGCAGGGACGATGTTTG	
3542-2F	GCTCTAGACCTTAACGGTGAACTGTAATCCTGT	
3542-2R	CCCAAGCTTTGAAGGCTGTGGGTATGA	
0092-1F	CGGAATTCTTCCAGCGTTCGGCATTG	To construct mutant strain Δ0092
0092-1R	CGGGATCCACGGGACGCTCACTACGC	
0092-2F	GCTCTAGACTCCTTAGAAACCACCCA	
0092-2R	CCCAAGCTTTGTCGCTAAACATCCCTC	
1260-1F	CGGAATTCCGTGGTGGTCAGCGAATA	To construct mutant strain Δ1260
1260-1R	CGGGATCCCTGGCGTGGGCAGAAGAA	
1260-2F	GCTCTAGACCGGCTCTGCATCCAGACCCACTGG	
1260-2R	CCCAAGCTTTGAGCAAGCGGCACAGAA	

toothpick, the verified colonies were dipped onto both the LA (Rif + Km) and LA (Rif + Gm) selective plates. A positive colony could not survive on the LA (Rif + Km) selective plate because the *loxP-Km-loxP* cassette was deleted by Cre recombination.

Eliminating the Cre expression plasmid through sucrose reverse selection

The above-mentioned strains were inoculated into LB medium without sucrose and cultured for 20 h in a shaker at 28 °C. A 1:100 dilution of the overnight cultured bacteria solution was transferred to fresh LB medium containing 5% sucrose and cultured in a shaker at 28 °C. The transfer to fresh LB medium with 5% sucrose was repeated and cultured in a shaker at 28 °C when the bacteria solution showed turbidity. A sterilized inoculation ring was dipped into the bacterial solution from the previous step and spread on LA (Rif + 10% sucrose) resistant selective medium to facilitate shedding of the vector. The obtained single colony was verified by Gm, and the resistant medium containing both Gm and Km antibiotics was used for reverse screening. Finally, a mutant strain with the Km resistance fragment deleted and no Cre expression vector was obtained. Based on this principle, this study modified the strong promoter P_{lpp} of vector pEX18 carrying *sacB* sucrose sensitive gene, so as to construct Cre expression vector that can be normally expressed in *Pectobacterium* strains.

Virulence assay

Virulence assay of the bacteria in the host plant was performed as previously described (Wang et al. 2018; Jiang et al. 2017).

Southern blot

Southern blot of the bacteria in the host plant was performed as previously described (Guo et al. 2013). The genomic DNA of wild-type and mutant strains was extracted for testing. Specific digestion sites containing the target gene were selected, the extracted genome was enzymatically digested and electrophoresed. Then, the electrophoretic band was transferred to a nylon membrane. The membrane was stained and photographed for analysis.

Result

Multiple-markerless gene deletion in *Pectobacterium carotovorum*

In this study, we aimed to investigate the feasibility of using this system for obtaining marker-less gene deletion mutants in *Pectobacterium*. To achieve this, we modified the strong promoter P_{lpp} into vector pEX18, which carries the *sacB* fragment, a gene of sucrose sensitive for selection, to construct Cre expression vector that can be normally expressed in PccS1 strain (Fig. 1B).

PccS1 strain harbors a total of 5 homologous *vgrGs*, and we selected three of them (*PccS1_0112*, *PccS1_0181* and *PccS1_3542*) as examples for gene deletion using the Cre-*loxP* system. Initially, we employed the traditional homologous exchange method, where we exchanged kanamycin resistance fragments (*Km*) and target genes of the homologous genes to obtain mutants containing the *lox71-Km-lox66* (*loxP-Km*) fragment (Fig. 1B). Subsequently, we introduced the pEX18-Cre plasmid into the mutants to delete the *loxP-Km* fragment and then obtained maker-less mutant with *lox72* site. In the later stage of this method, the vector was eliminated by adding sucrose into the medium (5% for LB, 10% for LA) for reverse screening, enabling to obtain marker-less target gene deletion mutants without the pEX18-Cre plasmid.

PCR and southern blot verification demonstrated the successful deletion of the *loxP-Km* fragment from the mutants (Fig. 2AB), and sequencing analysis further confirmed that the *loxP-Km* fragment was indeed deleted. These results clearly indicate the specific removal of the antibiotic marker through the Cre-*loxP* recombination system in vivo. Consequently, we were able to successfully delete three selected genes from the chromosome.

In terms of functional study, whether the *lox72* site affects the pathogenicity of the mutant is an important question. Therefore, virulence assay on Chinese cabbage was conducted. The result shows that mutant containing *lox72* residue macerate host plants at a level similar to the mutants containing *lox71-Km-lox66* cassette, (Fig. 2C and D). Furthermore, when three genes were deleted and virulence was significantly decreased compared to the wild-type strain (Fig. 2C and D), triple-gene deletion mutant containing the *lox72* residue shows pathogenicity similar to the mutants containing the *lox71-Km-lox66* cassette. All these results indicate that *loxP-Km* fragment deletion by Cre will not affect pathogenicity.

Multiple-markerless gene deletion in *Pectobacterium* brasiliense

To further explore whether this method is feasible in other strains of different species in the genus of *Pectobacterium*, we performed a multi-gene deletion based on the Cre-*loxP* system in *P. brasiliense* (*Pcb*). PCR verification shows that double genes (*Pcb_1260* and *Pcb_0092*) were successfully mutated and then *loxP-Km* fragment was deleted (Fig. 3A).

On the other hand, the virulence assay also indicated that *loxP-Km* fragment deletion by Cre did not affect pathogenicity in *Pcb* (Fig. 3B and C). These results demonstrate that multiple genes deletion method is functional in *Pcb*.

Discussion

Pectobacterium is a devastating plant pathogen that affects a variety of crops, vegetables, and ornamentals, causing wilt, rot, and black tibia in roots, stems, leaves, and fruit (Gijsegem et al. 2021). However, until recently, there was no effective technique available for deleting multiple genes in *P. carotovorum*.

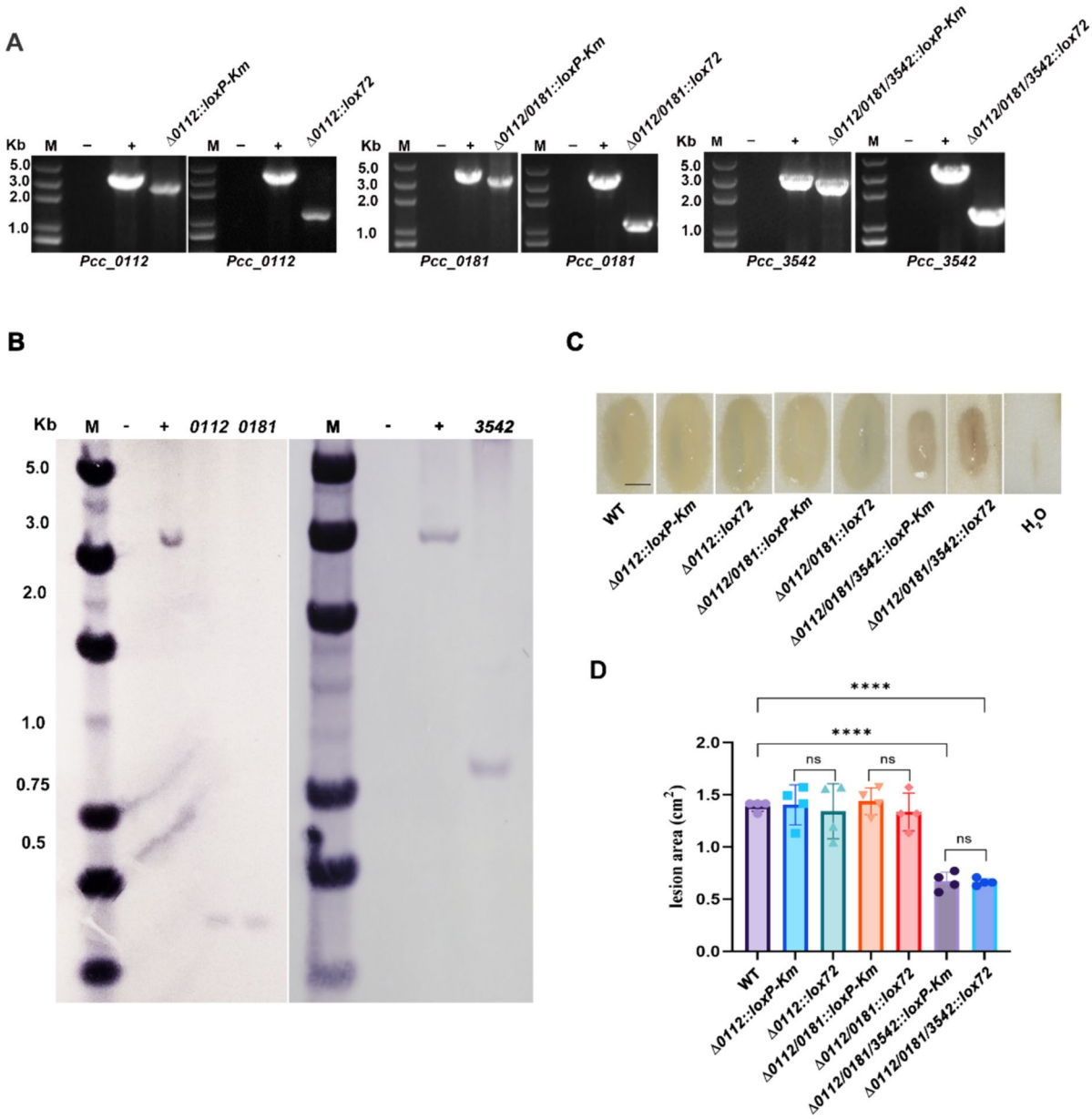


Fig. 2 PCR, Southern blot and virulence verification of *Pectobacterium carotovorum* and the mutants. **A** PCR analyses of single and double genes deletion in *P. carotovorum* using the Cre-*loxP* system. M, the maker; −, negative control; +, positive control. **B** Genomic southern blot analysis on *P. carotovorum* wild type strain and mutant Δ*0112/0181*::*lox72*. The genomic DNA of wild-type and mutant strains to be tested was extracted, specific digestion sites containing the target gene to be tested were selected, the extracted genome was enzymatically digested and electrophoresed, and then the electrophoretic band was transferred to a nylon membrane. The membrane was stained in the color and photographed for analysis. **C** Virulence assay. Images present tissue macerations caused by inoculation of *P. carotovorum* subsp. *carotovorum* strains PccS1 and the strains with a mutation in *vgrGs* with or without *loxP-Km* fragment onto the detached Chinese cabbage. The strains cultured overnight were transferred to fresh LB medium, ddH_2O suspension adjusted $OD_{600}=1.0$, and fresh Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) petiole parts were inoculated, competitively cultured at 28 °C for 16 h, the lesion sites were measured and photographed. Bar, 0.5 cm. **D** Bars represent the maceration areas measured 16 HAI (hours after injection), (****$P<0.0001$, ns, not statistically significant, versus the wild-type PccS1). Statistical analyses are carried out by GraphPad software and assessed by one-way ANOVA, followed by Dunnett multiple comparisons test post-hoc test

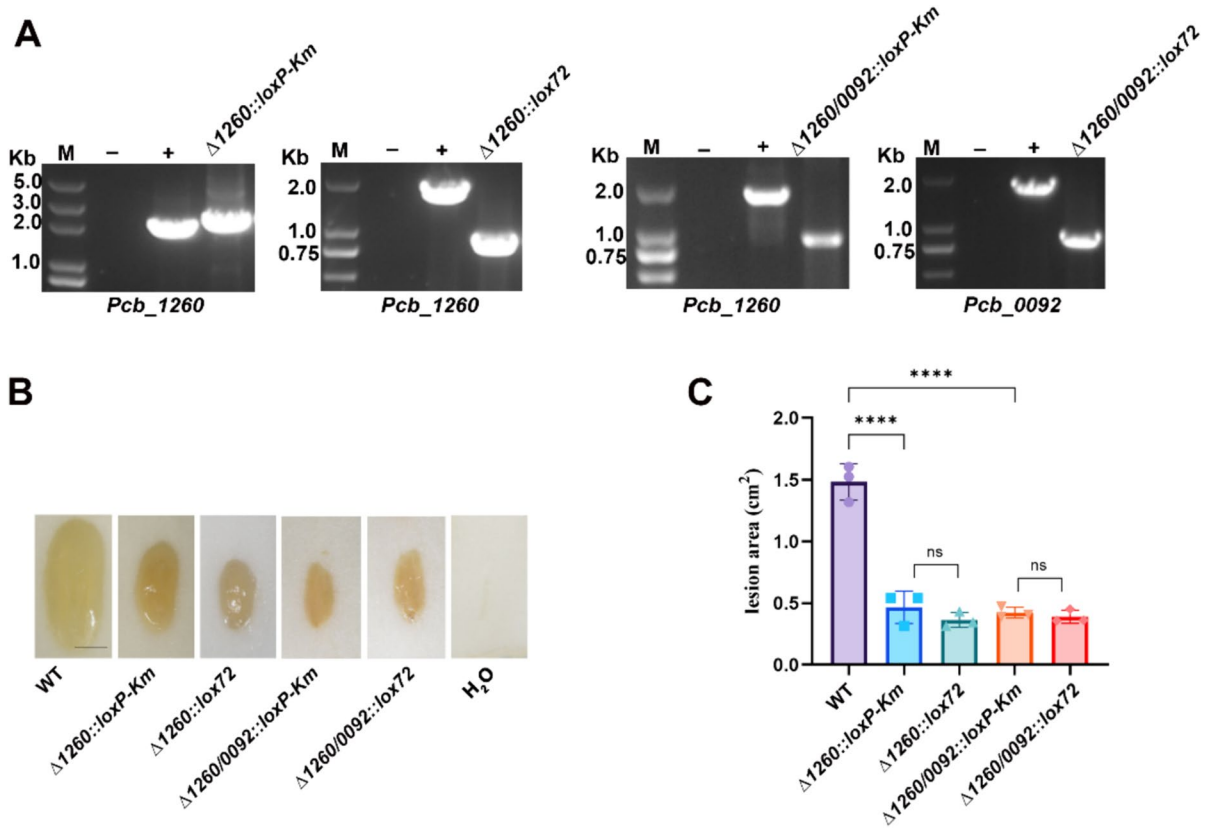


Fig. 3 PCR and virulence verification of *Pectobacterium brasiliense* and the mutants. **A** PCR analyses of single- and double gene deletion strains from the wild type of *P. brasiliense* using the Cre-*loxP* markerless gene-deletion system. M, Maker; −, negative control; +, positive control. **B** Virulence assay. Images present tissue macerations caused by inoculation of *P. brasiliense* strains and the mutants with or without *loxP-Km* fragment onto the detached Chinese cabbage. The strains cultured overnight were transferred to fresh LB medium, ddH_2O suspension adjusted $OD_{600} = 1.0$, and fresh Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) petiole parts were inoculated, competitively cultured at 28 °C for 16 h, the lesion sites were measured and photographed. Bar, 0.5 cm. **C** Bars represent the maceration areas measured 16 HAI (hours after injection), (**$P < 0.01$, ns, not statistically significant, versus the wild-type PccS1). Statistical analyses are carried out by GraphPad software and assessed by one-way ANOVA, followed by Dunnett multiple comparisons test post-hoc test

The main challenge was the inability to perform homologous double-crossover without resistance fragments. In our previous study, we found that the likelihood of obtaining a homologous double-crossover mutant without an antibiotic marker was less than 0.1%. Although substituting target genes with resistance fragments can screen out double-crossover mutants effectively, it is not conducive to the deletion of multiple genes. This problem can be addressed effectively by Cre-*loxP* system.

The Cre-*loxP* system has been successfully applied in various bacteria, including *Streptococcus* and *Bacillus*, as an efficient method for deleting antibiotic markers (Banerjee & Biswas. 2008; Guan et al. 2017).

While we have developed a multiple-gene deletion system based on the Cre-*loxP* system and homologous double-crossover, there are still some limitations. Although the Cre-*loxP* system is user-friendly, it does extend the time required for gene deletion by one week. Another limitation is the use of *loxP* sites, as directly deleting the target gene between *loxP* sites results in a *loxP* site remaining in the mutant after Cre-mediated deletion. If original *loxP-Km-loxP* is used for gene deletion, there will be two *loxP* sites

in the second gene deletion strain, which may cause confusion for Cre recognition.

To overcome these limitations, in the present study, we recruited variant *lox66* and *lox71* to replace the original *loxP* site surrounding the antibiotic marker (Lambert et al. 2007; Albert et al. 1995; Arakawa et al. 2001). *Lox66* and *lox71* are variant forms of *loxP* (Fig. 1A), with five bases changed at the right- and left- elements of *loxP* respectively. By employing Cre recombination to recognize and mediate the *lox71* and *lox66* sites, we achieved the *loxP* double mutant in the *lox72* site, containing mutations in both repeats and exhibiting reduced affinity for Cre recombinase as previous described (Albert et al. 1995).

Our method solves the most fundamental problem of marker-less homologous double-crossover and lays a good foundation for further functional studies of homologous genes.

This study presents the construction of a Cre-*loxP* system for marker-less gene deletion in *P. carotovorum,* which is the first application of this system in *Pectobacterium* (Fig. 1). Additionally, we have successfully used the Cre-*loxP* system to remove antibiotic markers in *P. brasiliense*, resulting in a double mutant (Fig. 2E). Furthermore, whether or not the deleted gene affects pathogenicity, *lox72* has no effect on the results of the experiment (Fig. 2C and D; Fig. 3B and C). Additionally, the significant decrease of pathogenicity after triple-gene deletion also reflects the necessity of researching the function of homologous genes (Fig. 2C and D). These findings suggest that the functionality of the Cre-*loxP* system extends beyond *P. carotovorum* and can be applied to other species of *Pectobacterium*. To the best of our knowledge, this is the first study reporting the use of the Cre-*loxP* system for marker-less gene deletion analysis in *Pectobacterium*. We believe that this approach will help us make more progress in the study of multi-gene function of *Pectobacterium*.

Author contributions All authors contributed to the experimental design, hands on work, discussions, and commented on the manuscript.

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Declarations

Conflict of interest The authors have declared that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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