

Construction of a new type of multi-gene plant transformation vector and genetic transformation of tobacco

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Abstract

A plasmid and two isocaudamer systems, namely, *NotI/Bsp120I* and *SpeI/XbaI/NheI*, were used to construct a new type of multi-gene plant transformation vector system. This system included a transformation vector containing the restriction enzyme cutting sites *Bsp120I* and *XbaI* as well as a cloning vector containing the restriction enzyme cutting sites *NotI*, *Bsp120I*, *SpeI*, and *NheI*. The open reading frame of the new target genes was connected to the transformation vector. The original restriction enzyme cutting site disappeared after connecting to the isocaudamer. The plant transformation vector p096871, which contained *Bacillus thuringiensis* (*Bt*) genes *CryIAc* and *Cry3A* as well as p09X6, which contained *mtlD*, *strD*, *betA*, *nhaA*, and *ostAB*, were constructed using this vector system. Resistant plants were obtained after tobacco was transformed by two vectors via the *Agrobacterium*-mediated method. Detection by PCR revealed that all exogenous genes were inserted into the genome of tobacco. Real-time fluorescence quantification PCR, reverse transcription PCR, and ELISA detections were performed on five transgenic lines transformed by two *Bt* genes. *CryIAc* and *Cry3A* were inserted into the genome with a single copy to transcribe and express *Bt* toxins. The proposed vector system reduced the number of operational procedures and minimized the difficulty of the experiment.

Additional key words: *Agrobacterium tumefaciens*, isocaudamer, *Nicotiana tabacum*, transgenic plants.

Introduction

The application of transgenic technology has greatly enhanced the plant genetic transformation process. Such process was developed from a single-character genetic transformation to a complex genetic transformation with different functions. This process can also be used to identify the biological characteristics of plants (Li *et al.* 2004). Single vector and multiple individual open reading frame (ORF) genes present some merits for plant genetic transformation, such as their simple operation, high efficiency, and non-recombination and separation of offspring. The single transformation vector is regarded as the major approach for multi-gene transformations. This vector mainly adopts the conventional enzyme connection method to construct another transformation

vector that uses a rare restriction enzyme cutting site or a homing endonuclease site (Belfort *et al.* 1997, Goderis *et al.* 2002). The ORF of the target gene is constructed through the gateway technology that is connected to the transformation vector (Hartley *et al.* 2000, Karimi *et al.* 2002, Curtis *et al.* 2003, Chung *et al.* 2005, Chen *et al.* 2006). A special homing endonuclease and Cre/LoxP recombination sequence can transform the artificial bacterial chromosome vector and the supporting auxiliary vector of the transformation system (Odell *et al.* 1990, Trinh *et al.* 2000, Halpin *et al.* 2001, Lin *et al.* 2003). However, these methods are hindered by a series of technical constraints, such as their need to avoid restriction enzyme cutting sites, their complex procedures

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Abbreviations: BA - 6-benzylaminopurine; Cef - cefotaxime; CTAB - cetyltrimethyl ammonium bromide; IBA - indole-3-butyric acid; Kan - kanamycin; MS - Murashige and Skoog; NCBI - National Center for Biotechnology Information; ORF - open reading frame; RT-PCR - reverse transcription PCR;

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and operations, their high cost, and the requirement of special vectors and strains. These challenges hinder the construction of a multi-gene plant expression vector. The creation of a simple and efficient technology for constructing a multi-gene vector is a breakthrough in the field of multi-gene transformation.

This study aimed to construct a new plant transformation vector system that includes an isocaudamer. This vector adopted two different isocaudamer systems, and the ORFs of new target genes were connected to the transformation vector. The original restriction enzyme

cutting site disappeared after the isocaudamers were connected. This method does not require special strains, expensive reagents, and redundant operational procedures; the method needs only a normal digestion and connection to construct a multi-gene plant transformation vector as well as to transform plant by using the *Agrobacterium*-mediated method. Therefore, the proposed system provides a new way for constructing multi-gene transformation vectors and achieving genetic transformation.

Materials and methods

Strains, vectors, plants, and basic reagents:

Escherichia coli strain JM109 and *Agrobacterium tumefaciens* L. strain EHA105 were used in this study. The plasmid vectors p209 and p1964A (Fig. 1) were used to construct the multi-gene plant expression vector. The p209 was the transformation vector that carried the restriction enzyme cutting sites *Bsp120I* and *XbaI*, whereas p1964A was the cloning vector that carried the restriction enzyme cutting sites *NotI*, *Bsp120I*, *SpeI*, and *NheI*. The target genes *CryIAC* [GenBank accession No. AF148644; it can express *CryIAC* toxin (Li *et al.* 1994)], *Cry3A* [acc. No. M84650; it can express *Cry3A* toxin (Kemp *et al.* 1992)], mannitol-1-phosphate dehydrogenase (*mtlD*, acc. No. NC_010473), sorbitol-6-phosphate dehydrogenase (*strD*, acc. No. NC_000913), choline dehydrogenase (*betA*, acc. No. EU032535), Na⁺/H⁺ reverse transportation A gene (*nhaA*, acc. No. NC_012947), and trehalose synthase (*ostAB*, acc. No. NC_010473) were used in this study. All these materials were provided by the Forestry Biotechnology Laboratory of the Agricultural University of Hebei.

The tobacco (*Nicotiana tabacum* L.) cv. Wisconsin 35 used for genetic transformation was provided by Professor Kai Xiao from the Agricultural College of the Agricultural University of Hebei.

Cloning the target genes: The primers for constructing the multi-gene plant transformation vector were designed on the basis of the related genes (information provided by the National Center for Biotechnology Information) and listed in Table 1 Suppl. The plasmid-carrying target gene was used as the template. *Taq* DNA polymerase mixed with *Pfu* DNA polymerase ((*Sangon*, Shanghai, China) was used for PCR. The PCR procedure was as follows: pre-denaturation at 94 °C for 10 min, denaturation at 94 °C for 30 s, annealing at 56 °C for 40 s, extension at 72 °C for 2 min and 20 cycles, and extension at 72 °C for 20 min. After this reaction, 1 % Tris base - acetic acid - ethylenediaminetetraacetic acid (TAE) agarose gel electrophoresis was performed to detect the amplified product fragment and to determine its length. The appropriate fragments were recovered through gel

digestion and by using a *SanPrep* column DNA gel extraction kit (*Sangon*).

Construction and detection of complete ORF: The p1964A plasmid was extracted by using a *SanPrep* column plasmid mini-preps kit (*Sangon*) and was then digested by the restriction enzyme *XcmI*. Afterward, 1 % TAE agarose gel electrophoresis was performed to detect the appropriate fragments, which were then recovered as T vectors by using a *pUCm-T* vector cloning kit (*Sangon*).

The amplified fragment was connected to the gel-reused fragments of the enzyme-digested products of the vector. *E. coli* strain DH10B was grown to competence through thermal activation (prepared using a competence preparation kit produced by *TaKaRa*, Dalian, China). *E. coli* DH10B was then applied on a 50 mg dm⁻³ ampicillin Luria-Bertani (Amp LB) solid medium and was cultured at 37 °C for 12 h when a single colony was created.

The 35# primer was designed based on the end sequence of the CaMV35S promoter, and served as the forward primer. The 35# primer and the corresponding reverse primer comprised a pair of test primers. A single colony was selected for colony PCR identification. If the amplified fragment length was consistent with the target gene length, then the amplified fragments were connected to the cloning vector at a forward direction, which would produce an ORF that comprised the CaMV35S promoter, target gene, and NOS termination sequence. Colonies that passed the test were shaken, and the bacteria were sent to *Sangon* for sequencing before further testing. The target fragment was connected to the vector to form a new vector that included the target gene ORF (Fig. 2).

The target fragment was connected to the vector to form a new vector that included the target gene ORF (Fig. 2). This new vector was named by connecting the abbreviation of the cloning vector with the serial number of amplified primer. For example, the amplified *CryIAC* gene that was based on the 68# primer and connected to p1964A was referred to as p6435+68, whereas the amplified *Cry3A* gene that was based on the 71# primer and connected to p1964A was referred to as p6435+71.

Construction of the plant multi-gene transformation vector: The vector system that was used in this experiment included two parts, namely, the transformation vector and the cloning vector. The transformation vector contained the restriction enzyme cutting sites *Bsp120I* and *XbaI*, whereas the cloning vector contained the restriction enzyme cutting sites *NotI*, *Bsp120I*, *SpeI*, and *NheI*. *Bsp120I* and *NotI* were isocaudamers that shared a 3' overhang CCGG cohesive terminus, whereas *XbaI*, *SpeI*, and *NheI* were isocaudamers that shared a 3' overhang GATC cohesive terminus. Isocaudamers can be interconnected to make the original enzyme digestion locus disappear.

The *Bsp120I* and *XbaI* double digestion transformation vector and the *NotI* and *NheI* double digested cloning vector carrying the ORF of the target gene were both used in the experiment. The plant transformation vector was constructed using the enzyme digestion fragments that were connected after recovering the appropriate fragments. The original *Bsp120I* and *XbaI* sites on the transformation vector as well as the *NotI* and *NheI* sites at both ends of the target gene ORF all disappeared. The *Bsp120I* and *SpeI* sites of the newly synthetic plant transformation vector were connected to the target gene ORF fragments with *NotI* and *NheI* sites through double digestion. The process was repeated to construct plant transformation vectors with multiple target genes (Fig. 3).

Detection of the multi-gene transformation vector: The testing method for the transformation vectors was similar to the ORF detection method. For each newly added target gene, the 35# primer that was designed on the basis of the end sequence of the CaMV35S promoter was used as the forward primer that constituted a pair of test primers. The test fragments served as the reverse primers. During PCR identification, if the amplified fragment length was consistent with the target gene length, then the amplified fragments were connected to the transformation vector at a forward direction.

Tobacco transformation by *Agrobacterium*-mediated leaf disc method: *E. coli* GV3101 competence was prepared by using the competent cell preparation kit (*TaKaRa*). The detected plant expression vector was converted into *E. coli* GV3101 competence via thermal activation. Tobacco was transformed using the *Agrobacterium*-mediated leaf disc method (Horsch *et al.* 1985). Sterile, robust, fresh, and green tobacco leaves were cut into 0.5 - 1 cm² leaf discs along their veins. These leaves were infected with the bacterial liquid that was diluted with 5 % (m/v) sucrose solution (1:1) for 8 min. The leaf discs were removed and dried using a sterile blotting paper and were then inoculated in the co-culture medium: Murashige and Skoog (MS) + 2.0 mg dm⁻³ 6-benzylaminopurine (BA) + 0.1 mg dm⁻³ indole-3-butyric acid (IBA). After dark culture for 2 d,

the leaves were transferred to the screening culture medium: MS + 2.0 mg dm⁻³ 6-BA + 0.1 mg dm⁻³ IBA + 50 mg dm⁻³ kanamycin (Kan) + 400 mg dm⁻³ sodium cefotaxime (Cef). The medium was replaced every two weeks. After growing to 2 cm, the resistant buds were transferred to the rooting medium: MS + 75 mg dm⁻³ Kan + 400 mg dm⁻³ Cef for screening. The samples were placed in a chamber to be cultured at a temperature of 25 ± 2 °C, an irradiance of 27 to 36 μmol m⁻² s⁻¹, and a 14-h photoperiod. The rooting plants were propagated and acclimatized before they were transplanted into the greenhouse.

PCR detection of transgenic plants: The leaves of lines screened by Kan and of the non-transgenic tobacco (control), were collected in the greenhouse. Genomic DNA was extracted using the modified CTAB (cetyltrimethyl ammonium bromide) method (Wang *et al.* 2002). With genome DNA as the template, the forward and reverse primers of *Cry1Ac* and *Cry3A* were used for the PCR of transgenic plants that were transformed by vector p096871, whereas the forward and reverse primers of *mtlD*, *strD*, *betA*, *nhaA*, and *ostAB* were used for the PCR of transgenic plants that were transformed by vector p09X6.

Detection of the copy number of exogenous genes in transgenic plants: Five transgenic lines were chosen from the transgenic tobacco that was transformed by vector p096871. Following the principle of relative quantification, the initial number of copies of the exogenous and housekeeping genes in transgenic tobacco was measured using the real-time fluorescence quantitative PCR technique. These values were then used to identify the copy number of the exogenous gene in transgenic plants.

Using the known single copy endogenous gene *RNR2* (ribonucleotide-diphosphate reductase subunit 2, GenBank acc. No. X92443) in tobacco nuclear genome as well as primer 129-0# that was designed based on its publicly known sequence, primer 129-1# was designed based on the *Cry1Ac* sequence for fluorescence quantitative PCR detection (Table 1 Suppl.). Related sequences were amplified using primers 129-0# and 129-1# from tobacco and plasmid, respectively, and were connected to the cloning vector pUCm-T. The constructed plasmids p1290 and p1291 carried the target fragments. Recombinant plasmid DNA was extracted by using a *SanPrep* column plasmid mini-preps kit (*Sangon*). The abscissa Ct value and the ordinate logarithm of the initial template were used to prepare the standard curve of real-time quantification. The following formula was used to calculate the plasmid copy number: copy number of plasmid DNA (copies cm⁻³) = [concentration of plasmid DNA (g cm⁻³) × volume of plasmid DNA solution (cm³) × 6.02 × 10²³] / {length of plasmid (bp) × 650 [Da (bp × mol)⁻¹] × (1 × 10⁻⁹ g Da⁻¹)}.

Transgenic tobacco genome DNA and gradient-diluted recombinant plasmid DNA were used as templates for the PCR reaction. Fluorescence quantitative PCR was performed three times for each sample by using the platinum *SYBR green* qPCR *super mix-UDG* kit. After obtaining the initial copy number of exogenous and housekeeping genes, the exogenous gene integration copy number was calculated by dividing the exogenous gene initial copy number by the specific housekeeping initial copy number.

RT-PCR detection of exogenous genes *Cry1Ac* and *Cry3A* in transgenic plants: The total RNA in transgenic tobacco lines was extracted using the *UNIQ-10* column total RNA extraction kit (*Sangon*). The reverse transcription of the first chain of cDNA was performed using the *RevertAidTM* first strand cDNA synthesis kit (MBI; *Fermentas*, Lithuania). Synthesized cDNA was used as template, and the *actin* gene (acc. No. NTU60495) was used as housekeeping gene. The RT-PCR that was amplified by the detection primers of *Cry1Ac*, *Cry3A*, and *actin* genes (105#, 106#, and 108#, Table 1 Suppl.) was used to detect gene expression. The RT-PCR reaction was

tested in a *Biometra T1* (Göttingen, Germany) thermocycler. The reaction process was described as follows: pre-denaturation for 5 min at 94 °C, denaturation for 30 s at 94 °C, renaturation for 40 s at 50 °C, extension for 60 s at 72 °C and 30 cycles, and extension for 5 min at 72 °C. After PCR, the amplified product fragment and its length were detected by 1 % TAE agarose gel electrophoresis.

***Bacillus thuringiensis* (Bt) toxin test of transgenic plants:** The third leaf from the top of the field grown tobacco seedlings was collected from each transgenic line and the control. The process for each line was repeated thrice. The Bt toxin of each line was detected using the Bt-Cry1Ab/1Ac and Bt-Cry3A ELISA kits (*Agdia*, Elkhart, USA). A kit was used as the positive control, whereas non-transgenic tobacco was the negative control according to the instructions of the manufacturer. Data were detected using a *BioRad 550* (Hercules, USA) microplate reader. The total protein content was detected using an ultramicro spectrophotometer *K5600* (*Kaiao Technology*, Beijing, China), and the proportion of the toxin to the total protein was calculated afterward.

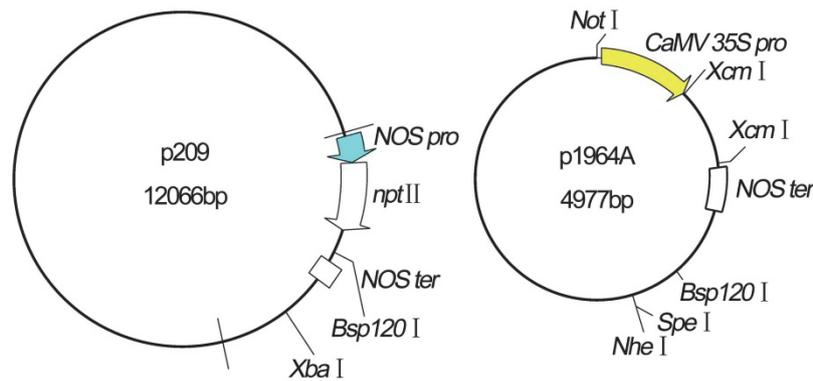


Fig. 1. Plant transformation vector p209 and cloning vector p1964A. The figure shows the structure of the two vectors and the target genes used in the experiment.

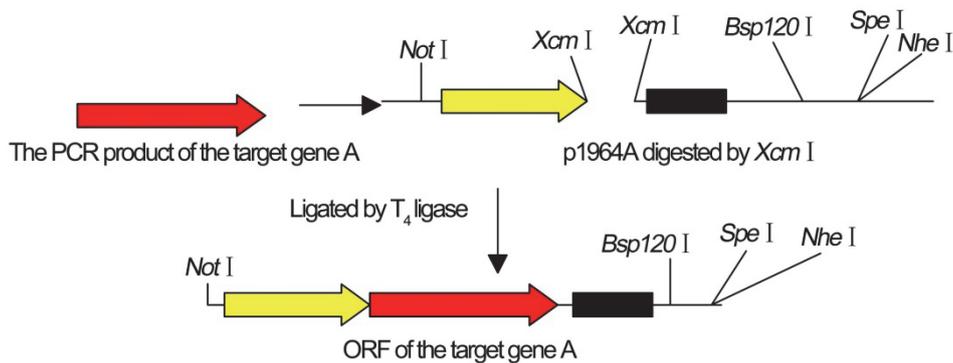


Fig. 2. Construction of a new vector that contains the ORF of the target gene A. The PCR product of the target gene A and the enzyme digestion fragment of p1964A are ligated by *T₄* ligase, then the ORF of the target gene A is constructed. The red arrow represents the target gene A, the yellow arrow represents the promoter, and the black bar represents the terminator.

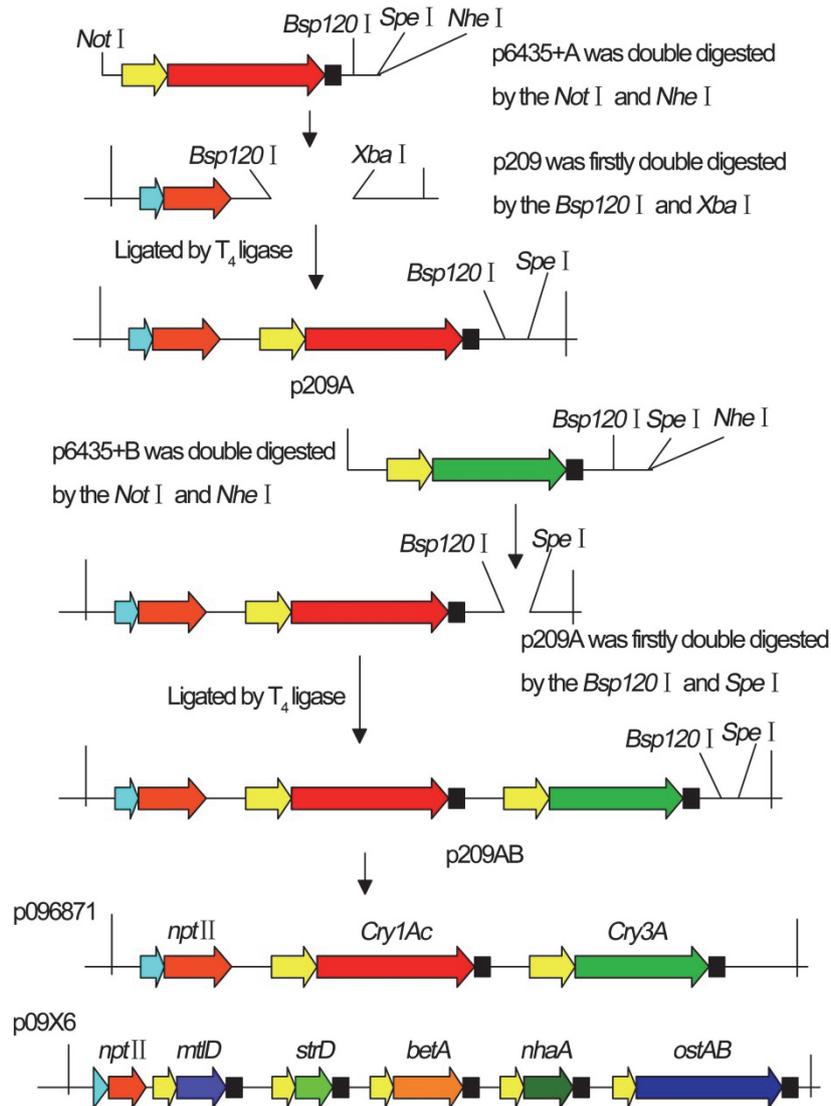


Fig. 3. Construction of a plant transformation vector with multiple genes. The ORF of target gene A ligated with the enzyme digestion fragment of p209 by ligase, and then the recombination vector was digested by two enzymes, so it can connect the ORF of another target gene. So repeatedly, the vectors p096871 containing two *Bt* genes and p09X6 containing five salt-resistant genes were constructed. In the construction scheme of p209AB, the *yellow arrow* represents CaMV35S promoter, *red arrow* represents the target gene A, the *black bar* represents the NOS terminator, the *blue arrow* represents the NOS promoter, the *orange arrow* represents *nptII* gene, and the *green arrow* represents the target gene B. In the construction process of p09X6, five salt-resistant genes are in different colours.

Results

The cloning vector p1964A that underwent enzyme digestion and gel recycling was connected to the PCR fragments of several target genes and was finally transferred into *E. coli* for culturing until the growth of a single colony. The 35# primer was taken as the forward primer, whereas the original reverse primer of the target gene was taken as the reverse primer for the colony PCR amplification. The length of the product fragment was similar to that of the positive control fragment. The

appropriate colonies were shaken, and the bacteria were sent to Sangon for sequencing. The target-gene-amplified fragment was completely forward-connected to the cloning vector, which constructed an ORF that contained the CaMV35S promoter, target gene, and NOS termination sequence.

When a new gene was added during the construction of the multi-gene plant expression vector, the 35# primer was used as the forward primer; the reverse primer of the

tested gene was used as the reverse primer; the *E. coli* total DNA was used as the negative control; and the PCR amplification of the tested gene was used as the positive control for PCR detection. Fig. 4 shows the PCR detection results for plant expression vectors p096871 with two *Bt* genes and p09X6 with five target genes. The positive control amplified the band of the gene fragment,

but the negative control had no band (data not shown). The length of each gene-amplified fragment was nearly similar to that of the positive control fragment, which indicates that the ORF of the target gene was connected to the plant expression vector and that the plant expression vector was constructed successfully.

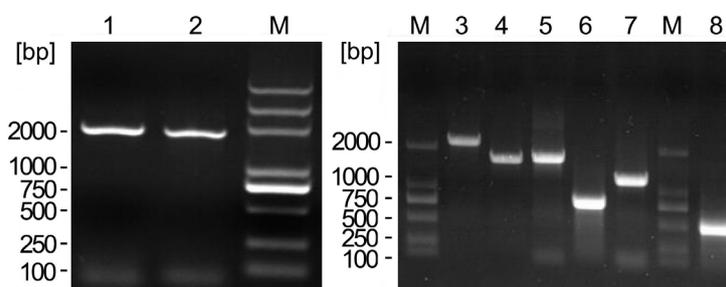


Fig. 4. The multi-gene plant transformation vectors analyzed by PCR: 1 - *Cry1Ac*, 2 - *Cry3A*, 3 - *ostAB*, 4 - *nhaA*, 5 - *betaA*, 6 - *strD*, 7 - *mtlD*, 8 - *nptII*; M - DNA marker DL2000.

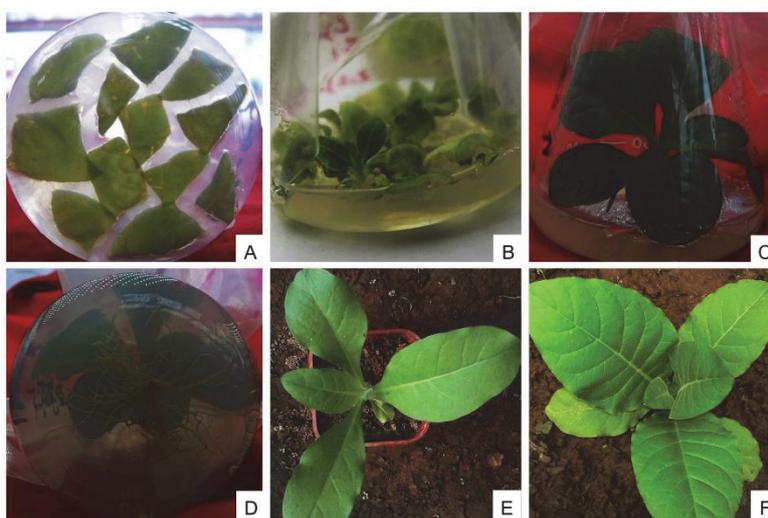


Fig. 5. Survey of culture of obtained transgenic lines. A - Leaves grew on selective medium after *Agrobacterium* infection; B, C - kanamycin-resistant shoots on agar medium; D - rooting of kanamycin-resistant shoot; E - kanamycin-resistant plant in a pot; F - plant transplanted to field.

When screening in the screening culture medium, the leaf discs of transgenic tobacco can be differentiated from the kanamycin-resistant shoots, but the control cannot be differentiated. The kanamycin-resistant shoots were transferred to screening rooting medium. The vast majority of the lines exhibited rooting, whereas the control did not exhibit any rooting. The rooting lines were transferred to the screening differentiation medium and were well differentiated. The re-differentiated resistant shoots were transferred to the rooting medium and then transplanted to the pots. After acclimatization, the rooting shoots were transplanted to the greenhouse. A total of 10 transgenic lines transformed by vector p096871 and 12 transgenic lines transformed by vector p09X6 were obtained *via* Kan screening (Fig. 5).

The DNA of five transgenic lines that were randomly picked from each transgenic series and untransformed tobacco (control, CK) were extracted before the PCR amplification of exogenous genes. Fig. 6 shows that the target bands of neomycin phosphotransferase II gene (*nptII*, 473 bp, the selective marker gene), *Cry1Ac* (546 bp), and *Cry3A* (667 bp) were amplified from five lines transferred by two *Bt* genes, which indicates that the target genes were inserted into the tobacco genome. Fig. 7 shows that the target bands of *nptII* (473 bp), *mtlD* (275 bp), *strD* (284 bp), *betaA* (241 bp), *ostAB* (203 bp), and *nhaA* (203 bp) were amplified from five lines transferred by two multiple genes (including *mtlD*), which indicates that the target genes were inserted into the tobacco genome.

The initial copy numbers of amplification products of the specific housekeeping and target gene sequences were obtained according to the standard curve (Table 1). Table 1 shows that the copy numbers of the exogenous gene *Cry1Ac* in five transgenic tobacco plants were 0.927, 0.886, 0.895, 0.856, and 0.863. In the integral function analysis, these numbers were all equivalent to 1, which indicates that the exogenous genes in five transgenic lines were single copies.

The total RNA of five transgenic tobacco lines was extracted for reverse transcription and for obtaining the first chain of cDNA. Fig. 8A shows the total extracted RNA of each transgenic plant, 28S and 18S were clearly visible. Fig. 8B shows the transcription abundances of *actin* genes in each line. There were obvious differences

in *Cry1Ac* gene expressions among five lines (Fig. 8C). Line 2 had the highest transcription abundance, followed by lines 3, 1, 4, and 5. There were also obvious differences in *Cry3A* gene expressions among five lines (Fig. 8D); the line 2 has the highest expression, followed by lines 1, 3, 4, and 5.

The toxins *Cry1Ac* and *Cry3A* were detected in all five lines (Table 2). The proportion of two toxins in line 2 was the highest, and in other four lines it was lower and with some differences among them. The change in the proportion of toxins to total protein in these five lines was consistent with the semiquantitative RT-PCR results. Table 2 also shows that the expression of *Cry3A* gene was higher than that of *Cry1Ac* gene.

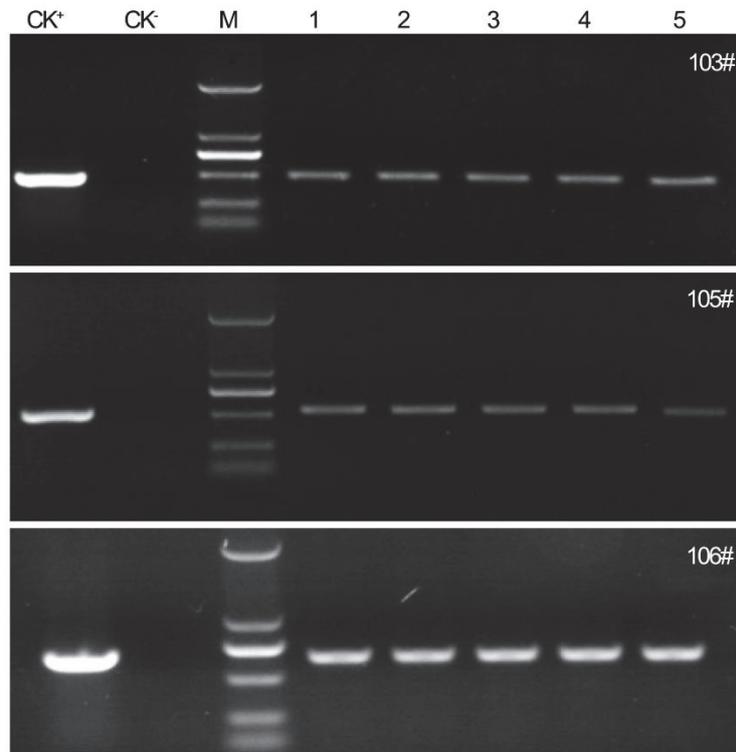


Fig. 6. PCR identification of the transgenic *Bt* plants: 103# - *nptII*, 105# - *Cry1Ac*, and 106# - *Cry3A*. CK⁺ - positive control, CK⁻ - negative control, M - marker DL2000 (the sizes of marker bands are 2000, 1000, 750, 500, 250, 100 bp from top to bottom); 1 to 5 - individual lines of transgenic plants.

Table 1. Exogenous gene copy numbers in transgenic plants.

Line number	Target gene <i>Cry1Ac</i>	Housekeeping gene <i>RNR2</i>	Exogenous gene copy numbers
1	$1.46 \times 10^5 \pm 2.20 \times 10^4$	$1.58 \times 10^5 \pm 2.89 \times 10^4$	0.927 \pm 0.028
2	$2.48 \times 10^5 \pm 2.47 \times 10^4$	$2.80 \times 10^5 \pm 2.29 \times 10^4$	0.886 \pm 0.016
3	$8.23 \times 10^4 \pm 2.30 \times 10^3$	$9.20 \times 10^4 \pm 4.10 \times 10^3$	0.895 \pm 0.015
4	$3.15 \times 10^5 \pm 2.00 \times 10^4$	$3.68 \times 10^5 \pm 2.25 \times 10^4$	0.856 \pm 0.050
5	$1.17 \times 10^5 \pm 8.72 \times 10^3$	$1.37 \times 10^5 \pm 1.64 \times 10^4$	0.863 \pm 0.129

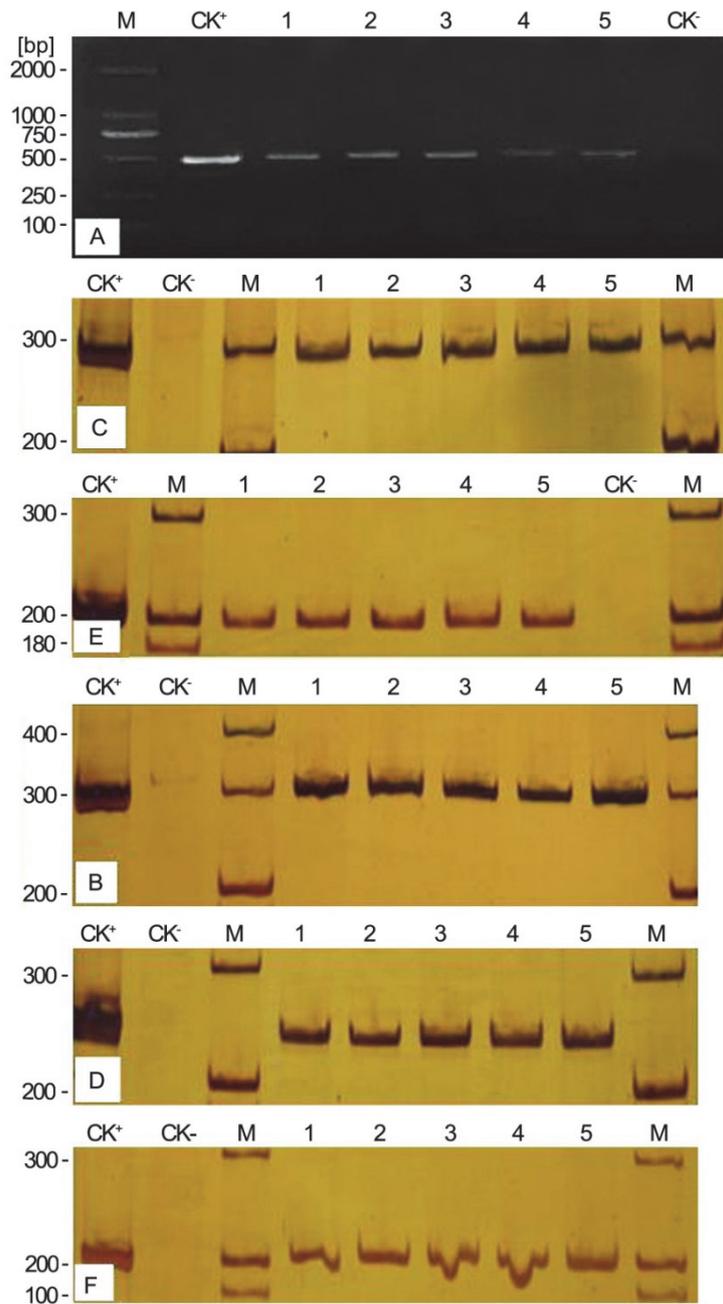


Fig. 7. PCR identification of the transgenic plants: A - *nptII*; B - *mtlD*; C - *strD*; D - *betA*; E - *nhaA*; F - *ostAB*. CK⁺ - positive control; CK⁻ - negative control; M - marker; 1 to 5 - individual lines of transgenic plants.

Table 2. Proportion of toxins to total protein in transgenic plants.

Sample	1	2	3	4	5
Cry1Ac toxin	7.27×10^{-8}	5.37×10^{-6}	2.72×10^{-6}	8.53×10^{-8}	1.51×10^{-7}
Cry3A toxin	1.78×10^{-3}	3.74×10^{-3}	1.92×10^{-3}	1.13×10^{-5}	4.10×10^{-5}

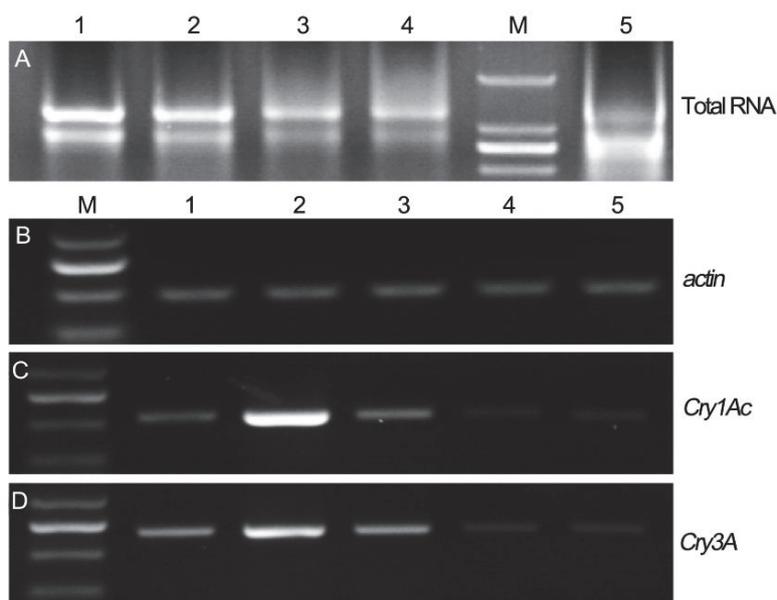


Fig. 8. RT-PCR detection of genes in transgenic tobacco plants. *A* - Total RNA; *B* - *actin* (481 bp); *C* - *Cry1Ac* (546 bp); *D* - *Cry3A* (667 bp). M - marker (marker bands in *A* are 2000, 1000, 750, and 500 bp, in *B*, *C*, *D* are 1000, 750, 500, and 250 bp); 1 to 5 - individual lines of transgenic plants.

Discussion

Construction of a multi-gene plant expression vector, carrying multiple genes into the plant genome, is considered as a highly efficient multi-gene transformation method, and has become a hot research topic (Fitzgerald *et al.* 2006, Underhill *et al.* 2007, Zeevi *et al.* 2012). However, the construction and assembly of multi-gene vectors is marred by several technical problems, such as the number of enzyme sites in the multiple cloning site, existence of the same enzyme site between the target gene and the vector, incomplete digestion, filling-in of cohesive ends, and the use of different enzymes for dephosphorylation treatment, all of which increase the difficulty of the experiment procedure.

A rare restriction enzyme cutting site or homing endonuclease site has been introduced to overcome the restrictions of the enzyme cutting site in vectors and target genes. These restriction enzyme cutting sites have long recognition sequences (12 to 40 recognition sequences), and the probability of isolating the exactly same sequence of these sites on the DNA fragment is minimal (Belfort *et al.* 1997, Goderis *et al.* 2002). The influences between the vector and the target gene can be avoided by using these enzyme cutting sites. However, the rare restriction enzyme cutting sites that are contained in a vector are limited and cannot be increased infinitely. An auxiliary vector is needed for each newly added target gene. However, the practical application of this vector is negatively influenced by its complex operations and the difficulty of obtaining the parts of homing endonucleases.

Gateway technology uses the two-way BP ($\text{attB} \times$

attP) and LR ($\text{attL} \times \text{attR}$) clonase reactions of a specific recombination system of λ phage locus. Enzyme digestion is not required after the target gene is connected to the preliminary vector. Recombinase can quickly and directionally recombine to any vector after gateway treatment. With certain suicide genes such as *ccdB* and *sacB*, the high-efficiency separation of recombination cloning can be guaranteed, and a cloning efficiency of over 95 % can be obtained (Karimi *et al.* 2002, Earley *et al.* 2006). Nevertheless, the gateway technology is influenced by fragments that contain a similar recombination or a conserved sequence or by fragments with complex structures. Aside from the high operating cost and complex procedure of this technology, a specific segment of the phage recombinase recognition sequence (about 23 bp) also exists between the promoter and the target gene, which generates an immeasurable uncertainty on the target gene expressions.

The proposed plant transformation vector system requires only a transformation vector and a cloning vector for constructing the ORF of the target gene. Two groups of isocaudamer systems, namely, *NotI/BspI20I* and *SpeI/XbaI/NheI*, are adopted for this vector. After the characteristics of the original restriction enzyme cutting site disappeared after the isocaudamer is connected, new genes and their ORFs are connected constantly between the transformation and cloning vectors. By using two different isocaudamer systems, the vectors and fragments after digestion always have different cohesive ends, which prevent intralooping during the vector connection

process. The proposed vector does not require a dephosphorylation treatment after digestion, which simplifies the operating procedure and reduces the test difficulty. The system also has a mature and reliable traditional digested connection technology, simple operation, and low cost. Two multi-gene plant transformation vectors are constructed by using this vector system. Two vectors are used, of which one carries two *Bt* genes and the other carries five exogenous genes, including *mtlD*. Transgenic tobacco is obtained using the *Agrobacterium*-mediated method (Fig. 5). PCR detection shows that the exogenous genes are inserted into the tobacco genome, which proves the applicability of this vector system for constructing a multi-gene transformation vector that could transform plants (Figs. 6, 7).

RT-PCR is used to certify the transgenic tobacco lines that have been transformed by two clear function *Bt* genes. *CryIAc* and *Cry3A* are both single copies in all five transgenic tobacco lines. The expression of *Bt* toxin was studied using ELISA technology, and although both *CryIAc* and *Cry3A* could express toxins, the toxin content varied across each line (Table 2). Such variation may be attributed to the position of the exogenous gene that is inserted into different lines. In the same transformation event, the exogenous gene expression also varies among different lines, which is certified by many transgenic studies (Li *et al.* 2005). After comparing the two *Bt* gene expression conditions, the transcription and protein expressions of *Cry3A* are apparently higher than those of *CryIAc* (Table 2, Fig. 8). Wang *et al.* (2012) obtained a transgenic poplar and inserted two *Bt* genes into different positions in the poplar genome through a secondary reforming process. They showed that the two genes have high expression efficiency; the *Cry3A* toxin content in the genome was 10 times higher than the

CryIAc content.

As a practical application value, the proposed vector can carry multiple target genes and simultaneously transform control elements to meet the demand of multi-gene character production. This vector can also carry different kinds of target genes and achieve simultaneous improvement of several characters. The vector has also a simple, convenient, and low-cost operation. However, this vector system has its own shortcomings, the most prominent of which is the existence of only a single species of promoters and termination sequences that form ORF (*i.e.*, CaMV35S promoter and NOS termination sequence). This limitation has led to several repeated sequences in the vector after construction. The gene expression may also be inhibited or silenced after the transformation of the plant. This limitation can be addressed by cloning those vectors that carry different promoters and those ORFs that carry different promoters. The proposed vector system also applies two groups of regular isocaudamers of restriction enzymes, which may appear in the target gene sequence and further affect enzyme digestion and construction. The *SpeI/NheI* sites that are formed by six nucleotides have high probability to generate recognition sequences. Therefore, the author has arranged *SpeI* and *NheI* together in the sequence design (Fig. 1). In accordance to the restriction enzyme cutting site of the target gene, the enzyme cutting site that couples with *NotI* can be selected in the experiment (with the premise that *SpeI/NheI* will not appear in the target gene simultaneously), which greatly reduces the limitation of enzyme cutting sites. The problem involving the enzyme cutting site can be solved by introducing multiple isocaudamer systems and by constructing a series of vectors that carry different isocaudamers (currently in progress).

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