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A novel double T-DNA system for producing stack and marker-free transgenic plants

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Abstract

This study aimed to develop a new vector system to remove selection genes and to introduce two or more genes of interest into plants in order to express them in a coordinated manner. A multigene expression vector was established based on pCamBIA2300 using a selectable marker gene (SMG)-free system based on the combination of the isocaudamer technique and double T-DNA. The vector DT7 containing seven target genes was constructed and introduced into tobacco using *Agrobacterium*-mediated transformation. Twenty-one of 27 positive transgenic plants contained both T-DNA regions. The co-transformation frequency was 77.8 %. The frequency of unlinked integration of two intact T-DNAs was 22.22 % (6/27). The frequency of removal of SMG from transgenic T1 plants was 19.10 %. These results suggest that this vector system was functional and effective for multigene expression and SMG-free transgenic plant cultivation. At least seven target genes can be co-expressed using this system. Overall, these findings provide a new and highly effective platform for multigene and marker-free transgenic plant production.

Additional key words: *Agrobacterium tumefaciens,* isocaudamer technique, multi-gene stack, *Nicotiana tabacum.*

Introduction

Two factors have limited the development and use of biotechnology in agriculture. One is the concern over a selectable marker gene (SMG) remaining in the engineered crops, and the other is the need to transform multiple genes in order to express several interacting proteins involved in a specific agronomic trait. Developing new techniques that remove the SMG and/or introduce two or more genes of interest (GOIs) and express them in a coordinated manner has become an important research area.

Co-transformation is a frequently used strategy to produce multigene or SMG-free transgenic plants. The linked genes strategy includes all of the GOIs and SMGs within a single transfer DNA (T-DNA). This approach allows the simultaneous introduction of multiple GOIs and SMGs to a plant cell in one generation, often with all target genes integrated at the same chromosome locus (Ambros *et al.* 1986, Chyi *et al.* 1986, Komari *et al.* 1996, McCormac *et al.* 2001, Miller *et al.* 2002, Zhou *et al.* 2003, Breitler *et al.* 2004, Chen *et al.* 2005, Yau and Stewart 2013).

Some multigene co-transformation vector systems have been developed using recombination-based cloning strategies (Cheo *et al.* 2004, Sasaki *et al.* 2004, Karimi *et al.* 2005, Wakasa *et al.* 2006, Chen *et al.* 2006, 2010), a combination of engineered zinc finger nucleases (ZFNs) and homing endonucleases (Zeevi *et al.* 2012), the isocaudamer technique (Sun 2008), and different combinations of traditional methods (Wu *et al.* 2005, Fujisawa *et al.* 2009).

Multigene or SMG-free transgenic plants have been obtained with high efficiency in tobacco, soybean, maize, and rice using the double T-DNA method (Miller *et al.* 2002, Zhou *et al.* 2003, Breitler *et al.* 2004, Chen *et al.* 2005, Lu *et al.* 2009, Lin *et al.* 2011, Kapusi *et al.* 2013). Nevertheless, the rigid design of the existing double T-DNAs system and the limitations of type II restriction enzyme sites on the multi cloning site (MCS) hinder its

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Abbreviations: cn - copy number; CF - co-transformation frequency; CT - cycle threshold; GOI - gene of interest; GUS - β-glucuronidase; Kan^R - kanamycin resistant; Kan^S - kanamycin sensitive; LB - left border; MCS - multi cloning site; MS - Murashige and Skoog; Nos P - nopaline synthase promoter; Nos T - Nos terminator; PPT^R - phosphinothricin (PPT)-resistant plant; PPT^S - PPT-susceptible; RB - right border; SMG - selectable marker gene; YEB - yeast extract broth; ZFNs - zinc finger nucleases.

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ability to carry many more genes than a single transformable unit.

Isocaudamers are restriction enzymes that come from different resources but have similar recognition sequences and form the same sticky end. Using this characteristic of isocaudamers, Sun (2008) constructed an expression vector containing four genes that are related to the biosynthesis of the polyunsaturated fatty acid EPA. Meng *et al*. (2009) expressed three peptide-linked β2-microglobulin genes in *E. coli*. Therefore, the isocaudamer technique is a useful strategy to solve the difficulties finding appropriate unique restriction sites when three or more genes need to be linked to make a multigene construct.

In this paper, we describe a vector assembly system that is suitable for cultivation of multigene and SMG-free transgenic plants in that it supports the construction of vectors not only by the isocaudamer technique but also by the double T-DNA method. We describe the design of this system and demonstrate its use by cloning seven different genes into a double T-DNA transformation vector. We also show that this vector can be used for the production of multigene and SMG-free transgenic plants by *Agrobacterium*-mediated transformation and offspring selection.

Materials and methods

Materials: The vector pCamBIA2300 was purchased from *Cambia* (Canberra, Australia). A taq polymerase enzyme, DNA purification kit, T-vector, *E. coli* strains, and other reagents were purchased from *TakaRa* (Beijing, China). The restriction enzymes were purchased from *New England Biolabs* (Ipswich, MA, USA). Primer syntheses and DNA sequencing were done by *Shanghai Sangon Biotech* (Shanghai, China). Bivalent gold-labeled test strips Cry1Ab/1Ac/cp4 were kindly provided by Lin lab of the Biotechnology Research Institute of CAAS.

Tobacco plants (*Nicotiana tabacum* L. cv. NC89) were grown in a greenhouse.

Construction of the double T-DNA plant expression vector: Two primers that reverse-complement each other, DT2300-F (5'-AGCTACTAGTGGGCCCAGATCTCC ATGG-3') and DT2300-R (5'-TCGACCATGGAGATC TGG GCCCACTAGT-3'), were synthesized according to the sequence of the multiple cloning site. They included the *Xho*I and *Hin*dIII sticky ends at the 5'-end of DT2300-F and 3'-end of DT2300-R, respectively. The MCS2 vector was obtained by an equimolar mix of DT2300-F and DT2300-R at the following reaction conditions: initial denaturation at 98 °C for 5 min, denaturation at 94 °C for 40 s, and renaturation at 60 °C for 5 min. The MCS2 was inserted between the *Xho*I and *Hin*dIII sites of pCamBIA2300 to form the intermediate vector p2300N.

The primers DT2300-sacII-F (5'-GTACCGCGGTGA TCACAGGCAGCAACGCT-3') and DT2300-sacII-R (5'-CGACCGCGGACATACAAATGGACGAACG-3') were designed and synthesized according to the left and right border sequences of the T-DNA. A DNA fragment containing the T-DNA borders and MCS2 was amplified using DT2300-sacII-F/DT2300-sacII-R as the primer and p2300N as the template. The PCR conditions were: 98 °C for 10 min followed by 30 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 60 s, with a final cycle of 72 °C for 10 min. The amplification product, containing T-DNA and MCS2 (TMS), was ligated into the cloning vector pMD18-T to form the intermediate vector pT-1. The TMS DNA fragment was then inserted into the *Sac*II site of pCamBIA2300 to form the double T-DNA plant expression vector pDT2300.

Two primers that reverse-complement each other, 2300M-F (5'-AGCTGGATCCGTCGACAAGCTTAC TAGTGGTACCCCCGGGCTGCAGTCTAGAGAGCTC GAATTCCTCGAGAGATCT-3') and 2300M-R (5'-TCGAAGATCTCTCGAGGAATTCGAGCTCTCT AGACTGCAGCCCGGGGGTACCACTAGTAAGCTTG TCGACGGATCC-3'), were synthesized according to the sequence of the multiple cloning site. They had the *Xho*I and *Hin*dIII sticky ends at the 5'-end of 2300-F and 3'-end of 2300-R, respectively. Multi cloning site 1 (MCS1) was obtained by an equimolar mixture of 2300-F and 2300-R at the following reaction conditions: an initial denaturation at 98 °C for 5 min, then denaturation at 94 °C for 40 s and renaturation at 70 °C for 5 min. MCS1 was inserted between the *Xho*I and *Hin*dIII sites of pCamBIA2300 to form the cloning vector p2300M.

Primers were designed based on the sequences of the target genes *gus* (β-glucuronidase gene), *bar* (bialaphos resistance gene), *gfp* (green fluorescent protein gene), *hptII* (hygromycin phosphotransferase II gene), *Cry1Ab/1Ac*, *cp4-epsps*, *nptII,* promoters Nos, CaMV35S, and Gacab5 (promoter of chlorophyll *a*/*b* binding protein from *Gossypium arboretum*), and Nos terminator. Corresponding restriction enzyme sites were introduced at the ends of the primers for convenient vector construction (Table 1 Suppl.). Target DNA fragments were amplified using corresponding primers and templates. The amplification products were ligated with pMD18-T and sequenced.

The CaMV35S promoter was inserted between the *Bam*HI and *Hin*dIII sites of the cloning vector p2300M to form 2300M-35S. The Nos terminator was inserted between the *Xho*I and *Bgl*II sites of 2300M-35S to form 2300M-35SN. Then, *nptII* was inserted between the *Kpn*I and *Pst*I sites of 2300M-35SN to form the single gene expression vector 2300MNPTII. A single *gus, bar, gfp, hptII,* and *cp4-epsps* gene expression vector was constructed using the same technique and corresponding restriction enzymes.

The vector 2300MNPTII was digested with *Spe*I and

*Xba*I. The *nptII* expression cassette was recovered and inserted to the *Spe*I site of DT2300 to form DT1. Then, *cry1Ab/1Ac* was inserted to the *Xho*I site of DT1 to form DT2. Afterward, *hptII*, *cp4-epsps*, *bar*, *gus,* and *gfp* expression cassettes were inserted into the *Bam*HI site to form the the multi-gene expression vector DT7 (Fig. 1).

Tobacco transformation: The final plant expression vector DT7 was mobilized into the *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method. The transformed bacteria were grown on yeast extract broth (YEB) medium containing 100 mg dm^{-3} kanamycin and shaken at a temperature of 28 °C and 150 - 250 rpm overnight. The cultures were diluted 1:1 with YEB and allowed to grow to an absorbance (measured at 550 nm) of approximately 0.8. Tobacco leaf discs from approximately 4-week-old shoot cultures were used for transformation with *A. tumefaciens.* After infection, the leaf discs were incubated on a co-cultivation medium with Murashige and Skoog (MS) salts, 3 % (m/v) sucrose, 2 mg dm^{-3} 6-benzylaminopurine, and 0.1 mg dm^{-3} α-naphthalene acetic acid at a temperature of 28 °C in the dark for 3 - 4 d and then selected on a co-cultivation medium containing 500 mg dm^{-3} cephalosporin and 100 mg dm^{-3} kanamycin. The selected transgenic plantlets were then allowed rooting on media containing MS salts, 3 % sucrose, 100 mg dm^{-3} kanamycin and 500 mg dm^{-3} cephalosporin. The rooted shoots were transferred to soil to allow them to blossom and have seeds.

Screening of tobacco co-transformants by PCR: The genomic DNA of transgenic tobacco was extracted from leaf tissue according to the instructions of the DNA extraction kit. PCR detection of different target genes was performed using the genomic DNA as template and the corresponding primers (Table 1 Suppl.); these were the same primers used for target gene cloning and suitable amplification conditions.

Analysis of two T-DNA integration in tobacco genomic DNA: According to the sequence of the *Cry1Ab/1Ac* and *nptII* genes that were located at different T-DNA regions, the primers nptIICry1Ab/1Ac1543-F (5'-CTCCAATAC AGTTCCAGCTACAGCT) and nptIICry1Ab/1Ac1543-R (5'-CTGCTTGCCGAATATCATGGTGGA) were designed and synthesized. Amplification by PCR was performed using the tobacco genomic DNA as template, nptIICry1Ab/1Ac1543-F/nptIICry1Ab/1Ac1543-R as primers at the following conditions: 94 °C for 8 min followed by 30 cycles of 94 °C for 40 s, 60 °C for 50 s, and 72 °C for 2 min, with a final cycle at 72 °C for 10 min.

Transgene insertion number analysis: Real-time quantitative PCR was used to assess the copy number of the inserted *nptII* gene in the transgenic plants. The primer pairs Ef (CTATCAGGACATAGCGTTGG)/Er (GCTCAGAAGAACTCGTCAAG) and Rf (GACGAA GCTTACTGAGGAAC)/Rr (CCAACAATCTATCAG CCACG) were designed according to the gene sequences of *nptII* and *rnr2*, which encode neomycin phospho-transferase (the most frequently used marker in plant transformation experiments) and ribonucleotide reductase (an endogenous reference), respectively. Real-time PCR was carried out individually with the genomic DNA from transgenic plants as templates on an *ABI 7500* real time PCR system (*Applied Biosystems*, Carlsbad, USA) with the following reaction conditions: 95 °C for 30 s followed by 45 cycles of 95 °C for 5 s, 52.8 °C for 34 s, and 70 °C for 40 s. The initial *nptII* and *rnr2* template copy numbers were derived from cycle threshold (CT) values, and the inserted gene copy number was estimated by the ratio of the initial template copy number of *nptII* to that of *rnr2*.

Phenotypic and molecular assays of T1 seedlings: To identify co-transformed lines that had segregated the SMG and GOI, about 100 T1 seeds were sterilized and inoculated onto a medium containing MS salts, 3 % (m/v) sucrose, and 12 mg dm^{-3} phosphinothricin (PPT) for germination. Seedlings with green leaves and growing vigorously were considered PPT-resistant plants (PPT^R) and otherwise defined as PPT-susceptible (PPT^S). Seedlings were evaluated by leaf color (green or yellow) after 20 d of culture. Leaf bleach assay was performed on the T1 seedlings of PPT^R to identify the expression of the *nptII* gene. The assay was performed by applying a 500 mg dm^{-3} kanamycin solution on a leaf using a brush. Plants that showed no bleaching were designated as kanamycin resistant (Kan^R), while those that bleached were defined as kanamycin sensitive (Kan^S). The results were recorded on the 10^{th} day after application. The marker-free plants identified based on phenotypic assays (PPT and kanamycin resistance) were advanced for confirmation through PCR as described previously and partial target protein detection. Histochemical assay for β-glucuronidase (GUS) activity was performed on leaf tissue as described by Jefferson *et al*. (1987). The Cry1Ab/1Ac and CP4 EPSPS were detected using bivalent gold-labeled test strips according to the manufacturer's (Aochuangjinbiao Bio com, Beijing, China) instructions.

Results and discussion

In this study, we established a multigene expression and SMG-free system by combination of the isocaudamer technique and double T-DNA. The system contains the vectors DT2300 and 2300M. The DT2300 is a double T-DNA plant expression vector suitable for *Agrobacterium*-mediated transformation. On DT2300, there are two T-DNA regions: both the left border (LB) and the right border (RB) are from pCamBia2300 and in the LB2-RB2-LB1-RB1 order without any intervening sequence between the two adjacent T-DNAs. In the

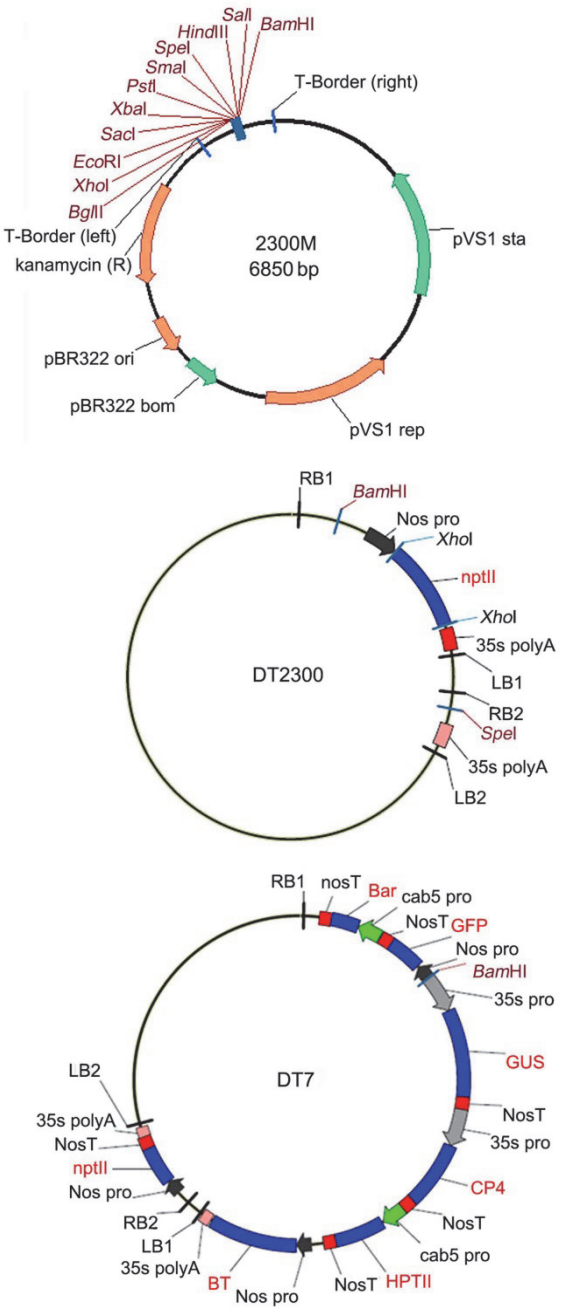

Fig. 1. Cloning strategy using the isocaudamer technique and double T-DNA. The seven target gene expression cassette. 35S Pro - CAMV35S promoter; Nos pro - nopaline synthase promoter; cab5 pro - promoter of chlorophyll *a*/*b* binding protein from *Gossypium arboretum*; Nos T - terminator of nopaline synthase, Bar - bialaphos resistance gene *bar*; BT-Cry1Ab/1Ac gene; CP4 - *cp4-epsps* gene; GUS - *gus* gene; HPTII - hygromycin phosphotransferase II gene; LB - left border; RB - right border; GFP - green fluorescent protein gene; nptII - neomycin phosphotransferase II gene; pBR322 ori - pBR322 origin of replication; pBR322 bom - bom site from pBR322; pVS1 rep - replication origin from pVS1; pVS1 sta - STA region from pVS1 plasmid; 35s polyA - 3' UTR (polyA signal) of CaMV35S; kanamycin (R) - kanamycin resistance gene *aadA* amplified from pIG121Hm.

T1-DNA region, a multi-cloning site comprising the *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI restriction sites is located between LB1 and RB1. In the T2-DNA region, there are multi-cloning sites containing the *Spe*I, *Apa*I, *Bgl*II and *Nco*I (Fig. 1). The 2300M is an auxiliary vector used for gene expression cassette assembly. On vector 2300M, there are the isocaudamers *Bam*HI/*Bgl*II, *Sal*I/*Xho*I, *Spe*I/*Xba*I, and *Hin*dIII, *Kpn*I, *Sma*I, *Pst*I, *Sac*I, and *Eco*RI, which are commonly used enzymes in genetic engineering (Fig. 1).

Two steps were needed to construct the plant expression vector. First, the target gene expression cassette was inserted between two isocaudamers of 2300M such as *Bam*HI and *Bgl*II. Then, the gene expression cassette was digested from 2300M using this pair of isocaudamers and inserted into a corresponding site in DT2300 (Fig. 1). Using the same strategy, other target genes can be inserted into DT2300 to form a multigene expression vector. The GOI and SMG were inserted into different T-DNA regions to allow selection of SMG-free transgenic plants.

A double T-DNA binary vector DT7 having the structure LB2-nptII-RB2-LB1-cry1Ab/1Ac-hptII-cp4 epsps-gus-gfp-bar-RB1 was developed for a quick evaluation of the functionality of the multigene and marker-free expression system. This could be achieved through assessment of the segregation of the SMG and GOI T-DNA regions by *nptII* leaf painting or *Basta* testing. In this experiment, we chose *hptII*, *gfp*, *gus*, *bar*, *cp4*-epsps, and *Cry1Ab/1Ac* as GOIs and *npt*II as SMG. All of the GOIs were inserted between RB1 and LB1 using the isocaudamers *Bam*HI and *Bgl*II. The SMG was inserted between RB2 and LB2 using the isocaudamers *Xba*I and *Spe*I. In order to avoid gene silencing results from one promoter being used to drive multi-genes, the CAMV 35S, Nos, and Gacab5 promoters were used to drive the expression of GOIs and SMG (Fig. 1). The *bar* and *hptII* genes were driven by the Gacab5 promoter; the *gus* and *cp4-epsps* genes were driven by the CaMV 35S promoter; and the *gfp, nptII,* and*Cry1Ab/1Ac* genes were driven by the Nos promoter. The structures of the vector and the progress of construction are summarized in Fig. 1. Different restriction enzyme digestion analyses show that the vector DT7 was correct.

The *A. tumefaciens* strain LBA4404 containing the co-transformation vector pDT7 was used for leaf disc transformation of tobacco. Twenty-seven independent kanamycin resistant plants were obtained from the explants surviving on the kanamycin selection medium. The transgenic lines were labeled as DT7-1 to DT7-27.

The putative transgenic tobacco plants produced using the pDT7 vector were analyzed for the presence of the *nptII* and the six target genes by PCR. An *nptII* PCR fragment of the expected size was obtained from the genomic DNA of all the 27 T_0 plants. Twenty-one plants (DT7-1 to DT7-21) that were positive for the *nptII* gene were found to possess one or more target gene fragments (Table 2 Suppl., Fig. 2). Therefore, 21 of 27 plants contained both the SMG and the GOI T-DNA for a co-

transformation frequency (CF) of 77.8 %. The CF varies depending on the co-transformation approach. For example, the CF ranges from 20.0 to 47.7 % and 54 % for the mixture of two strains of an *Agrobacterium* system (Zhou *et al*. 2003, Park *et al.* 2004). Daley *et al*. (1998) achieved a 52 % CF using two vectors in one strain of *Agrobacterium*. With two T-DNAs on one vector, a CF of 50 - 70 % has been reported (Komari *et al*. 1996, Zhou *et al*. 2003, Matheka *et al.* 2013). A key event in the initiation of DNA processing for T-DNA transfer resulting in the production of T-complexes is the site- and strand-specific cleavage at the T-border sequences (Zambryski 1992, Scheiffele *et al.* 1995). If the cleavage is made at a wrong site, it may lead to a failure of T-DNA integration (Yang *et al.* 2011). Of course, more than a half of the primary transformants will possess SMG and GOI at the same time. We obtained a relatively high CF (77.8 %) in this study.

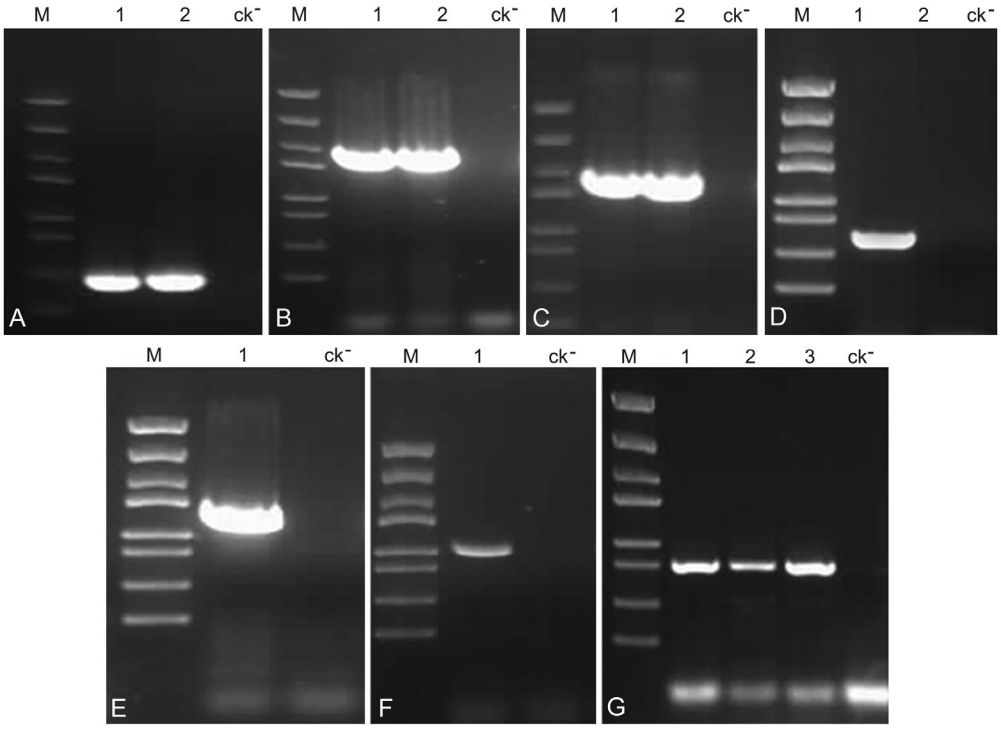

Fig. 2. Analysis of co-transformation of DT7 transgenic plants by PCR. *A* - *gfp*, 475 bp; *B* - *cry1Ab/1Ac*, 1 824 bp; *C* - *gus*, 1 812 bp; *D* - *bar*, 571 bp; *E* - *cp4*, 1 369 bp; *F* - *hptII*, 1 026 bp; *G* - *nptII*, 798bp. M - 5 kb DNA marker; 1,2,3 - transgenic tobacco lines; ck⁻ - non transgenic tobacco.

In *Agrobacterium*-mediated transformation, the integration site of the T-DNA appears to be random (Ambros *et al.* 1986, Chyi *et al*. 1986). Thus, through co-transformation, a separated T-DNA in a vector may integrate at either linked genome location or unlinked genome location. In order to analyze the insertion pattern of the two T-DNA regions, PCR detection of the transgenic plants was performed. The target DNA amplification was obtained from six transgenic tobaccos (DT7-2, DT7-7, DT7-8, DT7-9, DT7-10, and DT7-12). The separated T-DNA of 28.6 % (6/21) of the co-transformed plant lines were integrated at a linked genome location (Fig. 3). This may be related to the tandem nature of the two T-DNAs. In our vector DT7, the two T-DNAs were cascaded. If the middle RB or LB region was not recognized, read-through would allow the adjacent T-DNA regions to be transferred as a single linked unit during the T-DNA transfer progress, resulting in both the SMG and the GOI being inserted into the same site. McCormac *et al*. (2001) and Zhou *et al*. (2003) also found a read-through of separated T-DNAs with tobacco and rice transformation.

The other 15 co-transgenic tobacco lines included two T-DNAs inserted into the tobacco chromosome in an unlinked pattern. Nine lines contained partial target genes. There were different numbers of target genes missing during integration into the tobacco genome. The truncation in five plants occurred near the LB region. This coincides with the conclusion that a truncation mostly occurs at the LB site (Rossi *et al.* 1996). The truncation in the other four plants occurred at both the LB region and the RB region. We conjecture that this may be related to the size of the T-DNA region. In the T1-DNA region, there are six target genes, and the size is 12.7 kb. Although a precise upper limit for T-DNA transfer by *Agrobacterium* has not been established, it is more difficult to transfer an intact DNA fragment as the size increases, and it is a challenge to transfer more than 30 kb of DNA and keeping it intact (Yau and Stewart 2013). The detailed reasons need to be studied further in the

future. The other six transgenic lines had all the six target genes. These results show that the frequency of unlinked integration of the two intact T-DNAs was 22.22 % (6/27), and SMG-free transgenic plants could be obtained by selection from the offspring of these transgenic plants. These T_0 transgenic lines were grown in a greenhouse and the T1 seeds were harvested for further analysis.

In order to analyze the insertion number of target genes, real-time PCR was used to detect the copy numbers of the exogenous *nptII* gene in the transformed lines with six target genes and SMG (Table 3 Suppl.). The standard curve of the reference gene *rnr2* was CT = 33.953 - 3.069 log (cn), with r^2 and an amplification efficiency of 0.998 and 111.752 %, respectively, where CT is the cycle threshold and cn is the copy number. The standard curve of *nptII* was CT = 35.236 - 3.313 log (cn), with r^2 and an amplification efficiency of 0.999 and 100.382 %, respectively. The *rnr2* and *nptII* cn values were derived from standard curves and the CT values were obtained by real-time PCR. The results indicate a single copy insertion of *nptII* in the transgenic tobacco lines (DT7-4, DT7-5, and DT7-6).

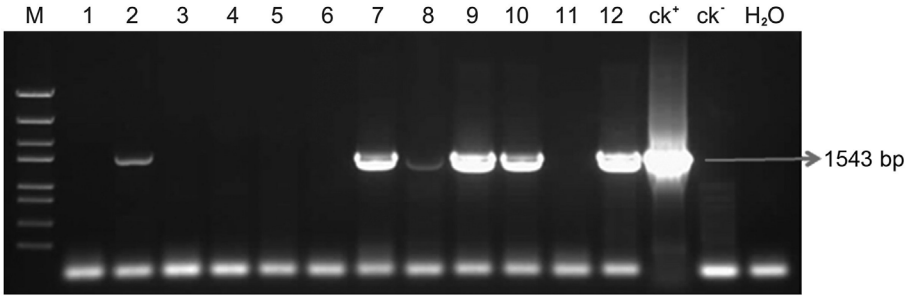

Fig. 3. Analysis of the insertion pattern of two T-DNA regions in transgenic tobacco by PCR. In transgenic lines DT7-2, DT7-7, DT7-8, DT7-9, DT7-10, and DT7-12, target DNA lines were amplified. This result indicates that the two T-DNAs were inserted into these six tobacco lines in a linked pattern. M - 5 kb DNA marker; 1 to 12 - different transgenic tobacco lines; ck^+ - plasmid DT7; ck^- - non transgenic tobacco NC89; *arrow* - the size of amplification product..

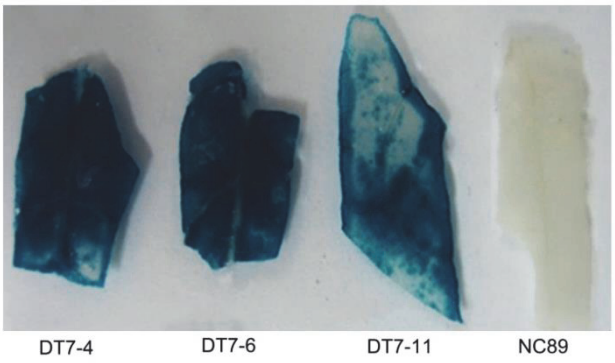

Fig. 4. Partial target protein detection in three transgenic tobacco lines (DT7-4,7-6,7-11) and in an untransformed plant (NC89). A β-glucuronidase histochemical assay.

Seeds from the co-transformed plant line DT7-4, with a single copy number and non-linked SMG and GOI, were germinated on a medium containing 12 mg dm^{-3} PPT. PPT-resistant tobacco plants could be distinguished from the sensitive ones after 14 d of culture on this selective medium. Kanamycin-sensitive tobacco plants could be distinguished from the PPT resistant ones after painting them with 500 mg dm^{-3} of kanamycin. Ten days after application, clear effects of the kanamycin were observed. Seventeen of the 89 T_1 seedlings derived from the co-transformed line DT7-4 showed herbicide resistance and kanamycin sensitivity. Analysis by PCR also revealed that all of these 17 T_1 plants were positive for the 6 target genes. The stable expression of the protein from the introduced partial target genes was confirmed by performing GUS histochemical assay, Cry1Ab/1Ac/cp4 bivalent gold-labeled test strip detection, and *Basta* leaf paint assay (Fig. 4 and Fig. 1 Suppl.). The results of these phenotypic assays and molecular analyses confirm that the two T-DNAs containing GOI and SMG separately segregated in the T1 plants. The frequency of removal of SMG from the transgenic T_1 plants was 19.10 %.

Overall, our findings provide a new and highly effective platform for multigene and marker-free transgenic plant production. While designing the study, we considered the interaction of multiple GOIs. Therefore, the GOI selection was based on the principle of avoiding an antagonism effect between GOIs. In addition, to avoid a gene silence effect between GOIs, we used different promoters at the adjacent GOIs when constructing a multi-gene expression vector. The scientific merit of this study is that we got a multi-GOI and SMG-free plant expression vector system, which is easy to operate and saves time and money.

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