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A new series of activation-tagging vectors: pKANACB, pKANACK and pKANACH

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Summary

The new activation-tagging vectors pKANACB, pKANACK and pKANACH were constructed. The constructs contain a tetramer of the cauliflower mosaic virus 35S enhancer sequence, a selection marker gene for plant transformation, an ampicillin resistance gene and the replication origin of *Escherichia coli* which can be used for plasmid rescue within the T-DNA. These vectors can be stably maintained in *Agrobacterium tumefaciens* strains GV3101, LBA4404 and AGL0. The plasmid selection in *E. coli* and *A. tumefaciens* was accomplished via an ampicillin resistance gene. When tomato cotyledons were infected with *A. tumefaciens* strain AGL0 harboring the activation-tagging vectors (AGL0-pKANACB, AGL0-pKANACK and AGL0-pKANACH), glufosinate-, kanamycin- and hygromycin-resistant plants, respectively, were generated. The sequences of the T-DNA regions of pKANACB, pKANACK and pKANACH have been deposited in GenBank under accession numbers AB775897, AB777654 and AB777653, respectively.

Key words: activation tagging, functional genomics, mutation, T-DNA, transgenic plant

Introduction

Activation tagging is a powerful tool that facilitates the functional analysis of genes in various plant species by producing dominant mutations through the random insertion of enhancer elements into the plant genome [1, 7, 10, 11, 12]. Many studies have employed activation tagging using the binary vector pPCVICEn4HPT [10] or its derivative (pPCVICEn4s). These vectors contain a tetramer of the tandemly repeated cauliflower mosaic virus 35S enhancer sequence (4×35S), the replication origin of *Escherichia coli* (ori) and an ampicillin resistance gene (amp) in the T-DNA region. The 4×35S activates the expression of genes located near the T-DNA insertion site, and the ori and amp can be used to clone the genomic region flanking the T-DNA insertion site by plasmid rescue [10].

Unfortunately, pPCVICEn4s cannot be maintained in the commonly used *Agrobacterium tumefaciens* strains, including AGL0, GV3101 and LBA4404. pPCVICEn4s contains only two factors (oriV and oriT) for the DNA replication origin of plasmid RK2, which is a broad-host-range plasmid. These conditional ori functions are active only when trans-acting helper functions for RK2 replication

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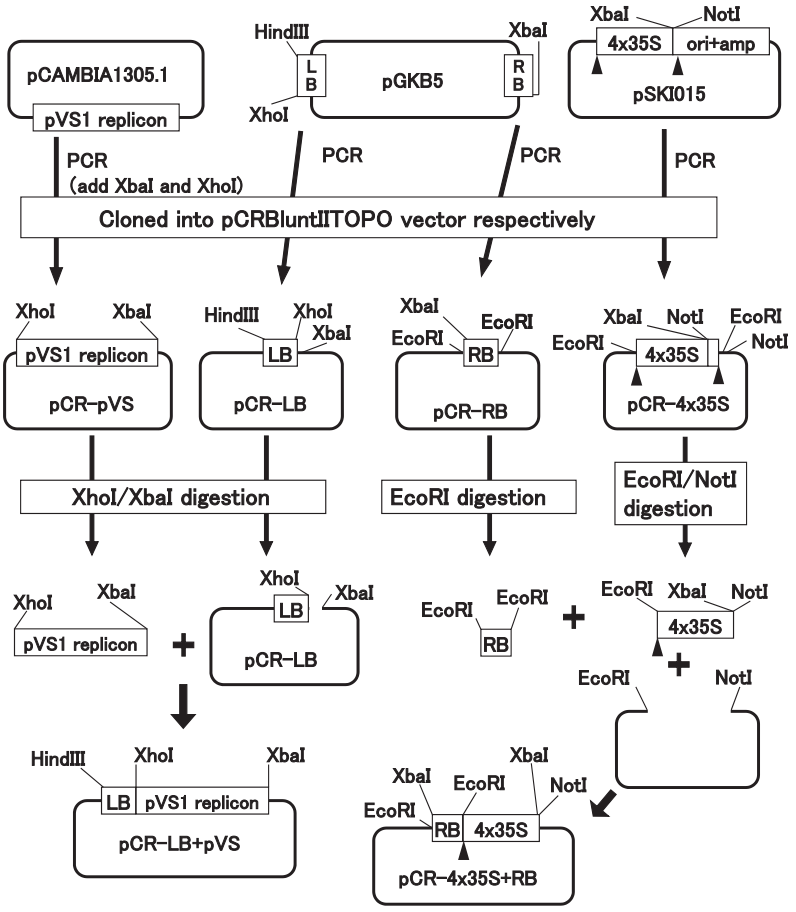

Figure 1. Construction of pCR-LB+pVS and pCR-4×35S+RB. LB: left T-DNA border sequence, RB: right T-DNA border sequence, 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, ori+amp: replication origin of *E. coli* and ampicillin-resistance gene, pVS1 replicon: replication sequence for *A. tumefaciens*. The black triangles indicate the annealing sites of the primer for PCR to evaluate the direction of the 4×35S in Fig. 2.

and conjugation (i.e., *trfa* and *Tra*) are present in *A. tumefaciens*. Thus, pPCVICEn4s can only be maintained in special *A. tumefaciens* strains, such as GV3101pMP90RK, which contains the helper plasmid pMP90RK harboring *trfa* and *Tra* [4, 6, 10].

Substituting the *A. tumefaciens* replication sequences in pPCVICEn4s for a small replicon such as the pVS1 replication sequence (pVS1 replicon), which functions in a broad range of *A. tumefaciens* strains [4], will facilitate the construction of a more convenient activation-tagging vector.

Transgenic plants with single T-DNA insertion are easier to analyze than those that contain many insertions. On average, 2.1 T-DNA inserts per line have been reported in the genome of pPCVICEn4HPT-transgenic plants [5]. On the other hand, the left and right T-DNA border sequences of pGKB5 [2] are presumed to be responsible for the low-copy-number integration of the T-DNA [8].

In the present work, we improved the activation-tagging vector pSKI015 [11], which is derived from pPCVICEn4HPT, and constructed new activation-tagging vectors based on the plasmid backbone. We substituted the *A. tumefaciens* replication sequences in pSKI015 for the pVS1 replicon and

substituted the border sequences of pSKI015 for those of pGKB5.

Materials and Methods

Cloning of components

A DNA fragment containing the pVS1 replicon was PCR-amplified using pCAMBIA1305.1 (http://www.cambia.org/daisy/cambia/585) as a template with the forward primer 5'-CTT GCC TCG AGG AAT GAA CGC CAA GAG GAA C-3' and the reverse primer 5'-CTT GCT CTA GAG TAG GCC AGC CAT TTT TGA G-3' to generate pCR-pVS (Fig. 1). Each primer contains the sequence CTTGC, followed by an XhoI (forward primer) or XbaI (reverse primer) restriction site at the 5' end. These CTTGC sequences added to the primers were designed for other experiments, in which both ends of the PCR product containing the pVS1 replicon are digested directly with XhoI/XbaI for subsequent cloning into an XhoI/XbaI-digested vector. These additional CTTGC sequences improve the digestion of the end sequences of the PCR product. Thus, these primers were also employed in the present work. All PCRs for cloning purposes were performed in 20 μL of PCR reaction solution containing 0.5 units KOD-plus (Toyobo), 1 × KOD-plus-ver.2 buffer, 0.2 mM each dNTP, 0.3 μM each of the forward and reverse primers and 1-5 ng plasmid DNA as the template. The thermal cycling conditions were 94°C for 120 s, followed by 25-35 cycles of 94°C for 30 s, 55-60°C for 30 s and 68°C for 60-180 s and a final step of 7 min at 68°C. All PCR products were cloned into the pCRBluntIITOPO (Invitrogen) vector according to the manufacturer's instructions. The functionality of the cloned pVS1 replicon was confirmed by the transformation of pCR-pVS into *A. tumefaciens*.

The right (RB) and left (LB) T-DNA border sequences were PCR-amplified using pGKB5 as a template with the primer set RB-forward (5'-ACC ATG ATT ACG CCA AGC TC-3') and RB-reverse (5'-CCC CAA GTG CAG CGT TTA AT-3') and the set LB-forward (5'-TTG CCT TTT CTT ATC GAC CA-3') and LB-reverse (5'- TAA AAC GAC GGC CAG TGA AT-3') to generate pCR-RB and pCR-LB, respectively (Fig. 1). The cloned RB and LB fragments were sequenced to identify any PCR errors. All sequencing was performed using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The DNA sequence analysis was conducted using the GENETYX program (Genetyx Co.).

A DNA fragment containing the 4×35S was amplified by PCR from pSKI015 using the forward primer 5'-TAA TAC GAC TCA CTA TAG GGC-3' and reverse primer 5'-CTG TCA AAC ACT GAT AGT TTC GG-3'. The fragment was subcloned into a vector to generate the plasmid pCR-4×35S (Fig. 1). The cloned 4×35S fragments were sequenced to identify any PCR errors.

Construction of pCR-LB+pVS and pCR-4×35S+RB

An XhoI/XbaI fragment from pCR-pVS (containing the pVS1 replicon) was ligated with an XhoI/XbaI fragment of pCR-LB to yield pCR-LB+pVS (Fig. 1).

An EcoRI/NotI fragment from pCR-4×35S (containing 4×35S) and an EcoRI fragment (containing RB) from pCR-RB were inserted into an EcoRI/NotI-digested pCR-4×35S vector to produce pCR-4×35S+RB (Fig. 1). The direction of the RB in pCR-4×35S+RB was evaluated by XbaI digestion.

Construction of pKANACB

A HindIII/XbaI fragment from the pSKI015 [containing a glufosinate-resistance selection cassette (BAR), ori and amp], an XbaI fragment from pCR-4×35S+RB and a HindIII/XbaI fragment from pCR-LB+pVS were ligated to yield the activation-tagging vector pKANACB (Fig. 2). The direction of the

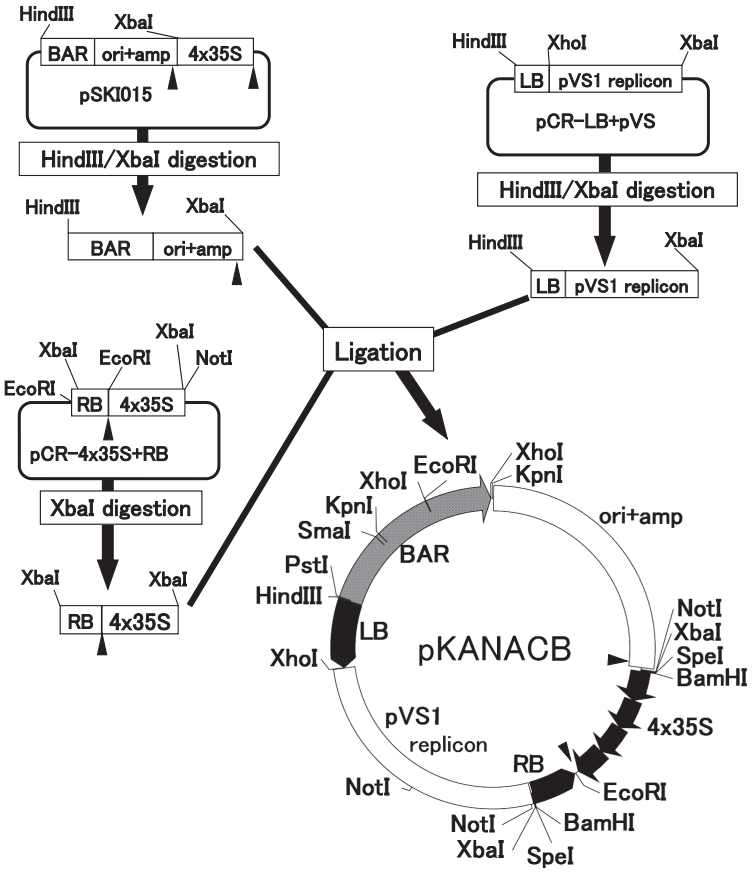

Figure 2. Construction of pKANACB. LB: T-DNA left border, RB: T-DNA right border, 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, ori+amp: replication origin of *E. coli* and ampicillin resistance gene, BAR: glufosinate-resistance selection cassette, pVS1 replicon: replication sequence for *A. tumefaciens*. The black triangles indicate the annealing sites of the primer for PCR to evaluate the direction of the 4×35S.

4×35S derived from the XbaI fragment of pCR-4×35S+RB was assessed by PCR using the same primer set that was used to construct pCR-4×35S. The locations of these primers are shown in Fig. 1 and Fig. 2.

Construction of pKANACK

The BAR of activation-tagging vector pKANACB (Fig. 2) was replaced with a kanamycin-resistance gene (KMR). The KMR-containing DNA fragment was isolated from pSKI074 [11] by HindIII/KpnI digestion and ligated with the HindIII/KpnI fragment of pKANACB (Fig. 3). Thus, the BAR of pKANACB was replaced with KMR to generate the activation tagging vector pKANACK (Fig. 3).

Construction of pKANACH

A HindIII fragment from plasmid pPCVICEn4HPT [10] containing a hygromycin-resistance cassette (HPT) was blunted using KOD DNA polymerase (Toyobo) and subcloned into

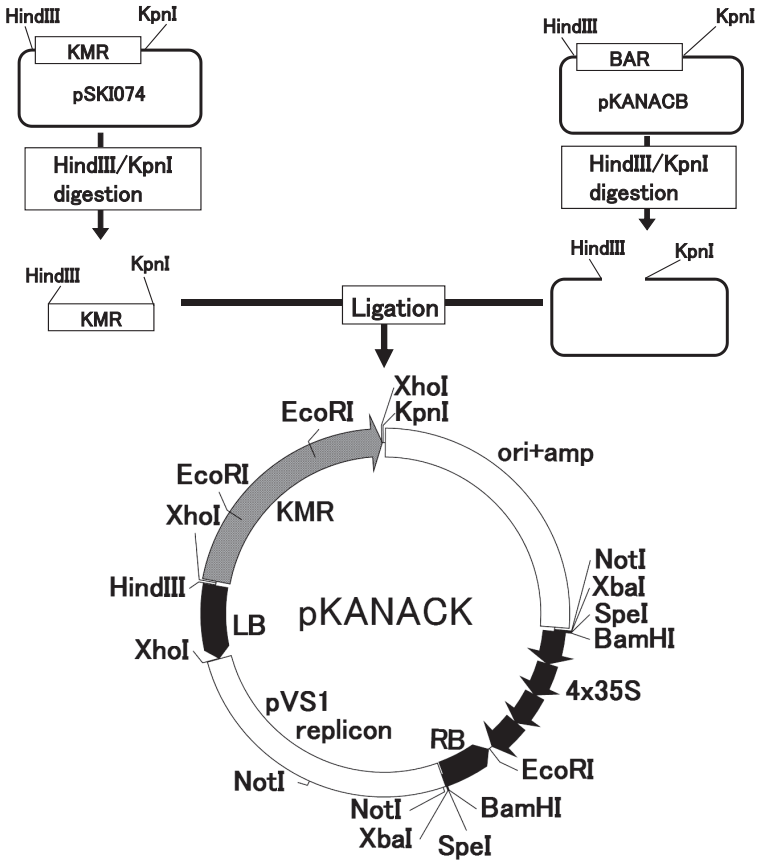

Figure 3. Construction of pKANACK. LB: T-DNA left border, RB: T-DNA right border, 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, ori+amp: replication origin of *E. coli* and ampicillin resistance gene, BAR: glufosinate-resistance selection cassette, KMR: kanamycin-resistance selection cassette, pVS1 replicon: replication sequence for *A. tumefaciens*.

pCRBluntIITOPO (Invitrogen), resulting in pCR-HPT (Fig. 4). A KpnI fragment from pKANACB was excised and self-ligated to produce pKANACB-kpnI (Fig. 4). A HindIII/EcoRV fragment from pCR-HPT (containing HPT) was ligated with the HindIII/SmaI fragment of pKANACB-kpnI (Fig. 4). Thus, the BAR of pKANACB was replaced with HPT to generate pKANACH (Fig. 4).

Results and discussion

The sequences of the T-DNA regions of pKANACB, pKANACK and pKANACH have been deposited in GenBank under accession numbers AB775897, AB777654 and AB777653, respectively. When these activation-tagging vectors were introduced into *E. coli* or *A. tumefaciens*, Luria-Bertani (LB) medium with 100 mg/L carbenicillin was found to be more suitable than LB medium with 100 mg/L ampicillin for selection because the former allowed for less satellite colony formation, enabling the transformed colonies to be more easily selected.

The *A. tumefaciens* strains AGL0, GV3101, GV3101pMP90RK and LBA4404 were successfully transformed with the plasmids pKANACB, pKANACK and pKANACH. Weigel *et al.* [11] reported

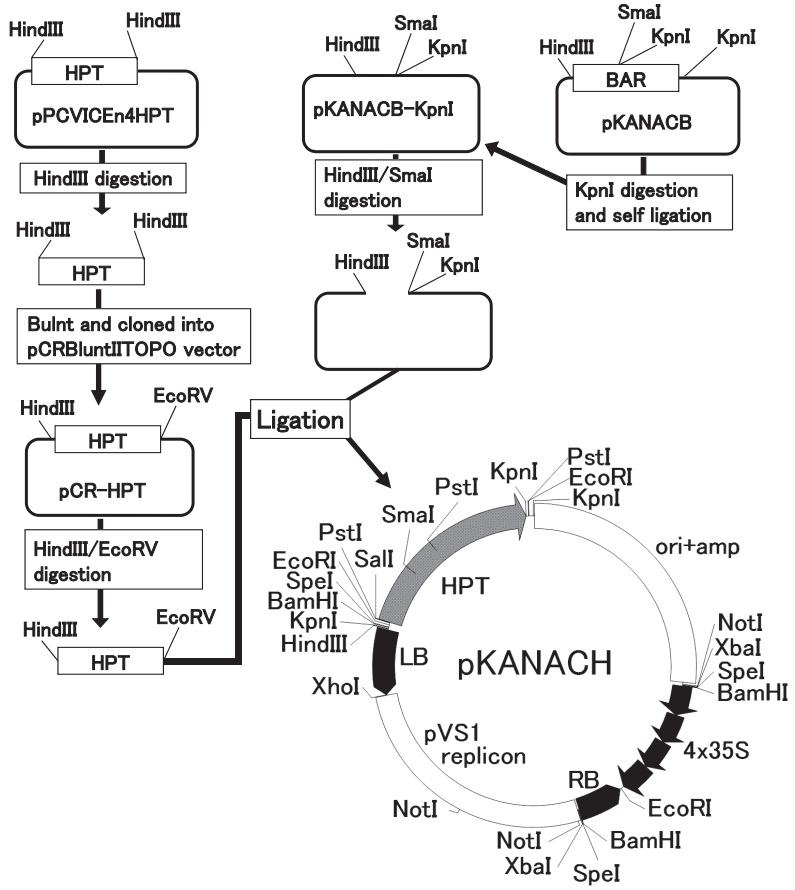

Figure 4. Construction of pKANACH. LB: T-DNA left border, RB: T-DNA right border, 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, ori+amp: replication origin of *E. coli* and ampicillin-resistance gene, BAR: BASTA-resistance selection cassette, HPT: hygromycin-resistance selection cassette, pVS1 replicon: replication sequence for *A. tumefaciens*.

that 4×35S enhancers are unstable in *E. coli* and *A. tumefaciens* and that the storage of *A. tumefaciens* strains carrying activation-tagging vectors at 4°C leads to the progressive loss of the copies of the cauliflower mosaic virus 35S enhancer. Weigel recommended that *A. tumefaciens* strains harboring activation-tagging vectors should always be inoculated from stocks stored at -80°C (http://www.weigelworld.org/resources/plasmids/activationtagging/pski015). Thus, we always inoculated the cultures from -80°C stocks; however, in one case, we noted an *E. coli* colony with a reduced number of CaMV 35S enhancer copies. Therefore, we occasionally confirmed the number of CaMV 35S enhancer copies by colony PCR with the primer set used to amplify the 4×35S fragment for cloning purposes.

The *A. tumefaciens* strain AGL0 harboring the activation tagging vectors (AGL0-pKANACB, AGL0-pKANACK and AGL0-pKANACH) was used to transform the tomato (*Solanum lycopersicum*) cultivar 'Micro-Tom' according to the methods described by Dan *et al.* [3] and Sun *et al.* [9]. When the 'Micro-Tom' cotyledon explants were infected with AGL0-pKANACB, AGL0-pKANACK and AGL0-pKANACH, glufosinate-, kanamycin- and hygromycin-resistant plants, respectively, were

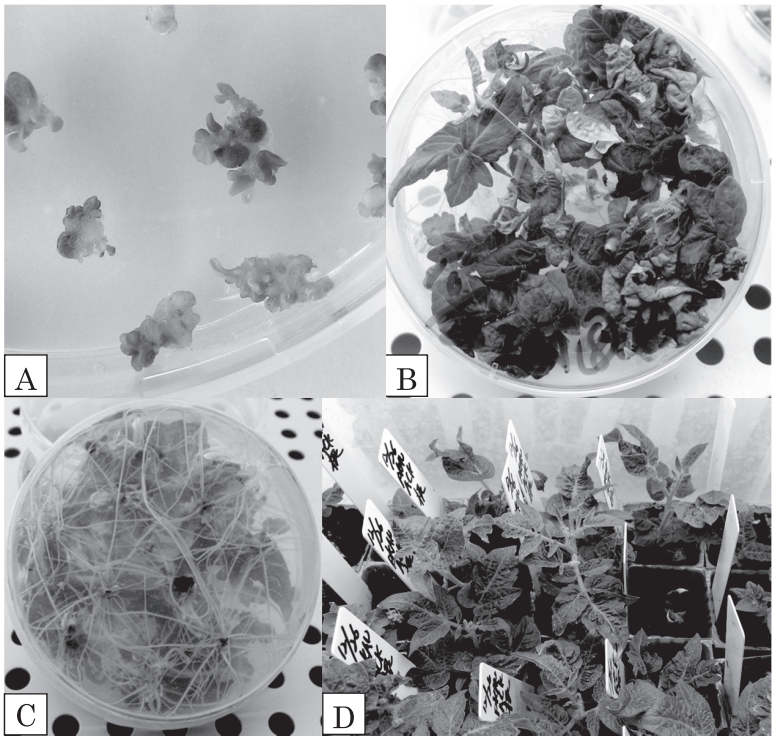

Figure 5. Regeneration of kanamycin-resistant plants from the 'Micro-Tom' cotyledons infected with AGL0-pKANACK. A: shoot formation from cotyledon explants on the medium containing kanamycin, B and C: rooted shoots on the medium containing kanamycin, D: acclimatized kanamycin-resistant plants.

generated. Fig. 5 shows the kanamycin-resistant plants regenerated from the 'Micro-Tom' cotyledons that were infected with AGL0-pKANACK. A comparison of the transformation efficiencies of these vectors is now underway.

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新規アクティベーションタギングベクターpKANACB, pKANACK 及び pKANACH

清水圭一† ・橋本文雄

要　約

新規の植物のアクティベーションタギングベクターpKANACB，pKANACK 及び pKANACHを構築した。これらのベクターはT-DNA領域中にエンハンサー配列，植物形質転換のマーカー遺伝子とプラスミドの構成要素配列を持っており，多くのアグロバクテリウム中で維持できる。バクテリアでの選抜にはアンピシリン耐性遺伝子が利用できる。アグロバクテリウムの系統AGL0に，これらのベクターを導入してトマトの子葉に感染させると，pKANACBではグルホシネート耐性，pKANACKではカナマイシン耐性，pKANACHではハイグロマイシン耐性の植物体が再生した。これらベクター配列のGenBankにおけるアクセッション番号はpKANACB：AB775897，pKANACK：AB777654，pKANACH：AB777653である。

キーワード：アクティベーションタギング，機能ゲノム科学，突然変異，T-DNA，形質転換植物

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