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The Construction of two new Activation-Tagging Vectors, pKANAC2H and pKANAC2K

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Summary

We reported on the construction of two new activation-tagging vectors, pKANAC2H and pKANAC2K. These vectors have a higher number of recognition sites in the T-DNA region for the popular restriction enzymes of plasmid rescue than the more established activation-tagging vectors pPCVICEn4HPT or pSKI074. The sequences of the T-DNA regions of pKANAC2H and pKANAC2K have been deposited in GenBank under accession numbers AB889495 and AB889496, respectively.

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

Introduction

Activation-tagging is a powerful tool that facilitates the functional analysis of genes in various plant species. This process involves a random insertion of enhancer elements into the plant genome, which results in dominant mutations [1, 3, 4, 7, 8, 9]. Many studies have conducted activation-tagging using the binary vector pPCVICEn4HPT [7] or its derivative, pSKI074 [8]. These vectors contain a tetramer of the tandem-type 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 sequence activates the expression of genes located near the T-DNA insertion site. Ori and amp sequences are used to clone the genomic region flanking the T-DNA insertion site during plasmid rescue. This is a technique used to recover bacterial plasmids from eukaryotic transgenic genomic DNA. In other words, the genomic DNA of the T-DNA inserted into the plant is digested by restrictive enzymes, and the digested DNA self-ligates; this circular DNA fragment containing both the ori and amp sequences is used to transform *E. coli* [7].

In the present study, we improved the activation-tagging vector pPCVICEn4HPT and put together the new activation-tagging vectors pKANAC2H and pKANAC2K. The new vectors have a higher number of unique restriction sites in the T-DNA region than do pPCVICEn4HPT, or pSKI074, both of which are frequently used for activation-tagging. A large number of unique restriction sites in the T-DNA region enable efficient plasmid rescue [3].

Materials and Methods

Cloning of 4×35S and replication origin of *E. coli*

A DNA fragment containing the 4×35S was amplified by PCR from pPCVICEn4HPT using the forward primer 5'-CAG GAA ACA GCT ATG AC-3' and reverse primer 5'-CTG TCA AAC ACT GAT AGT TTC GG-3'. The fragment was sub-cloned into a vector to generate the plasmid pCR-4×35S-2 (Fig. 1). All PCR analyses 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 for each dNTP, 0.3 μM for each of the forward and reverse primers, using 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, 55-65 °C for 30 s, and 68 °C for 60-180 s, with a final step at 68 °C for 7 minutes. All PCR products were cloned into the pCR®-BluntII-TOPO® (Invitrogen) vector according to the manufacturer's instructions. The cloned 4×35S fragments were sequenced to identify

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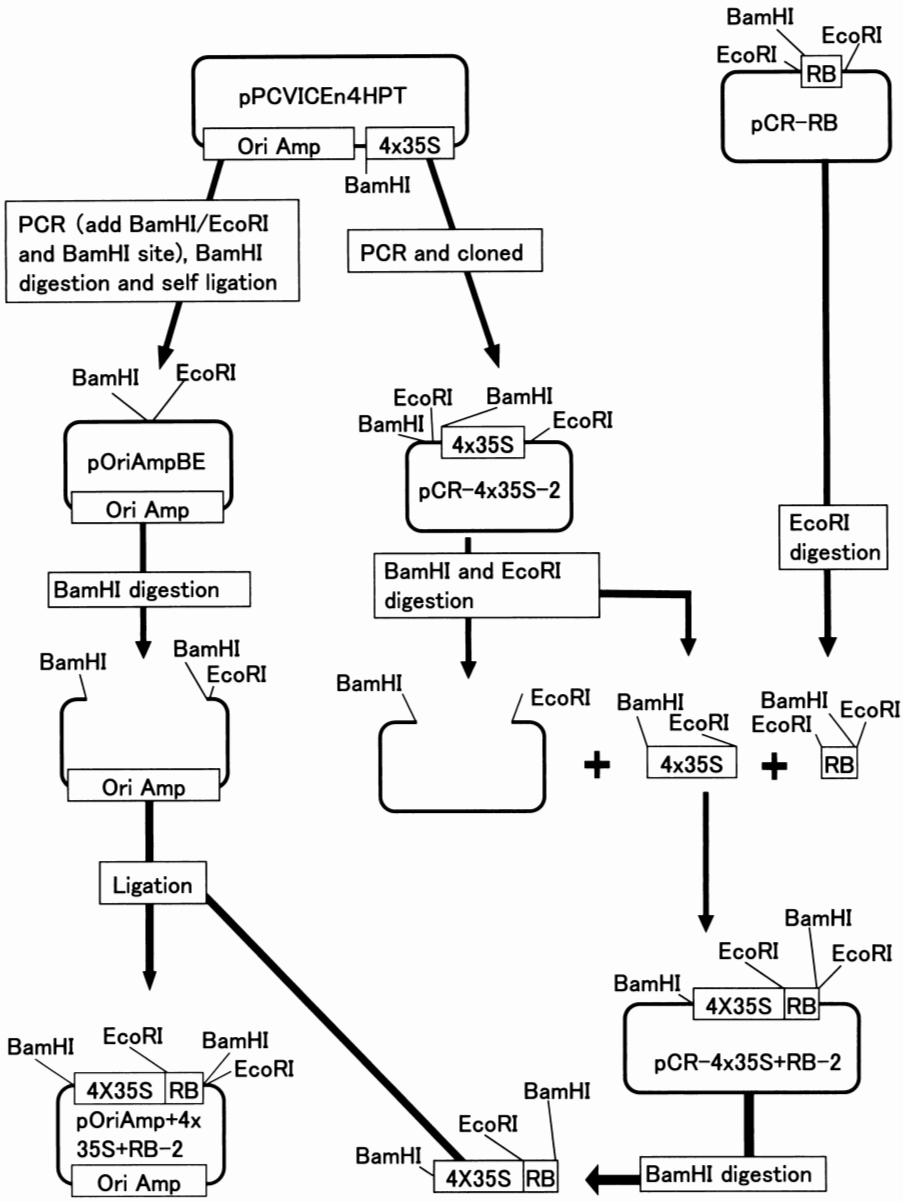

Figure 1. Construction of pOriAmp+4×35S+RB-2. LB: left T-DNA border sequence, RB: right T-DNA border sequence, 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, Ori Amp: replication origin of *E. coli* and ampicillin-resistance gene.

any errors introduced during PCR. 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 replication origin of *E. coli* and an ampicillin-resistance gene was PCR-amplified using pPCVICEn4HPT as a template with the forward primer 5'-GAA GGA TCC GAA TTC AAC GTC GTG ACT GGG AAA AC-3' and the reverse primer 5'-GAA GGA TCC AGC TCA CTC AAA GGC GGT AA-3'. Each primer contained the sequence GAA, followed by a BamHI/EcoRI (forward primer) or BamHI (reverse primer) restriction site at the 5' end. These additional GAA sequences improved the digestion of the end sequences of the PCR product. The PCR product containing the ori and amp was digested with BamHI and self-ligated to generate a pOriAmpBE (Fig. 1). The functionality of the cloned replication origin and the ampicillin-resistance gene were confirmed by the transformation of pOriAmpBE into *E. coli*.

Construction of pCR-4×35S+RB-2

A BamHI/EcoRI fragment from pCR-4×35S-2 (containing 4×35S) and an EcoRI fragment from pCR-RB [5] [containing the right T-DNA border sequences (RB)] were inserted into a BamHI/EcoRI-digested pCR-4×35S-2 vector to produce pCR-4×35S+RB-2 (Fig. 1). The direction of the RB in pCR-4×35S+RB-2 was evaluated by BamHI digestion.

Construction of pOriAmp+4×35S+RB-2

A BamHI fragment from the pOriAmpBE and a BamHI fragment from pCR-4×35S+RB-2 (containing 4×35S and RB) were ligated to generate pOriAmp+4×35S+RB-2 (Fig. 1). The direction of the 4×35S+RB in pOriAmp+4×35S+RB-2 was evaluated by EcoRI digestion.

Construction of pKANAC2

An EcoRI fragment of pKANACH [5] [containing RB, the left T-DNA border sequences (LB), and pVS1 replicon; a replication origin for *Agrobacterium*] and EcoRI fragment of pOriAmp+4×35S+RB-2 (containing 4×35S, ori, and amp) were ligated to generate pKANAC2 (Fig. 2). The directions of the RB, pVS1 replicon, and LB in pKANAC2 were evaluated by BamHI digestion.

Construction of pKANAC2H

A SacI fragment from plasmid pPCVICEn4HPT containing a hygromycin-resistance cassette (HPT) was blunted and sub-cloned into pCR®-BluntII-TOPO®, resulting in pCR-HPT-2 (Fig. 3). A SalI/blunted-EcoRI fragment from pCR-HPT-2 (containing HPT) was ligated with the SalI/PvuII fragment of pKANAC2 to generate pKANAC2H-s (Fig. 3).

A polylinker of XhoI, SpeI, SacI, NsiI, SphI, NotI, SalI, and HindIII (annealed oligonucleotides of 5'- AGC TGC TCG AGA CTA GTG AGC TCA TGC ATG CGG CCG CGT CGA CAA GCT TA -3' and 5'- TCG ATA AGC TTG TCG ACG CGG CCG CAT GCA TGA GCT CAC TAG TCT CGA GC -3') was inserted into the HindIII/SalI site of pKANAC2H-s by linker ligation to produce pKANAC2H (Fig. 3). The HindIII and SalI sites derived from pKANAC2H-s were eliminated by replacing AAGCTT (HindIII) with AAGCTG and GTCGAC (SalI) with ATCGAC.

Construction of pKANAC2K

The HPT of the activation-tagging vector pKANAC2H (Fig. 3) was replaced with a kanamycin-resistance gene (KMR). A DNA fragment containing the KMR was PCR-amplified from pBI121 (Clontech), using the forward primer 5'-GGT ACC GAT CAT GAG CGG AGA ATT AAG G-3' and reverse primer 5'-AAG CTT CAC CAG AAC CAC CAC CAG A-3'. Each primer contained the KpnI (forward primer) or HindIII (reverse primer) restriction site at the 5' end. The fragment was sub-cloned into a vector to generate the plasmid pCR-KMR (Fig. 4). The cloned KMR fragments were sequenced to identify any errors introduced during PCR. The KMR-containing a DNA fragment was isolated from pCR-KMR by HindIII/KpnI digestion and ligated with the HindIII/KpnI fragment of pKANAC2H (Fig. 4). Thus, the

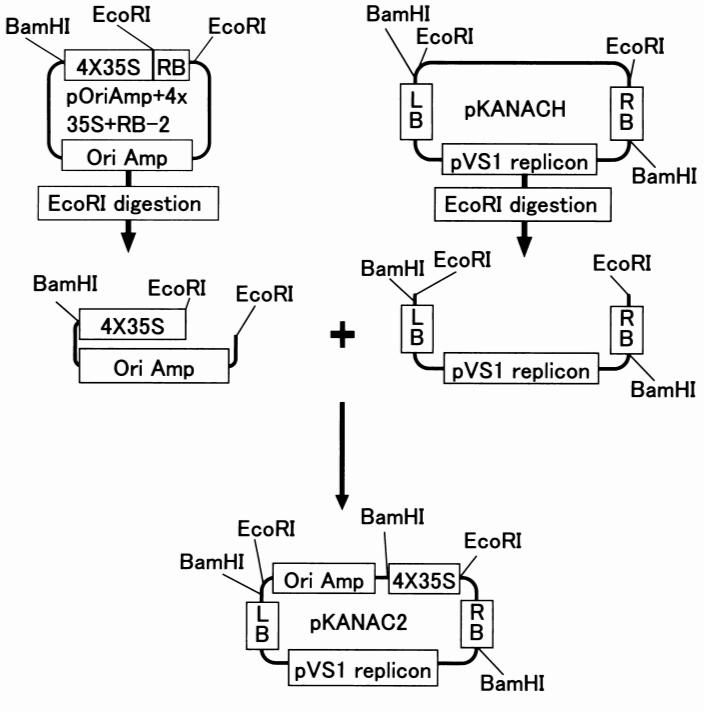

Figure 2. Construction of pKANAC2. LB: T-DNA left border, RB: T-DNA right border, 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, Ori Amp: replication origin of *E. coli* and ampicillin resistance gene, pVS1 replicon: replication sequence for *A. tumefaciens*.

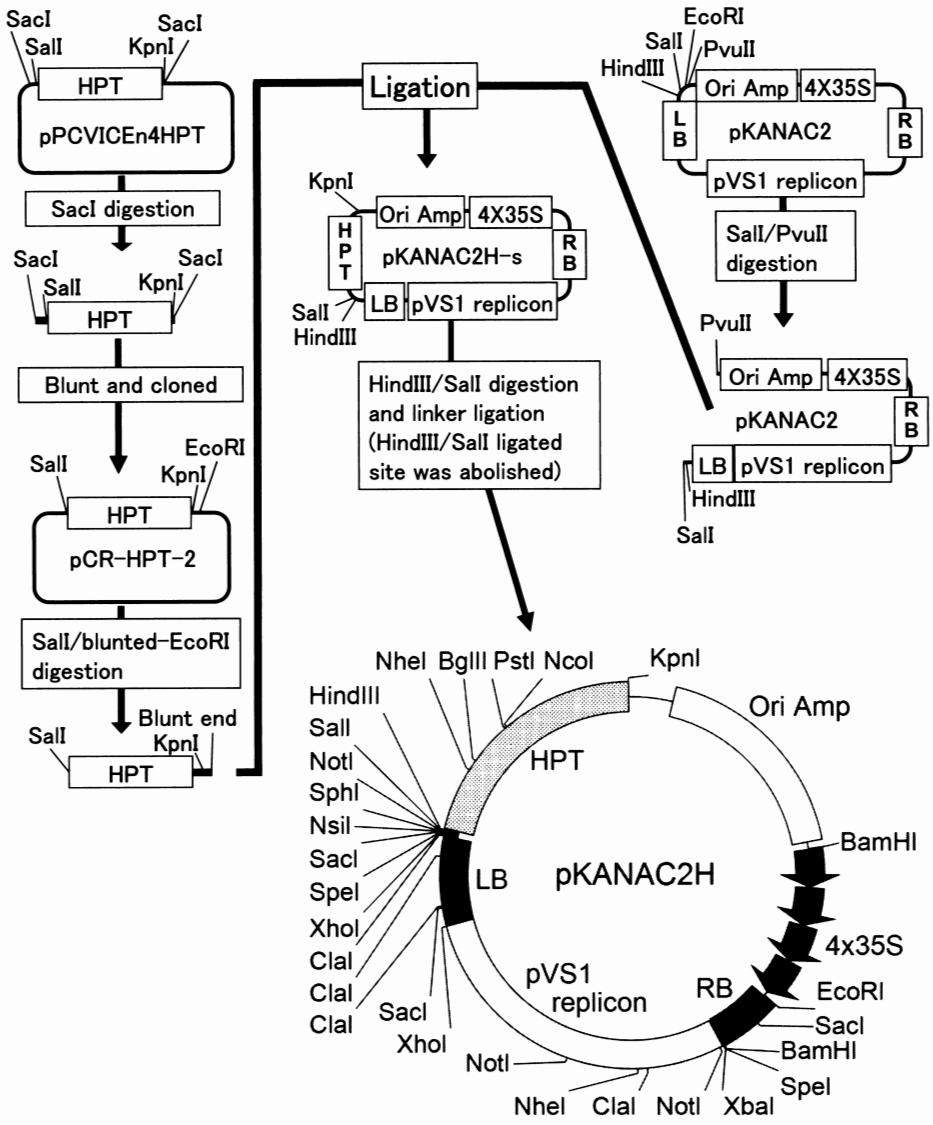

Figure 3. Construction of pKANAC2H. LB: T-DNA left border, RB: T-DNA right border, 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, Ori Amp: replication origin of *E. coli* and ampicillin resistance gene, HPT: hygromycin-resistance selection cassette, pVS1 replicon: replication sequence for *A. tumefaciens*.

HPT of pKANAC2H was replaced with KMR to generate the activation-tagging vector pKANAC2K (Fig. 4).

Results and Discussion

The sequences of the T-DNA regions of pKANAC2H and pKANAC2K have been deposited in GenBank under the accession numbers AB889495 and AB889496, respectively. Seventeen and fifteen types of popular restriction enzymes were available for plasmid rescue in pKANAC2H and pKANAC2K respectively, while ten were available in pPCVICEn4HPT and pSKI074 (Fig. 5). The ligation of the blunted EcoRI (GAATT) and PvuII site (CTG) was expected to generate the EcoRI (GAATTC) site when the HPT fragment was inserted into pKANAC2 (Fig. 3). However, the *Eco*RI site was eliminated by a single nucleotide deletion mutation during cloning, causing the expected GAATTCTG sequence to be changed to GAATTTG. *E. coli* and *Agrobacterium tumefaciens* strains were successfully transformed using pKANAC2H and pKANAC2K.

The *A. tumefaciens* strain AGL0 harboring the activation-tagging vectors AGL0-pKANAC2H and AGL0-pKANAC2K was used to transform the tomato (*Solanum lycopersicum*) cultivar 'Micro-Tom,' following the methods described by Dan *et al.* [2] and Sun *et al.* [6]. When the 'Micro-Tom' cotyledon explants were infected with AGL0-pKANAC2H and AGL0-pKANAC2K, the respective hygromycin- and kanamycin-resistant shoots, were generated. A comparison of the transformation efficiencies of these vectors is now underway.

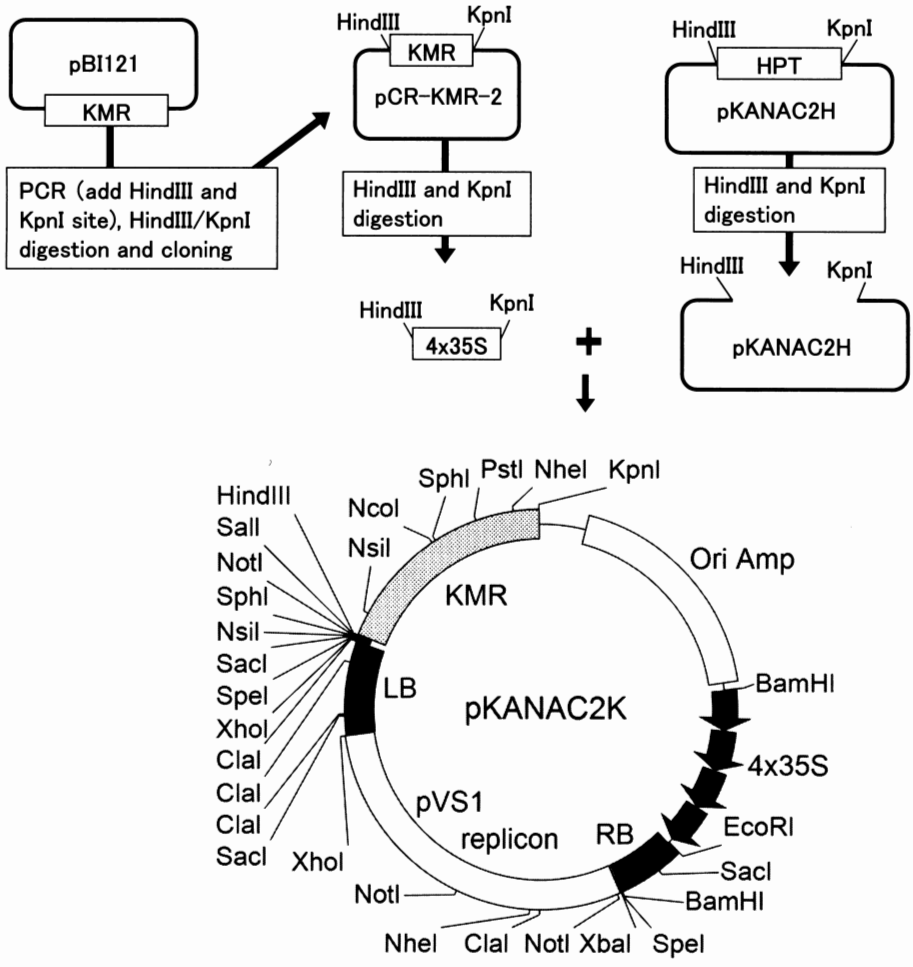

Figure 4. Construction of pKANAC2K. LB: T-DNA left border, RB: T-DNA right border, 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, Ori Amp: replication origin of *E. coli* and ampicillin-resistance gene, KMR: kanamycin-resistance selection cassette, HPT: hygromycin-resistance selection cassette, pVS1 replicon: replication sequence for *A. tumefaciens*.

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References

[1] Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A. and Lamb, C.: Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell, 12, 2383-2394 (2000)

[2] Dan, Y., Yan, H., Munyikwa, T., Dong, J., Zhang, Y. and Armstrong, C. L.: MicroTom-a high-throughput model transformation system for functional genomics. Plant Cell Rep., 25, 432-441 (2006)

[3] Gou, X. and Li, J.: Activation tagging. Methods Mol. Biol., 876, 117-133 (2012)

[4] Mathews, H., Clendennen, S.K., Caldwell, C.G,. Liu, X.L., Connors, K., Matheis, N., Schuster, D.K., Menasco, D.J., Wagoner, W., Lightner, J. and Wagner, D.R.: Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell, 15, 1689-1703 (2003)

[5] Shimizu, K. and Hashimoto, F.: A New series of activation-tagging vectors, pKANACB, pKANACK and pKANACH. Mem. Fac. Agric. Kagoshima Univ., 63, 39-47 (2013)

[6] Sun, H-J., Uchii, S., Watanabe, S. and Ezura, H.: A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol., 47, 426-431 (2006)

[7] Walden, R., Fritze, K., Hayashi, H., Miklashevichs, E., Harling, H. and Schell, J.: Activation tagging: a means of isolating genes implicated as playing a role in plant growth and development. Plant Mol Biol., 26, 1521-1528 (1994)

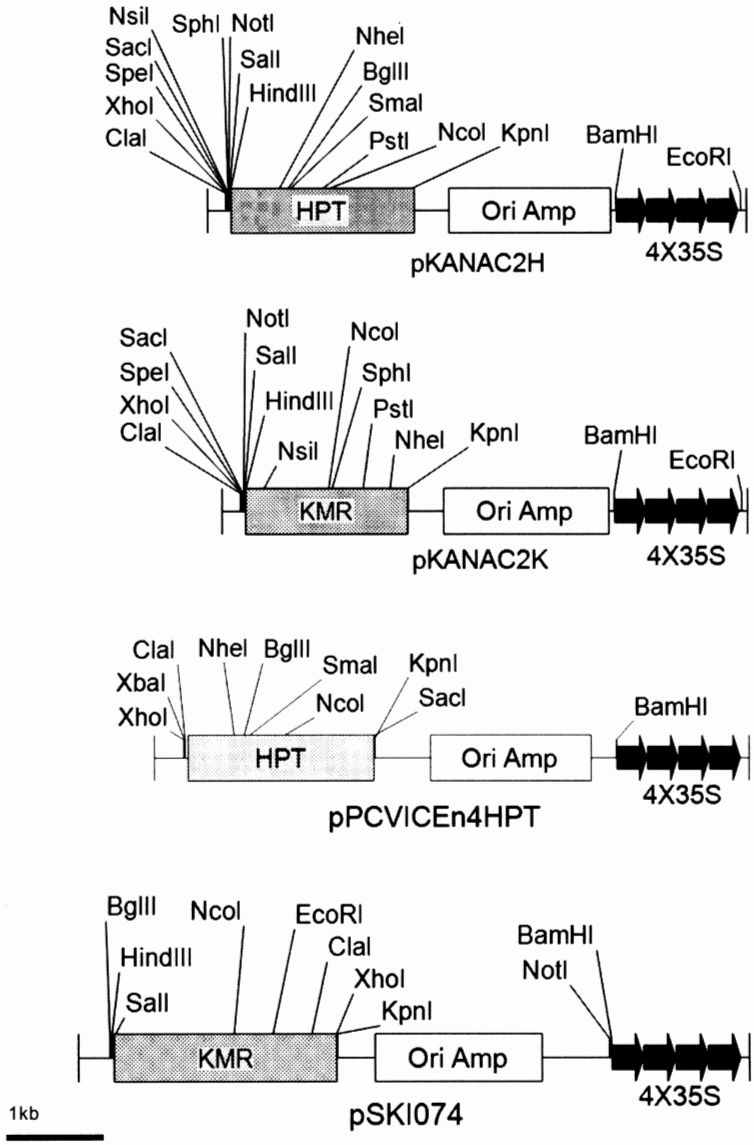

Figure 5. Sites of restriction enzyme available for plasmid rescue. 4×35S: tetramerized cauliflower mosaic virus 35S enhancer sequence, Ori Amp: replication origin of *E. coli* and ampicillin-resistance gene, KMR: kanamycin-resistance selection cassette, HPT: hygromycin-resistance selection cassette.

[8] Weigel, D., Ahn, J.H., Blázquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrándiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., Nguyen, J.T., Sato, S., Wang, Z.Y., Xia, Y., Dixon, R.A., Harrison, M.J., Lamb, C.J., Yanofsky, M.F. and Chory J.: Activation tagging in Arabidopsis. Plant Physiol., 122, 1003-1014 (2000)

[9] Zubuko, E., Adams, C.J., Machaekova, I., Malbeck, J., Scollan, C. and Meyer, P.: Activation tagging identifies a gene from Petunia hybrida responsible for the production of active cytokinins in plants. Plant J., 29, 797-808 (2002)

新規アクティベーションタギングベクターpKANAC2H及びpKANAC2Kの構築

清水圭一[†]・橋本文雄

要　約

新規の植物用アクティベーションタギングベクターpKANAC2H及びpKANAC2Kを構築した。これらのベクターは，一般的に使われているpPCVICEn4HPT及びpSKI074などのアクティベーションタギングベクターと比べて，T-DNA領域により多くのプラスミドレスキューに使える制限酵素部位を持っている。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pKANAC2H：AB889495，pKANAC2K：AB889496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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