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T-DNA Integration Patterns in Transgenic Tobacco Plants

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To investigate the various integration patterns of T-DNA generated by infection with *Agrobacterium*, we developed a vector (pRCV2) for the effective T-DNA tagging and applied it to tobacco (*Nicotiana tabacum* cv. Havana SR1). pRCV2 was constructed for isolating not only intact T-DNA inserts containing both side borders of T-DNA, but also for partial T-DNA inserts that comprise only the right or left side. We also designed PCR confirmation primer sets that can amplify in several important regions within pRCV2 to detect various unpredictable integration patterns. These can also be used for the direct inverse PCR. Leaf disks of tobacco were transformed with *Agrobacterium tumefaciens* LBA4404 harboring pRCV2. PCR and Southern analysis revealed the expected 584 bp product for the *hpt* gene as well as one of 600 bp for the *gus* gene in all transformants; one or two copies were identified for these integrated genes. Flanking plant genomic DNA sequences from the transgenic tobacco were obtained via plasmid rescue and then sequenced. Abnormal integration patterns in the tobacco genome were found in many transgenic lines. Of the 17 lines examined, 11 contained intact vector backbone; a somewhat larger deletion of the left T-DNA portion was encountered in 4 lines. Because nicking sites at the right border showed irregular patterns when the T-DNA was integrated, it was difficult to predict the junction regions between the vector and the flanking plant DNA.

Keywords: *Agrobacterium tumefaciens*, flanking plant DNA, tagging, T-DNA integration

A. tumefaciens-mediated transformation is the most popular and powerful technique for introducing foreign genes into plants (Ryu et al., 1998; Jin et al., 2002). This method requires that *vir* genes be activated to generate T-DNA that are transferred to the plant cells and integrated into the genome. Two proteins, virD1 and virD2, play key roles in recognizing the T-DNA border sequences and nicking the T-strand at each border. Due to the positions of the nick sites, T-strands theoretically embrace only 2 bp of the right border (RB), but 22 bp of the left border (LB) when T-DNA integrates into a plant genome (Pansegrau and Lanka, 1991). However, analysis of the junction regions has shown various unpredictable patterns between the T-DNA border repeats and the flanking DNA after integration (van der Graaff et al., 1996; Kim et al., 2003). Not only the T-DNA but also the vector backbone are sometimes transferred from *Agrobacterium* carrying such a binary vector (Martineau et al., 1994). Furthermore, the nicking positions at the RB are very complex (Sha et al., 2003). Approximately 10% of the T-DNA inserts also contain an intact LB that is still linked to vector sequences outside of the T-DNA (Mathur et al., 1998). Nevertheless, these often problematic irregular patterns can also serve as mutagens in order to discover gene functions.

Insertional mutagenesis using T-DNA is a valuable tool in the identification and isolation of genes now that several methods have been developed for screening T-DNA in a known gene and for recovering sequences flanking the insertions (Koes et al., 1995; Liu and Whittier, 1995; Cooley et al., 1996; Frey et al., 1998; Couteau et al., 1999; Krysan et al., 1999). In these methods, plasmid rescue and inverse PCR are generally used for sequencing the adjacent fragment (Feldmann, 1991). However, the success of fragment sequencing is usually limited under the unstable integration of T-DNA because plasmid rescue and inverse PCR can be successful only if one assumes that T-DNA border repeats are cut exactly. In particular, inverse PCR reactions are difficult when the position of the primer set, i.e., the one most adjoining the flanked plant DNA is uncertain.

Therefore, the objectives of this study were to investigate the various integration patterns of T-DNA and to develop an effective method for isolating the flanking plant DNA under irregular integration of T-DNA into the genome.

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MATERIALS AND METHODS

Construction of the T-DNA Tagging Vector

We constructed the T-DNA tagging vector, pRCV2, by ligating pCAMBIA 1301 with pBluescript II KS(+) (Stratagene, USA). pCAMBIA 1301 with the *hpt* gene (hygromycin resistance gene) and pBluescript II KS(+) were doubly digested with *Bam*HI and *Hin*dIII. Afterward, the *Bam*HI/*Hin*dIII fragment of pBluescript II KS(+) containing the ampicillin resistance gene and bacterial replication origin was inserted into the *Bam*HI/*Hin*dIII site of pCAMBIA 1301. The proper orientation and reading frame of the rescue-cloning vector were confirmed by restriction enzyme mapping, PCR, and DNA sequencing.

Transformation of Tobacco with *A. tumefaciens* Containing pRCV2

Tobacco (*N. tabacum*) leaf disks were first infected with *A. tumefaciens* containing the plasmid pRCV2 as described by Park and Kim (2000). To regenerate hygromycin-resistant transgenic plants, shoots were induced from the leaf disks in a selection medium containing 1.5 mg L^{-1} BA, 200 mg L^{-1} cefotaxime, and 20 mg L^{-1} hygromycin. The green shoots were then transferred to a root induction medium containing 200 mg L^{-1} cefotaxime and 20 mg L^{-1} hygromycin.

Plant Genomic DNA Isolation

Plant DNA was isolated as described by McCouch et al. (1988). Briefly, 0.5 g of tobacco leaf tissue was ground with liquid nitrogen in a well-chilled mortar and pestle. The fine powder was then mixed with extraction buffer (0.5 M NaCl, 0.1 M Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 1.25% SDS) and was maintained at 65°C for 20 min. The mixtures were extracted with phenol and chloroform, and precipitated in isopropanol. Precipitated DNA was picked out and washed in 70% EtOH, and dried to measure concentration.

PCR Confirmation of Transgenic Tobacco Plants

To detect the T-DNA fragments in the genome of transformed plants, 50 ng of genomic DNA was amplified by PCR using primer sets that can be amplified in several important regions of the vector, e.g., *hpt, gus,* or the vector backbone fragment. Each amplification was performed in a 50 µL reaction mixture with *Taq* polymerase and a thermocycler (Biometra, USA). In all, 35 PCR cycles were performed, each comprising a 1 min denaturation step at 95°C, 1 min annealing at 55°C, and 2 min extension at 72°C.

Southern Hybridization

Procedures for restriction enzyme digestion, electrophoresis, and Southern blot analysis were carried out according to Cho et al. (1994). Briefly, 20 µg of genomic DNA was digested with *Eco*RI, separated by electrophoresis in a 1% agarose gel, and transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech, USA). The plant DNA was then probed with a ^{32}P-labeled 0.7-kb fragment from the *hpt* gene to confirm integration of pRCV2 into the plant genome. Random primer labeling of the probe was conducted as stipulated for a Ladderman TM Labeling Kit (Takara, Japan).

Rescue Cloning and Inverse PCR

Plant genomic DNA was isolated from 17 transgenic lines carrying T-DNA of the pRCV2 vector. Twenty micrograms of genomic DNA was digested with *Pst*I, *Bam*HI, and *Hin*dIII, followed by ligation and extraction with phenol-chloroform. One half of the circularized DNA was used to transform electroporation-competent cells (Epicurian Coli SURE, Stratagene) with a Bio-Rad *Escherichia coli* Pulser Unit (Bio-Rad, USA) according to manufacture's instructions. The other half was used for inverse PCR, which was performed in a 50 µL reaction mixture with *EF-Taq* polymerase (Solgent, Korea) and a thermocycler (Biometra, USA). PCR comprised 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 60°C, and 2 min extension at 72°C.

PCR Analysis of Rescued Clones

Ten ng of plasmid DNA from each of the rescue clones was amplified by PCR using primer sets prepared to confirm the existence of vector backbone within the rescued plasmid. Each amplification was performed in a 50 µL reaction mixture with *Taq* polymerase and a thermocycler (Biometra). The 35 PCR cycles each comprised 1 min denaturation at 95°C, 1 min annealing at 55°C, and 2 min extension at 72°C.

Sequence Analysis

Purified plasmids from the rescue clones were

sequenced using an adjacent primer of a near the flanking plant DNA. The products of the inverse PCR were inserted into the pGEM-T vector (Promega, USA) and sequenced automatically with T7 and SP6 primers at the Macrogen (Korea). The flanking sequences were then subjected to BLAST searches at the National Center for Biotechnology Information.

Progeny Segregation Test

R_1 seeds from the self-transformants were germinated on a MS medium containing 100 mg L^{-1} hygromycin. After 5 weeks, the number of seedlings resistant or susceptible to hygromycin was counted.

RESULTS AND DISCUSSION

Construction of T-DNA Tagging Vector

The T-DNA tagging vector, pRCV2, was constructed by ligating pCAMBIA 1301 with pBluescript II KS(+), a procedure similar to one described for pRCV3 (Kim et al., 2003). During the cloning procedure, the *Pst*I site was deleted within the pRCV2 T-DNA, which was then used with the plasmid-rescue technique to isolate the intact T-DNA insert containing both side borders. Because both *Bam*HI and *Hin*dIII can be used for the isolating partial T-DNA inserts, the former is involved with rescue-cloning, during which one obtains the right side of the T-DNA that contains the RB and the flanked plant DNA. With the latter enzyme, the left side of the T-DNA containing the LB and flanked plant DNA are obtained (Fig. 1).

A. tumefaciens-Mediated Transformation of Tobacco with pRCV2

Transgenic tobacco plants were obtained by first incubating leaf disks with *A. tumefaciens* that contained the pRCV2 vector, then inducing shoot development in a selection medium. After being transferred to the root induction medium, the transgenic shoots showed root formation. Those that were capable of rooting within three weeks, i.e. hygromycin-resistant, were considered most likely transformed, whereas those showing no root growth until four to five weeks after their transfer were probably escapes that survived until the hygromycin had broken down.

Southern blot analysis was performed on total genomic DNA of the 18 presumed transformants to confirm that they contained pRCV2 and to determine the copy number of the integrated transgenes. When the genomic DNA was digested with *Eco*RI and hybridized with the *hpt* probe, integrated T-DNA was observed with the vector-containing transgenic plants. In contrast, no hybridization signals were detected in the genomic DNA of either the non-transformed plant or the one from Line 3, which was considered an 'escape' (Fig. 2). Copy numbers varied from one to two, similar to the insertion pattern described for *Arabidopsis* (Akama et al., 1995).

Among the 17 T_0 lines confirmed by Southern blot analysis, 11 (having one or two transgene copies) were self-pollinated to obtain T_1 progeny. To examine the segregation ratio in these T_1 lines, we tested 150 seeds for their hygromycin resistance. Six transformants (Lines 2, 4, 6, 8, 11, and 16) manifested ratios of approximately 3:1, which is the expected Mendelian inheritance of one independent locus.

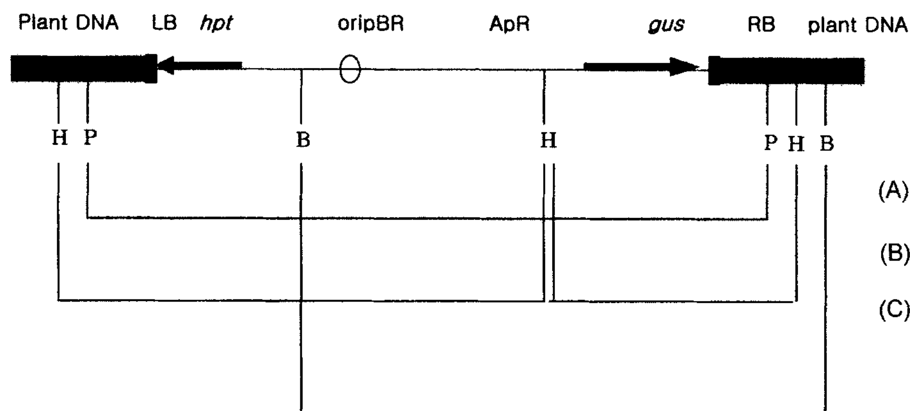

Figure 1. Strategy for isolating T-DNA insert that contains either both side borders of the T-DNA (**A**) or one side border of the T-DNA (**B, C**) during rescue-cloning and inverse PCR. LB and RB, left and right T-DNA 25-bp border repeats; ori_{bR}, pBR322 replication origin; Ap^R, ampilicillin resistance gene; *hpt*, hygromycin phosphotransferase gene; *gus*, β-glucuronidase gene; P, *Pst*I; B, *Bam*HI; H, *Hin*dIII.

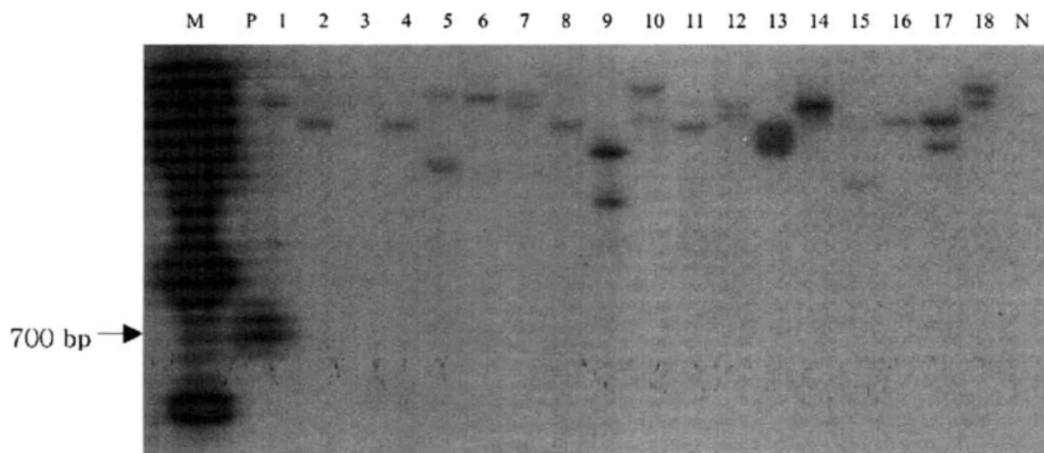

Figure 2. Southern hybridization of DNA from transformants with rescue-cloning vector RCV2. Genomic DNA was digested with *EcoR* I, and probed with a [^{32}P]-labeled 0.7-kb *hpt* fragment from pRCV2 plasmid DNA. M, size marker that is lamda DNA digested with *Hind*III; P, positive control that is *hpt* fragment from pRCV2; Lanes 1-18, transgenic tobacco lines; N, non-transgenic tobacco plant as a negative control.

The remaining T_1 lines, except Line 8, showed ratios of >3:1 (data not shown). This result is consistent with the copy numbers obtained via Southern blot analysis, except that Line 8 showed an abnormal segregation ratio of 1:1 for resistance.

To confirm the existence of the transgene in the progeny genomes, we conducted PCR of the T_1 lines, using specific primers of the *hpt* gene-coding region. This analysis revealed the expected 700-bp product for the *hpt* gene in all 11 T_1 lines (Fig. 3), thereby demonstrating that the transgene was stably inherited by the next generation.

Analysis of T-DNA Integration Pattern

To analyze the various integration patterns of T-DNA, we selected 17 transgenic lines with different patterns. In order to distinguish among them, genomic PCR was performed, using the primer set amplified in various regions of pRCV2, e.g., the T-DNA inside the region of the vector backbone. That backbone region of the RB was amplified in 11 transgenic lines, while another 2 lines showed amplification for both LB and RB. The remaining four lines had no amplification of the left portion of the T-DNA that contained the LB and the NOS terminator.

Based on these data, we selected four transgenic lines that showed distinct and abnormal integration patterns in the tobacco genome. For both the left and the right insert junctions, plant DNA is usually digested with an enzyme that does not cleave within the T-DNA but is self-ligated. Therefore, we digested our genomic DNA with *Pst*I, self-circularized it by ligation, and transformed it to *E. coli* by electroporation. These intact T-DNA tags, obtained by rescue-cloning from the four lines, were then fully sequenced to assess their integration patterns. The remaining 13 lines were also analyzed by either res-

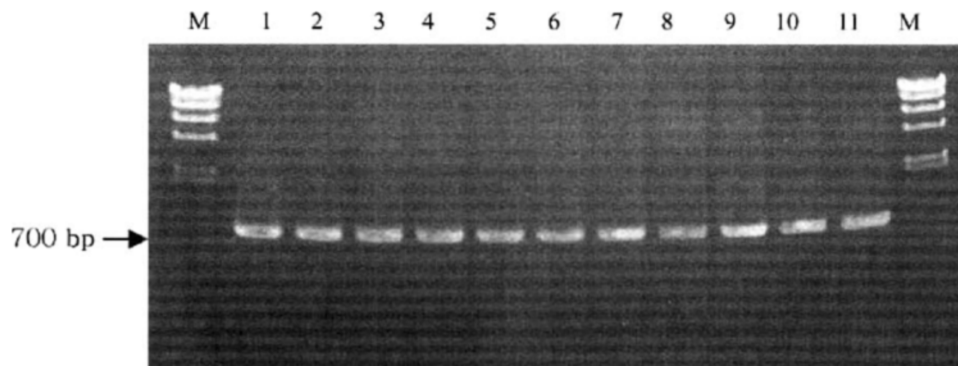

Figure 3. Detection of *hpt* gene in T_1 tobacco plants by PCR. Expected size of *hpt* (700 bp) of PCR products appeared. M, Lamda DNA digested with *Hind*III; Lanes 1-11, T_1 tobacco lines.

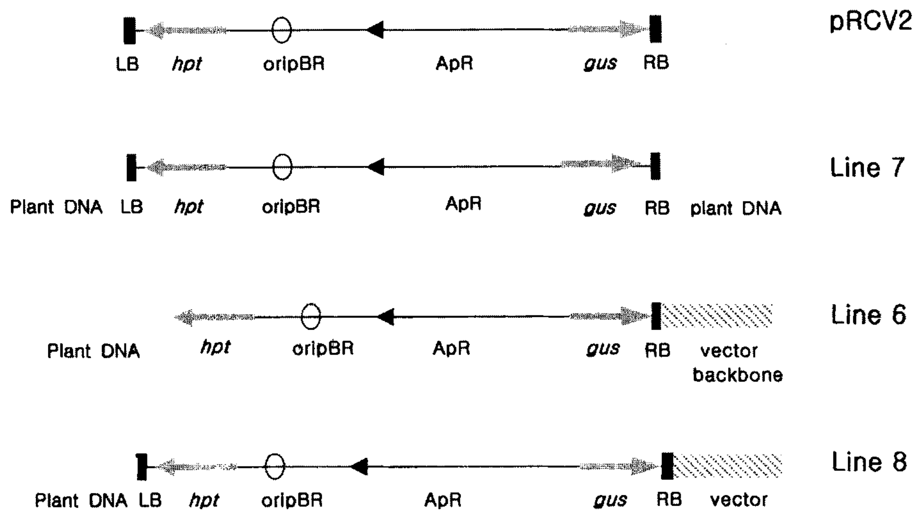

Figure 4. Schematic map of pRCV2 vector and isolated T-DNA structures from the lines 6, 7, and 8. LB and RB, left and right T-DNA 25-bp border repeats; ori_{pR}, pBR322 replication origin; Ap^R, ampilicillin resistance gene; *hpt*, hygromycin phosphotransferase gene; *gus*, β-glucuronidase gene.

cue-cloning or inverse PCR according to their T-DNA structures, as confirmed by genomic PCR. For example, in cases where the T-DNA would normally be cleaved in the RB, we used inverse PCR to isolate the insertion site of T-DNA and the flanking plant DNA. T-DNA tags were also partially retrieved by digestion with *Bam*HI or *Hin*dIII when rescue was not possible because of the large size of the intact tag.

From this intact T-DNA sequencing, we found that the transgene structure of Lines 6 and 8 had a vector backbone connected to the RB (Fig. 4). In particular, the full backbone of pRCV2 linking to the RB was co-integrated into the genome of Line 8. This indicates that transfer of the binary vector sequences flanking the RB probably resulted from T-DNA processing followed by skipping of the RB (van der Graaff et al., 1996). This skipping was also detected when partial rescue-cloning and inverse PCR was conducted with the other 13 transgenic lines (Fig. 5).

In this study, the nicking positions on the right side of the T-DNA were very complex out of the RB. Moreover, a somewhat larger deletion in the left portion of the T-DNA was seen in three transgenic lines. These results are similar to those from previously reported research, in which the LB was usually deleted, possibly because it lacked the protection of the *vir*D2 protein (Matsumoto et al., 1990; Mayerhofer et al., 1991; Tinland and Hohn, 1995). Gener-

T-DNA right border

pRCV2 ATTGGCGGGTAAACCTAAGAGAAAAGAGCGTTTATTAGAA--

Transgenic line

Line 6	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAATAA-
Line 10	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAATAA-
Line 11	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAATA--
Line 13	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TT--------------
Line 18	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAAT-----
Line 19	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAATAA-
Line 20	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAA-------
Line 23	aggaggccggcgcgggcctgcgtagcctggcagagccgtgggccgacaccacg-----------------
Line 26	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAATAA-
Line 27	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAATA--
Line 34	ATTGGCGGG**TAAACCTAAGAGAAAAGAGCGTTTA**TTAGAATAA-

Figure 5. Right-border fusion sequences of isolated T-DNA using gene-tagging approach. Nucleotides of the T-region are presented in capitals, with T-DNA right-border repeat sequences in bold and the whole-plant DNA sequences in lower case Sequence of right-border repeat of plasmid pRCV2 is shown on top for comparison.

ally, that protein is covalently bound to the nucleotide at the 5' end of the nick site; such binding prevents an exonucleolytic attack to the 5'-end of the single-stranded T-strand (Durrenberger et al., 1989; Tinland, 1996).

Strategy for Isolation of Flanking Plant DNA under Irregular Transgene Structures

Development of an effective method for isolating flanking plant DNA under irregular transgene structures requires that the actual integration patterns of the T-DNA in the plant genome be examined. This is because sequencing of the insert junctions is difficult when one is uncertain of the positioning of the primer sets that most closely adjoin the flanked plant DNA. Those inserts that contain the vector sequences located outside the T-DNA are discarded after hybridization with a probe from the vector backbone (Mathur et al., 1998). Therefore, the integration pattern of each transgenic line is analyzed by PCR, making this an effective strategy for isolating the flanking plant DNA and establishing individual patterns.

In our study, each transgenic line was amplified with PCR confirmation primer sets that were capable of amplifying in several important regions of the pRCV2 vector, e.g., the RB backbone. Based on this data, we could assign each transgenic line to one of two types: 1) having normal integration at the RB; or 2) having the T-DNA insert still linked to the vector sequence of the T-DNA outside. In the case of the normal type, genomic DNA from the transgenic plant was digested with *Hind*III and self-ligated. One half of the circularized DNA was then used for inverse PCR to isolate the flanking plant DNA that embraced the RB; the other half was used in rescue-cloning of the left portion (Fig. 1). In that second case, the genomic DNA was digested with *Bam*HI or *Hind*III, then self-ligated and transformed to *E. coli*. Based on the integration patterns of our transgenic lines, as confirmed by PCR, we could determine the most suitable primers, i.e., those most closely adjoined to the flanked

Table 1. Oligonucleotides used in sequence analysis and inverse PCR of pRCV2 vector.

Primer	Position[z] (bp)	Primer sequence
SQ1	2790	5'-AGCGCGCAAACTAGGATAAA-3'
SQ2	3530	5'-CCAAGCTGTTTTCCGAGAAG-3'
SQ3	4271	5'-GCTGCGTATATGATGCGATG-3'
SQ4	13952	5'-ATAATACCGCGCCACATAGC-3'
SQ5	12076	5'-GCGGCGAACTTACTTCTGAC-3'
SQ6	11079	5'-CGCGCGTAATACGACTCACT-3'
SQ7	9654	5'-TGTCTGTTGTGCCCAGTCAT-3'
SQ8	8879	5'-TCCCAGATAAGGGAATTAGGG-3'
SQ9	8421	5'-TAACGATGACAGAGCGTTGC-3'

[z]Position of pRCV2 map.

Table 2. BLAST analysis with sequenced T-DNA tags.

Transgenic plant line	Similarity to (accession number)	Identity (%)	Reference
2	*Oryza sativa* genomic DNA. Chromosome 1, BAC clone:OSJNBa001 (AP003210)	100	Sasaki et al., 2002
4	unknown	-	-
5	*Oryza sativa* genomic DNA. Chromosome 1, PAC clone:P0707D10 (AP002910)	31	Sasaki et al., 2002
5	unknown	-	-
6	unknown	-	-
8	unknown	-	-
10	unknown	-	-
10	unknown	-	-
11	*Arabidopsis* myosin heavy chain gene (U19616)	99	unpublished
15	unknown	-	-
15	unknown	-	-
16	unknown	-	-
17	unknown	-	-
17	unknown	-	-
18	unknown	-	-
18	unknown	-	-

plant DNA in each rescued clone, and use them for sequencing or inverse PCR (Table 1).

Both the products of inverse PCR and the plasmid DNA obtained by rescue-cloning were examined by sequence analysis; the resultant data were subjected to BLAST homology searches (Table 2). Here, the flanking plant DNA from Line 11 showed significant similarity to the *Arabidopsis* myosin heavy chain gene whereas that from Lines 2 and 5 was similar to *O. sativa* genomic DNA.

Rescue-cloning and inverse PCR are simple methods for isolating T-DNA-tagged chromosomal DNA segments and conducting sequence analysis of the plant genome by reverse genetics (Yanofsky et al., 1990). Both techniques can be performed by cutting the plant DNA that contains a T-DNA insert and following this with self-ligation (Feldmann, 1991). These self-ligated DNA can then be used with them for bidirectional sequencing (Mathur et al., 1998). Inverse PCR facilitates the isolation of sequences in the upstream or downstream region of a known DNA "sequence core". Historically, the RB or LB portions of the T-DNA usually served as that "core" when isolating plant flanking DNA. However, when applied, the rate of success was very low because the actual integration patterns were too varied. Therefore, bidirectional sequencing together with rescue-cloning and long-range inverse PCR have proven more effective.

To discover the functions of genes that do not display phenotypes when mutated, it is important to show that only a single insert exists in the transformed line. However, even transgenic plants with multiple gene copies can be used in such research because the disrupted genes can often be readily identified when compared with published sequences. Further more, transgenic plants with multiple inserts are useful in studies on gene silencing because the transgene copy number and the structure of the inserted T-DNA can be correlated with transgene expression (Park et al., 1996; Parinov et al., 1999). This was demonstrated in our current investigation, in which rescue-cloning was effective in re-cloning the inserted fragments from transgenic lines having multiple inserts.

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