Determination of Transgene Repeat Formation and Promoter Methylation in Transgenic Plants

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ABSTRACT

The integration of transgenes into a plant host genome following Agrobacterium tumefaciens*-mediated or direct transformation may occur as a single copy or in the form of tandem repeats. The latter has been associated with promoter methylation and silencing of transgenes. Thus, the early screening of such transgenic plants is desirable for ruling out future repeat-dependent transgene instability. We developed a simple PCR-based method in which primer pairs were specifically designed so that amplifications could only be obtained if the transgene was present in the form of multiple inserts in a transgenic line. The method was established using 35S-*rolC *transgenic aspen lines showing morphologically visible transgenic silencing. Later, it was possible to screen independent transgenic lines showing no visible marker gene expression. Furthermore, a method was developed in which positive PCR amplification was indicative of promoter methylation. The results were consistent and reproducible across different independent transgenic lines. The methods were quick, reliable, consistent and reproducible, and can be useful for routine screening of transgene silencing in lines derived from many different systems.*

INTRODUCTION

Epigenetic transgene inactivation occurs most often when multiple copies of a transgene are inserted at the same chromosomal site. This has frequently been associated with methylation of transgene promoters (1,6,7,9,12,13). Following *Agrobacterium tumefaciens*-mediated or direct plant genetic transformation, transforming plasmids may undergo rearrangements before or during integration into the host genome. Integrated transgenes may be arranged head-to-tail as a direct repeat (DR), and head-to-head or tail-to-tail as inverted repeats (IR) (11,19). These changes lead to a loss of integrity of the construct and may give rise to abnormal transcripts (aberrant RNA that may affect DNA methylation) (10). Early screening of such transgenic plants containing repeated transgenes is therefore important to rule out any future repeat-based instability of the transgene.

Transgene repeat formation in crop plants is routinely determined using Southern blot analysis (2,6,14,20). This method is labor intensive but satisfactory, particularly when a small number of samples is analyzed. However, it is relatively impractical for routine screening of large numbers of transgenic lines. Other procedures, such as inverse PCR, randomly primed PCR, vector ligation PCR and a recently reported method based on the amplification of a region flanking the transgene in a restriction fragment (18) have been developed for amplifying uncloned plant genomic DNA that flanks the transgene. These methods are useful and informative when there is either a single transgene insert or when multiple inserts are present at different loci. The determination of the transgene copy number becomes more complex when multiple inserts are integrated into the same locus (18).

Southern blot analysis using the isoschizomers *Hpa*II and *Msp*I is the most commonly used method to determine promoter methylation (1,16). Genomic sequencing of bisulfite-modified DNA is another way to determine methylation patterns in chromosomal DNA (15). However, both methods are time consuming, technically complex, expensive and are generally not used for routine analysis. New procedures are needed to allow fast and inexpensive analysis of the many samples encountered in transgenic plant research.

Here, we describe two simple one-step, PCR-based methods for easy selection of transgenic lines containing a single transgene copy, without promoter methylation. The first method uses a set of primers for genomic DNA to amplify different organizations of transgene repeat rearrangements. This method is quicker, inexpensive and more reliable than existing ones to determine multiple inserts at the same locus. Second, for promoter methylation, we propose digestion of genomic DNA with methylation-sensitive restriction enzymes followed by PCR amplification of promoter regions. Positive results indicate promoter methylation.

MATERIALS AND METHODS

Plant Materials and Transformation

A leaf disc co-cultivation method was used for *Agrobacterium*-mediated binary vector transformation of aspen (*Populus tremula* L.) and hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx) (4,5). The plasmid pPCV002-CaMVC (17) carries the chimeric *rol*C gene from the Ri-plasmid of *Agrobacterium rhizogenes* under the control of the 35S-promoter of cauliflower mosaic virus (3,17).

Plant Growth, Morphological Analysis and DNA Extraction

Transgenic plants propagated in vitro were transferred to and cultivated in a greenhouse under natural daylight conditions (4,5). Aspen plants transgenic for the 35S-*rolC* construct are characterized by a reduction in plant height associated with shortened internodes, smaller leaves and a lower chlorophyll content in leaves (4,5). For the detection of morphologically visible reversions indicating transgene inactivation, the transgenic plants grown in a greenhouse were compared to control plants for the *rolC* characteristic traits (4). Two hybrid aspen-based, single-copy transgenic lines (Esch5:35S-*rolC*#3 and #5) were used as controls (3). Extraction of genomic DNA was carried out as described (4,5).

Strategy to Determine T-DNA Repeats

The DNA isolated was used for long template PCR, which was performed with the Extend™ Long Template PCR kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's recommendations. PCR was conducted using five different pairs

Table 1. Determination of T-DNA Repeats in Transgenic Plants with PCR Using Reverse Primers

	PCR Amplifications Using Different Primer Pairs					Remarks
(A) Direct repeat[a]	1+2	1+5	1+3	2+6	2+4	
	-	-	-	-	-	No T-DNA repeat
	+	+	+	+	+	Complete repeat
	+	+/-	-	+/-	-	Incomplete repeat (both copies are truncated)
	+	+	+	-	-	Incomplete repeat (on left border of left copy T-DNA)
	+	-	-	+	+	Incomplete repeat (on right border of right copy T-DNA)
	-	+[b]	+[b]	-	-	Incomplete repeat (on left border of right copy T-DNA)
(B) Inverted repeat[c]						
(i) Head-to-head	+	+	+	+	+	Amplification due to one primer (see Figure 1C, i)
(ii) Tail-to-tail	+	+	+	+	+	Amplification due to one primer (see Figure 1C, ii)

[a]Other possible combinations that may be interpreted according to the PCR results obtained are not ruled out.
[b]Band size smaller than expected.
[c]The status (complete or truncated) and location of a repeat may be determined by the presence or absence of bands with different primer pairs and by their respective size, as in case of a direct repeat.

of primers (1+2, 1+5, 1+3, 2+6 and 2+4) (Figure 1A). Because these primer pairs face away from each other in the construct, it is not possible to achieve PCR amplification in case of a single-copy integration or when multiple copies are integrated at different chromosomal positions. However, PCR products will be observed in transgenic lines with direct or inverted repeats that may be present in complete or truncated forms. The PCR results obtained from different primer pairs may determine if there is a complete or truncated transgene repeat, and the direction of an incomplete repeat (Figure 1) (Table 1).

Promoter Methylation

The methylation-sensing ability of isoschizomeric restriction endonucleases was used to examine methylated cytosines in the 35S-promoter. The isoschizomeric pair *Hpa*II/*Msp*I cuts the sequence 5′-C/CGG-3′, but each enzyme differs in sensitivity to cytosine methylation. *Hpa*II cuts very weakly if the outer C is methylated but does not cut if the inner C is methylated; *Msp*I cuts if the inner C is methylated but cannot cleave if the outer C is methylated (8).

All restriction enzyme digestions were carried out with approximately 1 µg genomic DNA. The reactions were performed in 10-µL volumes using the manufacturer's buffers (Roche Molecular Biochemicals). The reactions were incubated overnight at 37°C. PCR was performed using primer pairs specific for amplifying the 35S-promoter region. As a positive control, a primer pair was added for the amplification of a small genomic region that had no restriction site for the enzymes used.

RESULTS

Based on morphological observation, two transgenic lines generated from wild aspen (W52:35S-*rolC*#9 and #12) and one line from hybrid aspen (Esch5:35S-*rolC*#2) were screened for *rolC* revertant features and tested for possible T-DNA repeat formation and promoter methylation. Table 2 summarizes the PCR results obtained from different transgenic aspen lines using reverse primer pairs. No amplification was observed in transgenic lines with a single-copy insert (Esch5:35S-*rolC*#3 and #5, Table 2). This was expected because the primer pairs faced away from each other (Figure 1A).

In contrast, transgenic lines revealing *rolC* revertant features showed positive results for reverse-primer PCR (Figure 2A). In the transgenic line, Esch5:35S-*rolC*#2, amplifications were obtained with all the primer pairs (Figure 2A) (Table 2), and the sizes of the bands were in line with expected sizes for a complete direct T-DNA repeat (Table 1) (Figure 1B). However, in two other transgenic lines, amplification products were obtained with some primer pairs but not with others, indicating that T-DNA repeats were present in truncated forms in these lines. In the transgenic line W52:35S-*rolC*#12, positive results were obtained with primer pairs 1+2 (Figure 2A), 1+5 and 2+6, but no amplifications were obtained with primer combinations 1+3 or 2+4 (Table 2). This suggests that the binding sites for primers 3 and 4 were missing in this transgenic line and that T-DNA copies were truncated.

The transgenic line W52:35S-*rolC*#9 showed positive results with primer pairs 1+5 or 1+3; however, the sizes of the bands were smaller than expected for a complete repeat (Figure 2A) (Table 2). This indicates that a part of the T-DNA had been lost in the repeat. Furthermore, no amplification could be obtained with primer pairs 2+6 or 2+4, which implies that the binding site for primer 2 was missing in

Table 2. T-DNA Repeats in Transgenic Aspen

Transgenic Line	PCR Amplifications Using Different Primer Pairs					Conclusions
	1+2	1+5	1+3	2+6	2+4	
Esch5:35S-*rolC*#3	-	-	-	-	-	No repeat
Esch5:35S-*rolC*#5	-	-	-	-	-	No repeat
Esch5:35S-*rolC*#2	+	+	+	+	+	Complete repeat
W52:35S-*rolC*#12	+	+	-	+	-	Both T-DNA copies are truncated
W52:35S-*rolC*#9	-	+[a]	+[a]	-	-	Incomplete repeat (on left border of right copy T-DNA)

[a]Band size is smaller than expected.

the T-DNA repeat.

The PCR results for the DNA digested with methylation-sensitive enzymes *Hpa*II/*Msp*I showed cytosine methylation in the promoter region of the three aspen transgenic lines containing T-DNA repeats (Figure 2B). Similar signal strength of bands digested with both restriction enzymes was observed in the line Esch5:35S-*rolC*#2. It indicates that both the outer and inner Cs in the restriction site-specific sequence 5'-C/CGG-3' were methylated in this transgenic line.

However, in the transgenic line W52:35S-*rolC*#9, a strong signal with *Msp*I-digested DNA and a weak signal with *Hpa*II-digested DNA were observed. This implies that the outer C is methylated in this line because *Msp*I was unable to cut and *Hpa*II cuts with reduced efficiency when the outer C was methylated. Similarly, positive results with *Msp*I-digested DNA and no amplification from *Hpa*II-digested DNA in the transgenic line W52:35S-*rolC*#12 suggest methylation of the outer C in this cell line. The transgenic line with a single T-DNA insertion (Esch5:35S-*rolC*#3) did not show any promoter methylation because negative results were obtained from DNA digested with both enzymes.

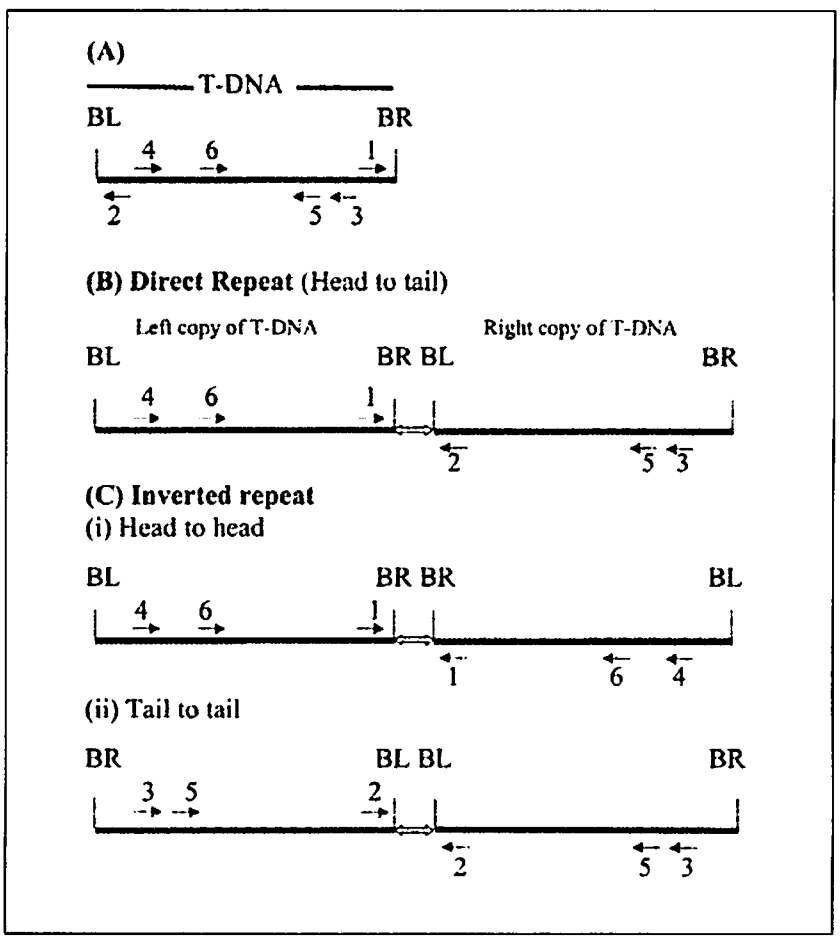

Figure 1. Schematic diagrams of possible transgene repeat arrangements in transgenic plants. (A) T-DNA with left (BL) and right (BR) borders. The numbers (1–6) represent the locations of different primers used and arrowheads indicate their respective directions. The primer pairs used (1+2, 1+5, 1+3, 2+6 and 2+4) are oriented in reverse directions, which rules out any possibility of PCR amplification when there is single-copy integration or when multiple copies are integrated into different chromosomal positions. (B) T-DNA direct repeat in the form of head-to-tail integration. Positive PCR results are obtained in this situation. The presence or absence of amplications with different primer pairs is described in Table 1. (C) Inverted T-DNA repeats in head-to-head (i) and tail-to-tail (ii) arrangements. PCR amplification is obtained with a single primer (1, 6 or 4 in the case of head-to-head and 2, 5 or 3 in tail-to-tail arrangement). The results with different primers and their sizes determine the status of the T-DNA repeat.

DISCUSSION

In this study, we applied simple PCR procedures for the determination of T-DNA repeats and promoter methylation in *A. tumefaciens*-derived transgenic aspen. Rearrangements of transforming plasmids may occur before or

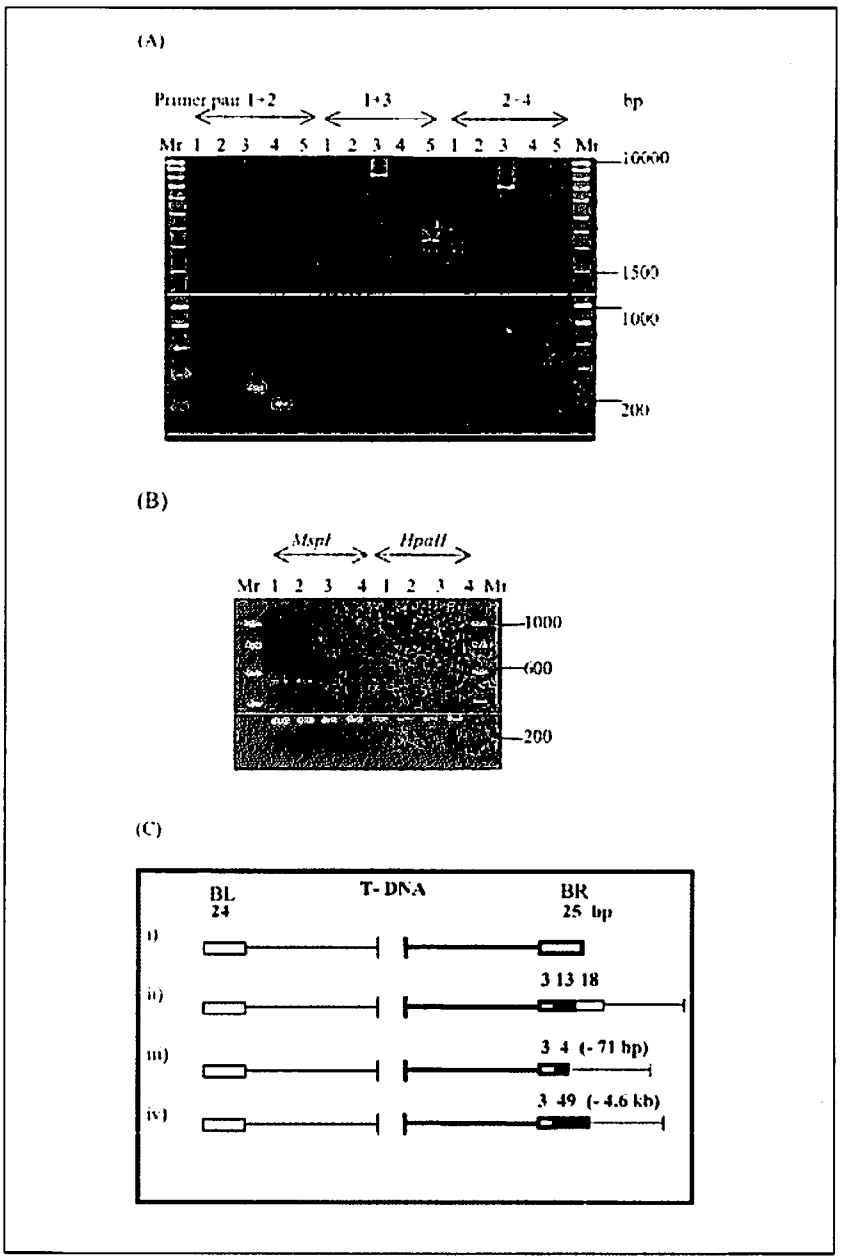

Figure 2. Molecular analyses for T-DNA repeats and promoter methylation in transgenic aspen. PCR was analyzed on ethidium bromide-stained 1.5% agarose gel. Mr is the Smartladder molecular weight marker (Eurogentec Bel SA, Seraing, Belgium). (A) Amplification products obtained from different transgenic aspen lines. 1: Esch 5:35S-*rolC*#3; 2: Esch5:35S *rolC*#5 (both single-copy T-DNA integrations); 3: Esch5:35S-*rolC*#2; 4:W52:355S-*rolC*#12; 5:W52 35S-*rolC*#9 (3–5: *rolC* gene silencing genotypes). Results are summarized in Table 2. (B) PCR analysis for 35S promoter methylation. The results obtained from *MspI*/*HpaII*-digested DNA are indicated by arrows. The lower band present in all the lanes is the positive control, and the upper band is the result of amplification of the 35S promoter. (C) Schematic drawing of observed T-DNA repeats. Open boxes: left border (BL) and right border (BR) regions; normal lines: BL and its adjacent region; thick lines: BR and its adjacent region; filled boxes: filler DNA. (i) Single T-DNA region: BL and BR regions of 24 and 25 bp, respectively (3, 17). (ii) Esch5:35S-*rolC*#2: the residual BR region is linked to the BL (18 bp) of the second T-DNA copy separated by a 13-bp filler region. (iii) W52: 35S-*rolC*#12: the BR residual sequence region is separated from the linked T-DNA copy by a 4-bp filler DNA region and a deletion of the BL of the linked copy along with a 71-bp region. (iv) W52:35S-*rolC*#9: as in (iii), but with a 49-bp filler DNA region and a 4.6-kb additional deletion. A conserved 3-bp region in the BR is common to these three transgenic lines (ii, iii and iv).

during the process of transformation, resulting in multiple transgene inserts that may have various organization. This makes analysis of multiple inserts difficult with existing methods. Our method for transgene repeat determination is based on five pairs of primers that cover most of the transgene region and amplify all possible multiple-copy inserts. The method worked well when more than two T-DNA copies were integrated into the same locus, although multiple bands were obtained in this case (data not shown). The results obtained from three aspen transgenic lines for T-DNA repeat formation were confirmed by sequencing T-DNA repeat junctions. These are shown schematically in Figure 2C.

For the present analysis, we used transgenic lines that showed morphological inactivation of the *rolC* gene. This method has already been extended to screen 31 independent aspen and hybrid aspen transgenic lines harboring four different gene constructs without visible markers. Six transgenic lines were observed with T-DNA repeat inserts (data not shown). Plants with a single transgene copy are desirable for stable transgene expression, and simple screening methods that eliminate plants with multiple insertions will be advantageous when a large number of transgenic lines are examined.

We also studied the methylation status of the transgene promoter and observed that the promoter was methylated in all three transgenic lines containing T-DNA repeats. Although it is possible that methylation alone does not repress transcription, promoter methylation has been a useful marker for transcriptional gene silencing (12). Therefore, a simple method to determine promoter methylation that can be used as an indicator of transgene silencing is desirable.

Our procedure to determine promoter methylation consisted of two steps: (*i*) digestion of genomic DNA with methylation-sensitive restriction enzymes and (*ii*) PCR using promoter-specific primers. We have tried eight different methylation-sensitive restriction enzymes (data not shown) and found that the isoschizomeric pair *HpaII*/*MspI* is best suited to study promoter methylation. We have also tested the Southern blot analysis method with DNA digested with methylation-sensitive restriction enzymes in transgenic lines containing multiple inserts. Ambiguous banding patterns were observed that were difficult to interpret (data not shown). On the other hand, the present method is simple to perform and easy to interpret because the PCR yields a positive display for promoter methylation.

In summary, we have developed a simple, quick and straightforward approach for screening unstably transformed plants from among transgenic plants that may become silent for future transgene expression. We established methods for transgene repeat determination and promoter methylation in transgenic aspen lines that carry a morphologically visible marker; the methods were successfully tested for transgenic lines without visible markers. This two-way approach can be used for the routine screening of transgenic lines from other plant systems to eliminate unstably transformed lines for transgene expression.

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Transient Host Range Selection for Genetic Engineering of Modified Vaccinia Virus Ankara

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ABSTRACT

Recombinant vaccinia viruses are extremely valuable tools for research in molecular biology and immunology. The extension of vaccinia vector technology to replication-deficient and safety-tested virus strains such as modified vaccinia virus Ankara (MVA) have made this versatile eukaryotic expression system even more attractive for basic and clinical research. Here, we report on easily obtaining recombinant MVA using stringent growth selection on rabbit kidney RK-13 cells. We describe the construction and use of new MVA vector plasmids that carry an expression cassette of the vaccinia virus host range gene, K1L, as a transient selectable marker. These plasmids allow either stable insertion of additional recombinant genes into the MVA genome or precisely targeted mutagenesis of MVA genomic sequences. Repetitive DNA sequences flanking the K1L gene were designed to remove the marker gene from the viral genome by homologous recombination under nonselective growth conditions. The convenience of this new selection technique is demonstrated by isolating MVA recombinants that produce green fluorescent protein and by generating MVA deletion mutants.

INTRODUCTION

Genetically altered vaccinia virus serves as a well-established expression system in the laboratory and has proven successful for the development of new candidate recombinant vaccines (9). The introduction of vector viruses based on the replication-deficient modified vaccinia virus Ankara (MVA) provided the capability for high-level gene expression while promising exceptional biological safety (15). Recombinant MVA has been successfully evaluated for vaccination against a variety of infectious diseases or cancer in animal models (1,4,12,17), and the first MVA vectors have now entered clinical investigation. While there is increasing demand for the evaluation of new viral constructs, the generation of MVA vectors is different from replication-competent recombinant vaccinia virus because of the growth deficiency of the virus and the diminished cytopathic effects.

Quick and easy methods for generating recombinant MVA are necessary to compare multiple candidate constructs. In addition, as the use of recombinant MVA vectors approaches clinical application, more accessible genetic engineering should enable the generation of virus vectors that contain multiple genomic insertions and modifications, offering possible advantages for specific prophylaxis or therapy. Previously, several protocols were used to isolate recombinant MVA, relying mainly on the co-production of reporter enzymes such as the *E. coli* β-galactosidase and β-glucuronidase, which allow screening of foci of MVA-infected cells by colorimetric assays (2,4,12, 15). Additional co-expression of the *E. coli gpt* gene encoding xanthine-guanine-phosphoribosyl-transferase has been shown to simplify purification of recombinant MVA by allowing selec-