



Cloning flanking sequence by single-primer PCR in transgenic plants

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ABSTRACT. The insertion position of exogenous genes in plant genomes is usually identified by adapter ligation-mediated polymerase chain reaction (PCR), thermal asymmetric interlaced PCR, and restriction site extension PCR in transgenic plant research. However, these methods have various limitations, such as the complexity of designing primers and time-consuming and multiple-step procedures. The goal of this study was to establish an easier, more rapid, and more accurate method to clone flanking sequence using single-primer PCR in transgenic plants. Unknown flanking genome sequences in transgenic plants, including those in tobacco, soybean, rice, and maize, were cloned using the single-primer PCR method established in this study, with the *Bar* gene as the anchor gene. The primer 1 (P1), P2, and P3 PCRs obtained 4 sequences, and the completely correct flanking sequence of 508 bp that was obtained in the P3 PCR was verified by sequencing analysis. The single-primer PCR is more rapid and accurate than conventional methods, justifying its application widely in cloning flanking sequences in transgenic plants.

Key words: Single-primer PCR; Unknown flanking sequences; Transgenic plants

INTRODUCTION

The insertion positions of exogenous genes in targeted plant genomes is usually identified by adapter ligation-mediated polymerase chain reaction (PCR) (Jones and Winistorfer, 1992), thermal asymmetric interlaced PCR (TAIL PCR) (Song et al., 2005; Liu and Chen, 2007), and restriction site extension PCR (RSE PCR) (Ji and Braam, 2010) in transgenic plant research. TAIL PCR, one of the most widely used methods, requires 3 anchor primers and 4-8 degenerate and random primers, takes 8-10 h to complete, and involves complex procedures to obtain a known flanking sequence through 3 rounds of asymmetric interlaced PCR. In contrast, to obtain a flanking sequence by the RSE PCR method, the genome has to be digested first by enzymes and then subjected to various procedures. There are various limitations to these methods, such as the complexity of designing primers and the need for time-consuming and multiple-step procedures. Therefore, easy, rapid, and accurate methods are needed to clone flanking sequences. It is possible to combine the degenerate and random primers into a single primer to simplify the primer design and to improve the efficiency of cloning unknown flanking sequences.

In this study, unknown flanking genome sequences in transgenic plants, including tobacco, soybean, rice, and maize, were cloned using single-primer PCR with the *Bar* gene as the anchor gene.

MATERIAL AND METHODS

Materials

Genomic DNA from *Bar*-transfected plants including tobacco, soybean, rice, and maize were used in this study. The KOD DNA polymerase kit with 10 mM dNTP, and 10X PCR buffer containing 12 mM MgCl₂, 100 mM KCl, 60 mM (NH₄)₂SO₄, 100 μL/mL bovine serum albumin, 1.2 M Tris-HCl, and 1% TritonX-100 was purchased from Genview (USA). Agarose, Tris, boric acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from AMRESCO (USA). The Genomic DNA Extraction Kit and DNA Marker were purchased from TaKaRa (Dalian, China).

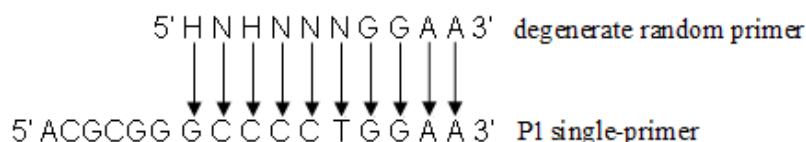
Primers

Five single primers specific for the *Bar* gene were designed as previously reported (NCBI database GenBank: KF206150.1) with some modifications, and the sequences are listed in Table 1.

Table 1. Primers.

Primer	Sequences (5'-3')	Primer length (bp)	Distance from the left border (bp)
P1	ACGCGGGCCCCTGGAA	16	602
P2	TGGCATGACGTGGGTT	16	348
P3	GCTTCAAGCACGGGAA	16	365
P4	GTCATCGGGCTGCCAA	17	446
P5	GACTTCAGCCTGCCGGT	17	320

All primers that were designed in this study served not only as anchor primers but also as random primers. All of the primers including P1, P2, P3, P4, and P5 were improved and performed better than those reported previously (Song et al., 2005; Liang et al., 2010; Ma et al., 2011a,b; Zhang et al., 2012), with characteristics consistent with those of single primers. When designing the P1 single primer based on the *Bar* gene with the vector pCAMBIA3300, we used the degenerate random primer sequence reported previously (Song et al., 2005; Liu and Chen, 2007; Ji and Braam, 2010) as a guide. The degenerate random primer contained the degenerate random sequence 5'-HNHNNNGGAA-3' (where N = A/G/C/T and H = G/C/A). The sequences of 5'-GGAA-3' in the *Bar* gene and the degenerate random primer were first located. Accordingly, three consecutive Ns toward the 5'-terminal orientation in the degenerate random primer were T, C, and C in the *Bar* gene, respectively. Then, H was C, N was C, H was G, and six bases were 5'-ACGCGG-3' toward the 5'-terminus. Thus, the single-primer P1 was 5'-ACGCGGGCCCCTGGAA-3'. The relationship between the P1 single primer and the previous degenerate random primer was shown below.



The same method was used to design primers P2-P5. Positions of each primer in the vector were shown in Figure 1. The above single primers could pair well with the *Bar* gene; thus, they could serve as the anchor primers. At the same time, they could serve as the random primers because the primer design considered the random primer strategy.

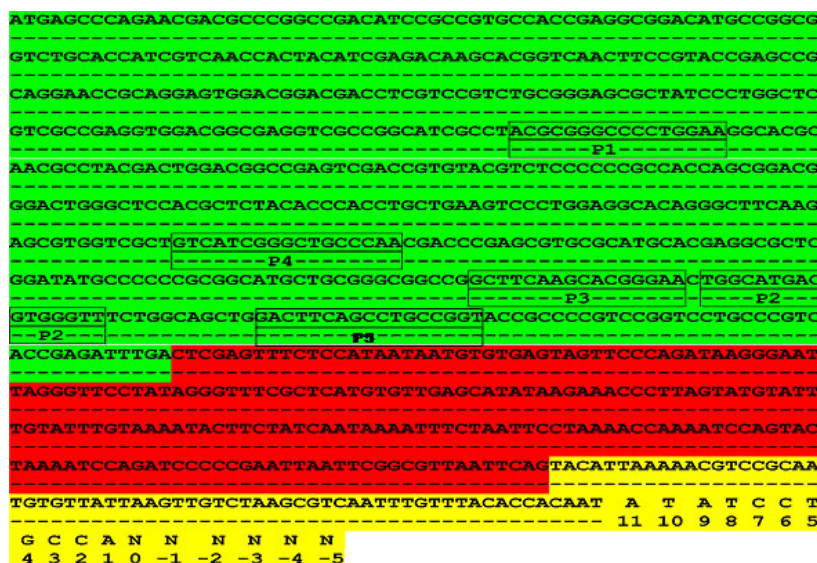


Figure 1. Positions of each primer in the T-DNA vector. Green color indicates the *bar* gene; red color is the 35SpolyA; yellow color is the left border of the vector.

Detection of the transgenic plants

Transgenic plants were identified as reported previously (Lu et al., 2011) using the QuickStix Kit 060591 (EnviroLogix Inc., USA) according to manufacturer directions. Briefly, 0.2 g leaves from transgenic plants was collected in 1.5-mL tubes. One hundred microliters of extraction buffer was added to the tubes, and the samples were ground for 5 min. Then, the test strips were incubated in the ground samples for 10 min to examine whether positive bands corresponding to the *Bar* product appeared to assess the expression of *Bar* in each transgenic plant.

PCR

PCR was conducted as reported previously (Jones and Winistorfer, 1992; Liu and Whittier, 1995; Liu and Chen, 2007; Ma et al., 2012). Total DNA of transgenic maize was extracted from plant leaves using the Universal Genomic DNA Extraction Kit (version 3.0; TaKaRa) according to manufacturer instructions. The reaction mixture (50 μ L total) for single-primer amplification with P1 contained 5.0 μ L 10X buffer, 1.0 μ L 10 mM dNTPs, 1.0 μ L 20 μ M P1 primer, 1.0 μ L genomic DNA template (<1 μ g), 1.0 μ L KOD DNA Polymerase (2.5 U/ μ L), and 32 μ L ddH₂O. The PCR was conducted using the following conditions: 98°C, 60 s; 5 cycles of 98°C, 30 s and 45°C, 30 s; 74°C, 120 s; 5 cycles of 98°C, 30 s and 54°C, 30 s; and 74°C, 120 s. The reaction mixture and cycling conditions for single-primer amplification of P2, P3, P4, and P5 were the same as those for the PCR with P1 as the single primer.

Agarose gel electrophoresis and purification of PCR products

The PCR products of reactions with P1, P2, P3, P4, and P5 as the single primers were resolved by electrophoresis on 0.8% agarose gels, and bands of lengths exceeding 602, 348, 365, 446, and 320 bp, respectively, were recovered routinely (Jones and Winistorfer, 1992).

Sequencing of PCR products

PCR products were recovered and sequenced routinely, which was followed by Basic Local Alignment Search Tool analysis with published homologous sequences of flanking sequences of the *Bar* gene in the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/nucleotide/>).

RESULTS

Detection of the transgenic plants

Based on the characteristics of random and degenerate primers in previous studies, it has been proposed that a single primer may serve not only as a random primer but also as a degenerate primer (Ma et al., 2011a,b, 2012). Thus, the single-primer PCR method was tested in this investigation.

In order to clone flanking sequence by single-primer PCR amplification in transgenic plants, including tobacco, soybean, rice, and maize, transgenic plants were first identified using Quickstix test strips. The test indicated that the *Bar* gene was integrated successfully into the genome of the transgenic plants and was expressed very well in the plants (data not shown).

Single-primer PCR in transgenic maize

To establish and identify an easy, rapid, and accurate method to clone flanking sequences by single-primer PCR, samples of the transgenic plants identified above were collected and subjected to single-primer PCR analysis with P1, P2, P3, P4, and P5 as primers. The PCR products were resolved using agarose gel electrophoresis. As shown in Figure 2, the P1 and P2 single-primer PCRs obtained the A and B bands of the *Bar* gene in the transgenic maize, respectively. The C and D bands were obtained in the P3 single-primer PCRs, and the P4 and P5 PCRs did not obtain obvious bands.

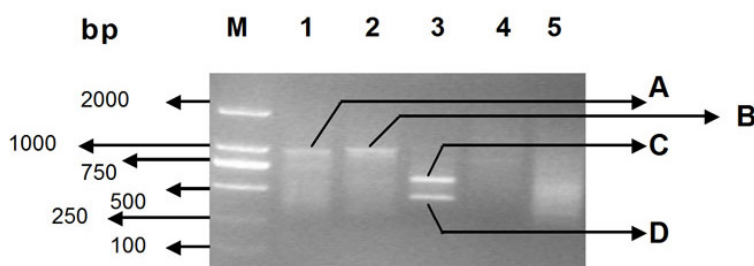


Figure 2. Single-primer PCR results in transgenic maize. Lane M = DL2000 molecular weight marker; lanes 1-5 = PCRs with P1, P2, P3, P4, and P5 as single-primers, respectively. Bands A-D = 1000, 1100, 508, 490 bp.

Sequencing results of PCR products from transgenic maize

Although PCR products were obtained with the single-primer PCR method in this study, the sequences of PCR products were unknown and needed further analysis. To test the efficiency of the single-primer PCR method, PCR products of transgenic maize were subjected to agarose gel electrophoresis and recovered. The recovered PCR products were then subjected to sequencing analysis. Inevitably, the PCRs (P1-A, P2-B, P3-C/D; Figure 2) that were obtained in the single-primer PCR reactions with P1, P2, and P3 as primers mismatched partially and, like the double-primer PCR, the obtained flanking sequences were not completely correct. Only a 508-bp band (Figure 2, P3-C) was the correct flanking sequence that was verified by sequencing analysis (Figure 3).

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AGAGACACAT TCTATAGGGGCGATTGAGCTGCCCTT GCTTCAAGCACGGG
AAGGGTGGCTGCTTCTAGGCAAACCTCCTGGCTGTCTTTGCACCCACCT
CCTTATCACTGAGCGGTCATTTAGGGGCC TTAGCTGGTATCCGGGCTGT
TTCCCTCTCGATGGCAGGATTTATATGTTGTAAACAAATTGACGCTTAGA
CAACTTAATAACACAT TGGGACGTTTTTAATGTA CTGAATTAACGCCGA
ATTAATTCGGGGATCTGGATTTAGTACTGGA TTTGGTTTTAGGAATTA
GAAATTTTATTGATAGAAGTATTTTACAAAATACAAATACATACTAAGGGT
TCTTATATGCTCAACACATGAGCGAAACCC TATAGGAACCCTAATTCCTT
ATCTGGGAACTACTCACACATTATTATGGAGAACTCGAGTCAAATCTCG
GTGACGGGCAGGACCGGACGGGGCGGTA CCGGCAGGCTGAAGTCCAGCT
GCCAGAAACCCACGTCATGCCAGTTCCTTGCTTGAAGCTCTGTTTCAGG
GTTCCAAACTCATAGTGGCAACTAAACACGAGGTAATGGCCGACGGG

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Figure 3. Sequencing results of band C in the PCR with P3 as the single-primer in transgenic maize. Sequences corresponding to the T vector pEASY-Blunt Simple cloning vector (blue), the *Bar* gene (green), 35S polyA (red), the left border of the expression vector (yellow) (the bases shown in red represent the two mutated bases), and the flanking sequence (gray) are shown.

Sequencing results of PCR products from transgenic tobacco, soybean, and rice

To further address the utility of the single-primer PCR system, PCR products from transgenic plants, such as tobacco, soybean, and rice, were also subjected to sequence analysis. The sequencing results of band C with P3, P5, and P2 as the single primer are shown in Figures 4, 5, and 6, respectively. The sequence of the flanking sequence of soybean was homologous with an entry in the NCBI database (NM_001250170.1), suggesting that it was successfully cloned in this study with the single-primer PCR method.

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AGAGACACATTCTATAGGGCGATTGAGCTGCCCTTGCTTCAAGCACGGGA
ACGGCGGCTCCATCTCCCGCTTCTTCCGCGTTGTCCAAAACCCAATCGCCT
TCCTCCTGCACATCCTCCACACTCCTCGCTAGATCAACCTTCCCTTGATCCG
GGCTGTTCCTCTCGATGCGAGGATTATTATGGTGTAACAATAATTGACGC
TTAGACAACCTAATAACAATTGCGGACGTTTTTAATGTAATGAAATACGC
CGAATTAATTCGGGGGATCTGGATTTTAGTACTGGATTTTGGTTTAGGAAT
AGAAATTTTATGATAGAAGTATTTTACAATACAAATACATACTAAGGGTTT
CTTATATGCTCAACACATGAGCGAAACCTATAGGAACCTAATCCCTTAT
CTGGAACTACTCACATATTATGGAGAACTCGAGTCAAATCTCGGTG
ACGGGACGACGGGGCGGTACCGGACGGCTGAAGTCCAGCTGCG
AGAAACCCACGTCATGCCAGTTCCCGTCTTGAAGCTCTGTTCAGGGTTC
CAAACTCATAGTGGCAACTAAACAGGATATGGCCGACGGG
    
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Figure 4. Sequencing result of band C with P3 as the single-primer in transgenic tobacco. Sequences corresponding to the T vector pEASY-Blunt Simple cloning vector (blue), the *Bar* gene (green), 35S polyA (red), the left border of the expression vector (yellow), and the flanking sequence (gray) are shown.

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GCGAGACACATTCTATAGGGCGATTGAGCTGCNCCCTGACTTCAGCCTGC
CGGTACCGCCCCGTCCGGTCTTGCCCGTCAACGAGATTGACTCGAGTTTC
TCCATAATAATGTGTGAGTATCCAGATAAGGGAATAGGGTTCTATAG
GGTTTCGCTCATGTGTGAGCATATAAGAAACCTTAGTATGATTTGATTT
GTAATAACTTCTATCAATAAATTTCTAATTCCTAAACCAAAATCCAGTA
CTAAATCCAGATCCCGCAATTAATTCGGCGTTAATTCAGTACATTAATAA
CGTCCGC AATGTGTTATTAAGTTGTCTAAGCGTCAATTGTTTACACCACAA
TATATCCTGCACGCTGTCTACAACTTTGACATGAGCAGATACTGACGTGA
GAAGCATCCTTGAGAGCACCCTGCAATGCCAATAAGTGGTGCTGCAAAGC
GTTTGAAGGATGACCGGCAGGCTGAAGTCCGCTCTGTTCAGGGTTCCAAA
CTCATAGTGGCAACTAAACAGGATATGG
    
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Figure 5. Sequencing result of band C in the PCR with P5 as the single-primer in transgenic soybean. Sequences corresponding to the T vector pEASY-Blunt Simple cloning vector (blue), the *Bar* gene (green), 35S polyA (red), the left border of the expression vector (yellow), and the flanking sequence (gray) are shown.

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CATCTATAGGGCGATTGAGCTGCCCTTGGCATGACGTGGGTTTCCCCG
GGCCGGAGGTGGCGACGACCGCCGACGCGCCGGACGCGCGGATT
GATCCGGGCTGTTCCCTCTCGATGGCAGGATTATTATGGTGTAACAATA
TGACGCTTAGACAATAACACATGCGGACGTTTTTAATGTAATGAAAT
TAAACCGCAATTAATTCGGGGATCTGGATTTAGTACTGGATTTTGGTTT
AGGAATTAGAAATTTATGATAGAAGTATTTACAATACAAATACTACTA
AGGGTTCTTATATGCTCAACACATGAGCGAAAACCTATAGGAACCTAAT
CCCTATCTGGAACTACTCACACATATTATGGAGAACTCGAGTCAATC
TCGGTACGCGGACGGACCGGCGGTACCGGACGGCTGAAGTCA
GCTGCCAGAAACCACTCATGCCAGCGTCTGTTCAGGGTTCCAACTCAT
AGTGGC
    
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Figure 6. Sequencing result of band C with P2 as the single-primer in transgenic rice. Sequences corresponding to the T vector, pEASY-Blunt Simple cloning vector (blue), the *Bar* gene (green), 35S polyA (red), the left border of the expression vector (mustard), and the flanking sequence (gray) are shown.

DISCUSSION

In this study, unknown flanking genomic sequences in transgenic plants, including those in tobacco, soybean, rice, and maize, were successfully cloned using single-primer PCR with the *Bar* gene as the anchor gene.

The methods that are currently used to identify the insertion positions of exogenous genes in plant genomes have various limitations (Liu et al., 1995; Liu and Whittier, 1995; Kim et al., 2004; Song et al., 2005; Liu and Chen, 2007; Liang et al., 2010). There is an urgent need for easier, quicker, and more accurate methods to clone the transgenic flanking sequences (Antal et al., 2004). In this study, we used the single-primer PCR method to successfully clone the flanking sequences in many transgenic plants, including maize, tobacco, soybean, and rice.

One of the most important innovations in this study was that the single primer served not only as a degenerate primer but also as a random primer, suggesting that it was a novel strategy for primer design that was applicable to the cloning of the transgenic flanking sequences. However, not all the single primers were successful in cloning new flanking sequences in transgenic plants. For example, the P4 and P5 primers did not yield satisfying PCR products. Furthermore, not all PCR products that were generated in our system included the unknown flanking sequences, such as bands A, B, and D. Therefore, these data indicated that the single-primer PCR system still needs to be improved and enhanced.

In conclusion, the single-primer PCR method is suitable to clone flanking sequences. Further studies are needed to optimize this method for efficient cloning of transgenic flanking sequences.

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