RESEARCH ARTICLE

Key Techniques and Efficiency Analysis of Amplification of Flanking Unknown Sequences by Inverse PCR

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Abstract

Inverse PCR (IPCR) is a reliable, straightforward, and effective technique for acquiring unknown sequences. In this study, we used the model monocot *Brachypodium distachyon* (ecotype Bd21) to standardize the conditions and materials necessary for the successful execution of IPCR. The analysis of the amplified sequences resulted in the following conclusions. First, the distance between the nearest primer and the boundary of the known-unknown sequence is crucial for determining whether the target sequence can be expanded in the second round of IPCR. Specifically, this distance should exceed 100 bp, ideally around 200 bp. Second, because the random cleavage of a 6 bp endonuclease occurs at a greater distance than that of a 4 bp endonuclease, the use of a 6 bp endonuclease in IPCR results in larger but often inconsistent bands, while maintaining good specificity. Therefore, if the goal is to amplify longer sequences or achieve high accuracy, it is advisable to select endonucleases with 6 bp restriction sites. Third, IPCR is a viable technique that can be effectively utilized to obtain unknown DNA sequences. The experimental conditions established in this study serve as a theoretical basis for the amplification of unknown genome sequences of Gramineae crops and other species.

Keywords: *Brachypodium distachyon*; inverse PCR; unknown sequence; flanking sequence

1 Introduction

Determining the locations of foreign genes and their flanking sequences in transgenic plants is of great significance for the study of gene function and genomics. A flanking sequence refers to a nucleotide sequence located on either side of a genomic region of interest within the chromosome [1]. Following the insertion of T-DNA or *Ds* transposon into the host chromosome, the sequence of the flanking region typically needs to be determined through a process known as chromosome walking [2]. Chromosome walking is a method employed to gradually ascertain the nucleotide composition of an unknown sequence adjacent to a known sequence within a biological genome or genomic library [3]. Following this, sequence alignment tools, such as NCBI Magic-BLAST [4], are utilized to efficiently match the relevant sequences and identify the position of the transgene

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within the host genome. Chromosome walking has been applied to determine sequences located both upstream and downstream of a region of interest [5, 6]; to identify the insertion sites of T-DNA or transposons [7, 8]; to ascertain fragment overlap in PAC, YAC, and BAC libraries [9]; and to locate the promoter and regulatory elements of known genes [10, 11]. Inverse PCR (IPCR) is one of the earliest techniques for chromosome walking based on polymerase chain reaction (PCR). The operational process of IPCR is both simple and convenient. Initially, the genome is digested using appropriate restriction enzymes. Subsequently, the digested fragments are self-ligated by ligase to form a loop. A pair of primers, complementary to the sequences flanking the core region, is then utilized to perform PCR, using the looped fragment as the template. Amplification begins at the core region and progresses along the circular molecules to the unknown sequences on both sides. Sequences upstream and downstream of the core region are obtained by cloning and sequencing the IPCR products [12]. In comparison to other methods, IPCR is straightforward and allows for the simultaneous acquisition of unknown sequences on both sides of the known sequence. To date, IPCR has been employed in several studies [13–15].

Table 1. Primer sequences used in this study.

Primer number	Sequence
IPCR-F1	GATATCACATCAATCCACTTGCTTTG
IPCR-R1	CGCACAATCCCACTATCCTTC
IPCR-F2	GTCTCAATCGCCAAGCTTATCG
IPCR-R2	ctactcgcaaaacaaattccgtattc

Given the substantial quantity of plant materials required for this experiment and the limitations associated with flanking unknown sequence cloning technology, as well as the absence of reported key technical points regarding the IPCR method, we have opted to develop a flanking unknown sequence cloning system tailored for processing a large volume of plant materials. The method established in this study offers novel insights for analyzing foreign genes in other plant species and agriculturally significant crops. The Primer sequences used in this study as shown in Table 1.

2 Materials and Methods

2.1 Material

2.1.1 Plant material and plasmids

Brachypodium distachyon ecotype Bd21 was used in this study. The plants were cultivated at 20$^{\circ}C$ under long-day photoperiod (18 h light/6 h dark). *Escherichia coli* DH5α and *Agrobacterium tumefaciens* strain EHA105 preserved in our laboratory were used for plant transformation, along with the plasmid backbone pCAMBIA1300.

2.1.2 Enzymes and biochemical reagents

Enzymes and other reagents were procured from various commercial vendors: conventional restriction endonucleases and *Taq* polymerases were obtained from Baoshan Biological Company (Dalian); T4 DNA ligase was sourced from Quanshi Gold Company (Beijing); Green Taq Mix was acquired from Nuoweizan Biological Technology Co., Ltd. (Nanjing); the plasmid extraction kit and fluorescence quantitative kit were supplied by Kangwei Century; the genomic reverse transcription kit was obtained from Quanshi Gold Company; the DNA gel recovery kit was sourced from Baitaike (Beijing); DMSO and β-mercaptoethanol were procured from Solaibao; NaOH, NaCl, and CH_3COOK were acquired from Tianjin Kaitong; and EDTA, Tris, $MgCl_2$, $CaCl_2$, along with other chemicals, were sourced from Shanghai Shenggong.

2.2 Method

2.2.1 DNA extraction

Fresh leaves, weighed to an appropriate amount, were placed in a 1.5 mL centrifuge tube, frozen in liquid nitrogen, and ground into a fine powder using a crusher. Subsequently, 700 μL of 2$\times$cetyltrimethylammonium bromide (CTAB) was added to each sample and mixed thoroughly. The samples were incubated in a water bath at 65 $^{\circ}C$ for 20 minutes and then allowed to cool to room temperature; alternatively, samples could be placed on ice for expedited cooling. Following this, 700 μL of chloroform was added to each sample, which were mixed gently by inverting the centrifuge tubes several times. Finally, the samples were centrifuged at 5,000$\times$g for 10 minutes at 4 $^{\circ}C$. The supernatant was transferred to a new centrifuge tube. Subsequently, 500 μL of isopropanol was added to each tube, mixed by inverting, and incubated at -20 $^{\circ}C$ for over 1 hour. The samples were then centrifuged at 15,000 $\times$ g for 10 minutes at 4 $^{\circ}C$, and the supernatant was discarded. Following this, 1 mL of 75% ethanol was added to each tube, and the tubes were inverted to ensure thorough mixing. After another centrifugation at 15,000 $\times$ g for 5 minutes at 4 $^{\circ}C$, the supernatant was removed. The tubes were then centrifuged for an additional minute, and the DNA that had deposited

at the bottom of each tube was resuspended in 50 μL of 1×TE after being dried at room temperature.

2.2.2 *Primer design*

Primers complementary to the known T-DNA sequences were designed using Serial Cloner version 2.6.1 and DNAMAN (a sequence alignment and primer design software that can be utilized offline). Unknown genomic sequences were amplified from the 3′ end of the T-DNA sequence. The binding site of the first primer (IPCR-R1) was located approximately 300 bp from the 5′ end of the T-DNA sequence, while the binding site of the second primer (IPCR-R2) was situated approximately 200 bp from the 5′ end. During amplification, primer IPCR-R1 was paired with primer IPCR-F1, and primer IPCR-R2 was paired with primer IPCR-F2. To enhance product specificity, the annealing temperature of the second PCR primer was set 3-5 $^{\circ}C$ higher than that of the first PCR primer.

2.2.3 *Enzymatic digestion, cyclization, and PCR amplification*

An appropriate restriction endonuclease, selected on the basis of the known sequence, was used to prepare the following reaction: The total volume of the enzyme cleavage reaction was 15 μL, comprising 1.5 μL of 10× Buffer, 2 U/μL of Enzyme, 3.5 μL (300 ng/μL) of Template DNA, and the remaining volume was supplemented with ddH_2O to reach a final total of 15 μL. Enzymatic digestion was conducted at 37 $^{\circ}C$ for a duration of 2-3 hours. The resulting enzyme digestion product was transferred to a 1.5 mL centrifuge tube, washed with deionized water (ddH_2O), and then diluted to a final volume of 250 μL. Subsequently, an equal volume of a phenol-chloroform mixture (1:1, v/v) was added to the sample, which was thoroughly mixed by vortexing and incubated at room temperature for 1 minute. The sample was then centrifuged at maximum speed (13,000 rpm) for 1 minute. The supernatant was carefully transferred to a new tube, and an equal volume of chloroform was added, followed by thorough mixing via vortexing. After another incubation at room temperature for 1 minute, the sample was centrifuged at maximum speed (13,000 rpm) for 2 minutes, and the supernatant was again transferred to a new tube. Subsequently, 2.5 volumes (≈500 mL) of absolute ethanol, prechilled at -20 $^{\circ}C$, and 0.1 (≈20 mL) volume of 3 M sodium acetate (pH = 5.2) were added to the sample and mixed gently by shaking. Following an incubation period of 5 minutes at room temperature, the sample was centrifuged at maximum speed (13,000 rpm) for 10-15 minutes at 4 $^{\circ}C$. The supernatant was discarded, and any residual liquid on the tube walls was removed as thoroughly as possible. Next, 1 mL of 70% ethanol, also prechilled at -20 $^{\circ}C$, was added to the tube, which was then inverted several times. The sample was centrifuged again at maximum speed (13,000 rpm) for 5 minutes, after which the ethanol was removed. To facilitate drying of the sample, the tube was placed horizontally on a clean surface, allowing the ethanol on the tube walls to volatilize. The enzyme-digested fragments were then cyclized overnight at 22 $^{\circ}C$ by preparing the following reaction. The ligase cyclization ligation system consists of a total volume of 5 μL, which includes 0.5 μL of T4 ligase buffer, 0.5 U/μL of T4 ligase, and 4.4 μL of ddHO.

Subsequently, polymerase chain reaction (PCR) was conducted as follows. In the first round, the cyclized product served as the template and was amplified under the following conditions: 95 $^{\circ}C$ for 5 minutes; followed by 35 cycles of 95 $^{\circ}C$ for 30 seconds, 55 $^{\circ}C$ for 30 seconds, and 72 $^{\circ}C$ for 2 minutes; a final extension at 72 $^{\circ}C$ for 5 minutes; and then a hold at 15 $^{\circ}C$. In the second round, the PCR product from the first round was utilized as the template and amplified under identical conditions: 95 $^{\circ}C$ for 5 minutes; 35 cycles of 95 $^{\circ}C$ for 30 seconds, 59 $^{\circ}C$ for 30 seconds, and 72 $^{\circ}C$ for 2 minutes; a final extension at 72 $^{\circ}C$ for 5 minutes; and a hold at 15 $^{\circ}C$. Following amplification, a 2 μL aliquot of the PCR products was analyzed via electrophoresis on a 1% (w/v) agarose gel at a voltage of 240 V and a current of 260 mA. After electrophoresis, the DNA was extracted from the gel and submitted to BGI for sequencing.

2.2.4 *DNA sequence analysis*

The sequence of the PCR product was analyzed using NCBI (https://blast.ncbi.nlm.nih.gov/), Phytozome (https://phytozome.jgi.doe.gov/) and Gramene (http://ensembl.gramene.org/) databases.

3 Results

3.1 Endonuclease selection and IPCR primer design

Theoretically, multiple randomLy selected restriction enzymes can be employed to perform inverse PCR (IPCR) [16, 17]. However, in practice, the selection of endonucleases must take into account the primer binding sites to prevent situations where suitable primer design sites are rendered unavailable following enzyme digestion, which could adversely affect subsequent experiments.

The design process of inverse PCR (IPCR) primers

A

GTAGGATGGGAAAATCCCGTACCGACCGTTATCGTATAACCGATTTTGTTAGTTTTATCC
CGATCGATTTCGAA***CCCGAGGTAAAAAACGAAAA***CGGAACGGAAACGGGATATACAAA
ACGGTAAACGGAAACGGAAACGGTA**GAGCTAGTTTCCCGACCGTTTC**ACCGGGAT
CCCGTTTTTAATCGGGATGAT***CCCGTTTCGTTACCGTATTT***TCTAATTCGG
GATG***ACTGCAATATGGCCAGCTC***CAACTCCCATCCATAACCACTGAGGCCCAGC
CCATGTAAGAAATACCTAGCGAACGCTGCTCTGCCTCTCTCCCAGGCG
GC**CAGGCACCACA*CGAGTAACAGCATCACACATTCAC***ACGCCGCCAC***GCGC***CCACGC
CGGAG***TCCGGA***CGCCGCCAGCCGCCATCTGCGGTCAGCGGTCCTCCAGACCTCCACTG
CTCGGCGCTCGCCCATCCGGCCAT***CCGCGG***TCTCCC**TGTCTCCACGGCTGCTCAC**AG
GCTCACAGCACTTAGCAGTACAGCACGTCAGCACCATTGCACCAAGCTGTTGTGTCAT
TTGTGTGCTGTCCAGGGGCTCTGCAACACCTGCTGATTGCTGTCCAGCCGTCCAGGTG
CTCACAAGTCACAGCAGTACAGCACCAAG**CTGATTGCTGAACACCTGCTG**TCCAGG
GCTCTGCTCTCCACTTCG***GCTAGC***CGGCTACGACTCCATTCCTCAGATGACGCCTCCGG
TTGGAAATAATCCTCCCTCAGGCTCAGCCATAAGATTGGCCAAGTTGATGTCTACCACA
AGAGCGCCTTCTACTCGCAAAACAAATTCCGTATTCTCTG***CATATG***CT.....CTT【1519bp】

B

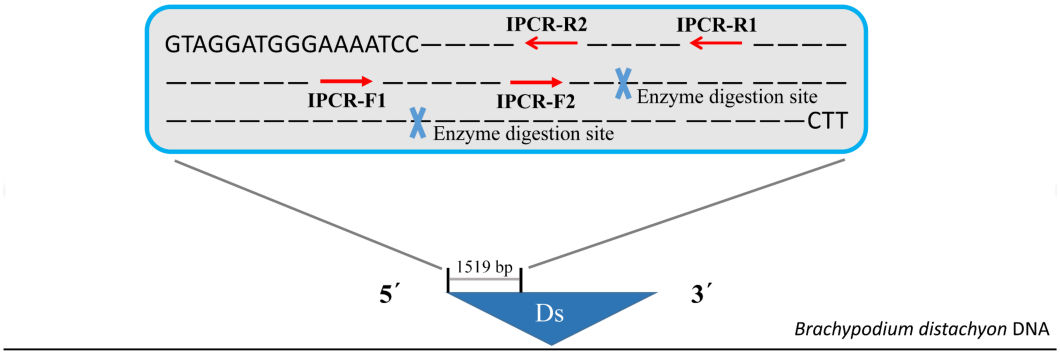

Figure 1. Known *Ds* insertion sequences and primer binding sites. A: Known sequence of the *Ds* transposon. Numbers 7, 8, 9, 12, and 13 (italicized letters with deep background and bold) indicate *Hha*I, *Acc*III, *Sac*II, *Nhe*I, and *Nde*I restriction enzymes, respectively. Skeleton letters 1, 3, 4, and 6 in the box indicate primers used for the *Hha*I restriction site; normal letters 2, 5, 10, and 11 with the underline represent primers used for the *Nde*I restriction site. B: Schematic representation of the IPCR primer binding sites. GTAGGATGGAAAATCC is the outermost end of the known *Ds* sequence, and the left outer DNA sequence of the known *Ds* sequence is unknown sequence of IPCR pre-amplification.

must take into account several factors, including the length, annealing temperature, GC content, and binding specificity of conventional primers, as well as the distance between the primers and the boundary of the known and unknown sequences. In this study, we observed that amplification is suboptimal when the distance between the primer and the known-unknown sequence boundary is either too short or too long. Such conditions not only complicate primer design but can also render the amplification of the target sequence impossible. Therefore, to ensure adequate sequence availability for primer design, it is recommended that the cleavage site of a restriction endonuclease be positioned at least 200 base pairs away from the boundary, as shown in Figure 1. Considering this rule, we selected five restriction endonucleases (from 5′ to 3′ end): *Hha*I, *Acc*III, *Sac*II, *Nhe*I, and *Nde*I, as shown in Figure 1.

3.2 Effect of different endonucleases on PCR amplification

Through multiple experiments employing five pre-selected restriction enzymes, we observed that the final effects of *Acc*III, *Sac*II, and *Nhe*I were not ideal, and the effect of PCR amplification using the product digested by *Acc*III and *Nhe*I enzyme is

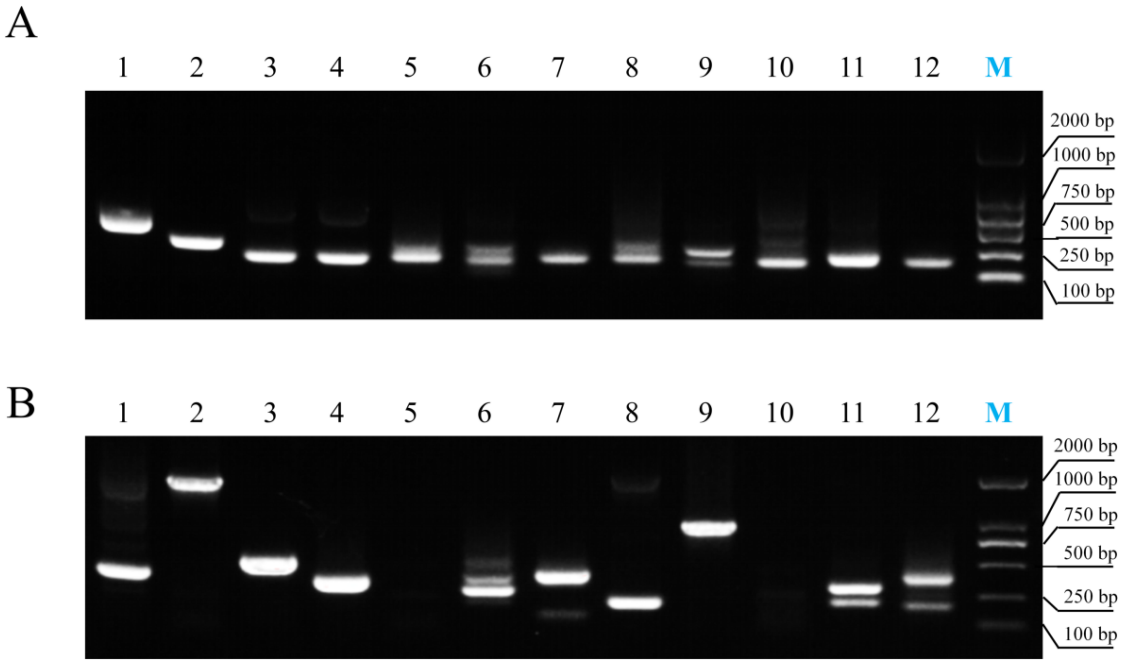

Figure 2. Effect of different restriction endonucleases on amplification. A, B: Effect of endonucleases with 4 bp restriction site, such as *Hha*I (A) and 6 bp restriction site, such as *Nde*I (B) on IPCR amplification. Lanes 1-12 indicate PCR amplification of the digested product of the same endonuclease. Lane M indicates the 2 kb DNA marker.

unstable. When PCR was amplified by 96 samples and gel electrophoresis was performed, sometimes only 3 samples had obvious bands, sometimes about 20 samples had obvious bands. *Sac*II produced miscellaneous bands when amplified. Only *Hha*I and *Nde*I produced ideal results. Generally, 60–87 out of 96 samples (per plate) digested with *Nde*I or *Hha*I could be successfully amplified by IPCR. Therefore, *Hha*I and *Nde*I were used in all subsequent analyses.

Using *Hha*I-digested samples as the template, IPCR amplification generated relatively short (150–750 bp) and consistent bands, with poor specificity, as shown in Figure 2 A. After *Nde*I digestion, IPCR produced large but mostly inconsistent bands, with good specificity, as shown in Figure 2 B. Gel electrophoresis strip gradient is diversified, and the size of the bands ranged from 150 to 2,000 bp.

3.3 Effect of primer–boundary distance on PCR amplification

Through extensive experiments with *Hha*I and *Nde*I restriction endonucleases, we found that the distance between the nearest primer and the unknown–known sequence boundary is key to determining whether the target sequence could be expanded in the second round of IPCR. Because of the limitations of the current Sanger sequencing method [18], dozens of base pairs, or even more than 100 bp, may be mismatched or missing during the sequencing process. When primers were located too close to the boundary (less than 100 bp) in the second round of PCR (IPCR-R2), it was difficult to obtain the complete, or even partial, sequence of the boundary region adjacent to the *Ds* transposon, as shown in Figure 3 A. Therefore, when designing the second round of nested PCR primers (IPCR-R2), the distance between the primers and the boundary region should be greater than 100 bp (ideally 200 bp; Figure 3 B), which can effectively expand to the unknown sequence of the flanking, such as Lanes4, 5, 7.

3.4 Analysis of sequencing results

A total of 327 sequences were amplified: 118 from *Hha*I-digested samples and 209 from *Nde*I-digested samples, as shown in Table 2. We examined the length distribution range of the obtained sequences. The results showed that sequences generated by the 4 bp cutter *Hha*I were mainly concentrated in the range of 100–200 bp (57 out of 118 sequences; 48%), and the lowest distribution range was in the ≥500 bp interval, with only 4 out of 118 sequences (3%). Among the sequences obtained using the 6 bp cutter *Nde*I, most were concentrated in the range of 200–500 bp (94 out of 209 sequences; 45%), and the lowest distribution range was in the ≤30 bp interval (8 out of 209 sequences; 4%). The final sequencing results indicated that the sequences amplified from *Hha*I-digested samples were relatively short, while

Table 2. Length distribution of the amplified sequences.

	Number of sequences within a specific length range				
	≤30 bp	30–100 bp	100–200 bp	200–500 bp	≥500 bp
*Hha*I	13	32	57	12	4
*Nde*I	8	34	63	94	10

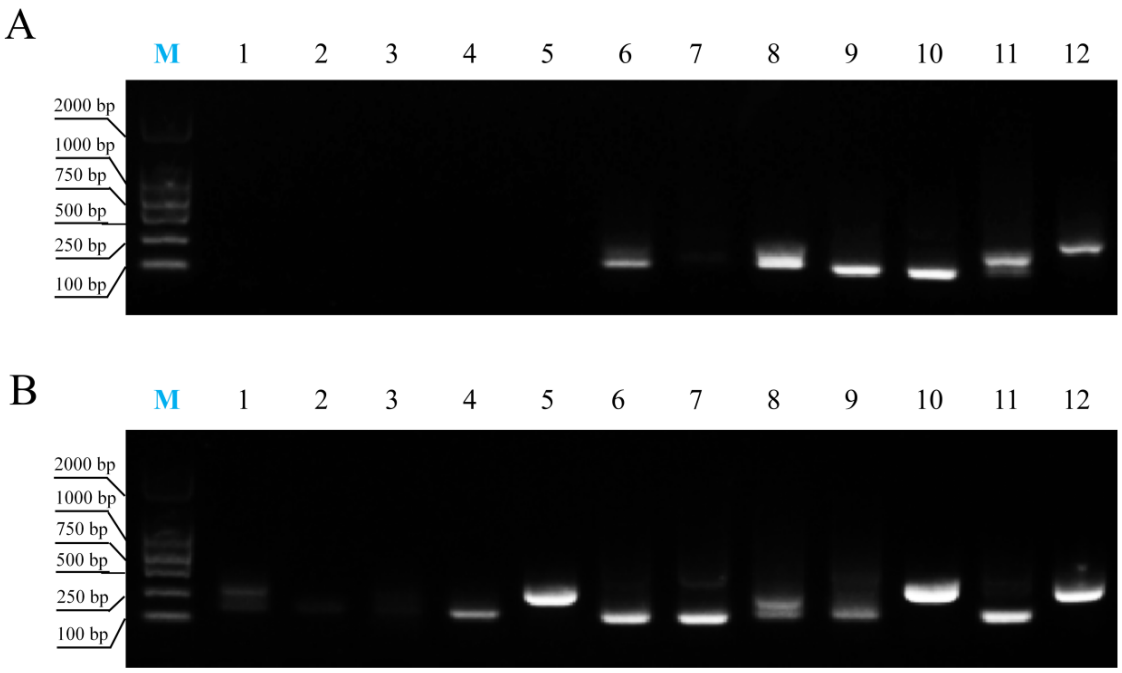

Figure 3. Effect of the distance between the primer binding site and the boundary region on IPCR amplification. A.B: Amplification using primers located approximately 100 bp from the boundary sequence (A) and approximately 200 bp from the boundary sequence (B). Lanes 1-12 indicate PCR amplification of the digested product of the same endonuclease. Lane M shows the 2 kb DNA marker.

those amplified from *Nde*I-digested samples were considerably longer. Therefore, if the objective is to amplify longer sequences or achieve high accuracy, it is advisable to select endonucleases with 6 bp restriction sites.

3.5 PCR validation of flanking sequences

Some amplified sequences could not be successfully aligned with the sequences available in the target species database, as shown in Figure 4 A. To verify the accuracy of the sequence in the target DNA, we designed primers relevant to the sample and verified all sequences obtained by sequencing. One primer targeted the *Ds* transposon (IPCR-R1 or IPCR-R2), while the other primer was specific to the unknown flanking sequence obtained by amplification. After amplification verification, all verification sequences could again be amplified to the expected target band. Sequence alignment showed that some of the sequences were amplified from the *Ds* transposon, and some were amplified from genomic DNA, as shown in Figure 4 B, thus proving that IPCR is a feasible technique that can be effectively used to obtain unknown DNA sequences.

4 Discussion

Numerous PCR-based chromosome walking techniques have been developed to date [19–21]. The IPCR method established in this study is particularly effective for determining unknown sequences, as it is a relatively simple, low-input, and cost-effective approach [22, 23]. This method facilitates convenient primer design and can be applied to a large number of experimental materials simultaneously.

The application of IPCR technology in *Brachypodium distachyon* led to several key conclusions. First, the genomic DNA extraction method affects the results of IPCR and the self-ligation of enzyme-digested DNA fragments [24]. Among the various methods evaluated, the traditional plant DNA extraction method, specifically the improved CTAB method,

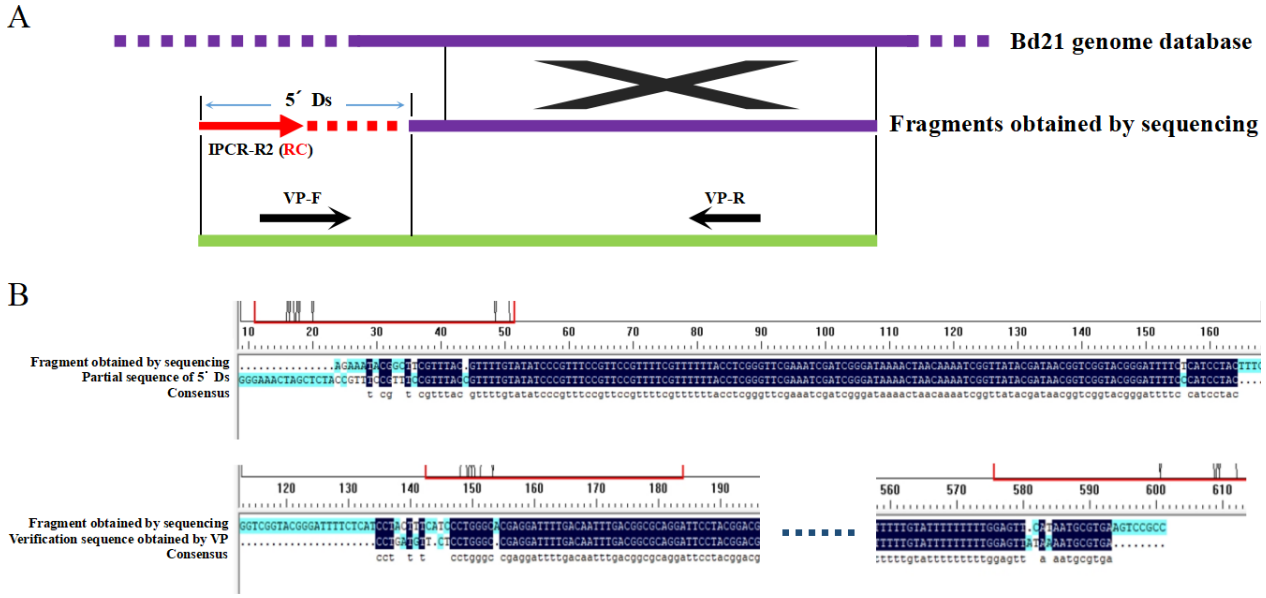

Figure 4. Comparison and verification of amplified sequences. A: Analysis of the amplified sequence using Gramene BLAST (length, 450 bp; score, 438; e-value, 0.0; sequence identity, 99.3%). RC: Reverse complementarity. B: Validation of the amplified sequence. Alignment of the amplified sequence with the *Ds* element (top) and with the verified sequence (bottom). VP: Validation primer.

proved to be the most suitable template for IPCR, as it yielded DNA of appropriate purity that could be effectively digested by different restriction endonucleases and successfully cyclized in subsequent experiments. Second, the selection of restriction endonucleases is critical for successful IPCR amplification. The distance between the cleavage site of the restriction enzyme and the left and right boundaries significantly impacts DNA amplification. A larger distance tends to reduce the amplification efficiency of standard polymerases, while a shorter distance may result in either multiple amplification products due to random binding to fragments or no amplification at all. Additionally, factors such as cost, availability, and potential star activity should be taken into account when selecting a restriction enzyme. Lastly, effective primer design and careful site selection are crucial for the success of Inverse PCR (IPCR). Nested PCR is usually used for chromosome walking, and the credibility of IPCR products can be greatly increased through two or three rounds of nested PCR and by comparing the PCR product size and concentration. During the primer design phase, in addition to the annealing temperature, length, GC content, and other attributes of conventional primers, the distance between two amplification primers should also be fully considered.

In the current study, we established a convenient and feasible IPCR method through the amplification and analysis of 327 *B. distachyon* sequences. The screening of different restriction endonucleases and primers and the analysis of sequencing results revealed that *Hha*I and *Nde*I restriction endonucleases were suitable for the digestion of *B. distachyon* DNA, and *Nde*I was a better choice than *Hha*I as it produced condensed bands of different sizes with good specificity. The experimental method and technical scheme developed in this study could be used to design IPCR primers in other studies, and will serve as a reference for the acquisition of unknown sequences and gene mapping in other species.

Data Availability Statement

Data will be made available on request.

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Conflicts of Interest

The authors declare no conflicts of interest.

Ethical Approval and Consent to Participate

Not applicable.

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