Establishment of Event-Specific Detection Method for Transgenic Rice BPL9K-2 Post-print

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Abstract

Using the hiTAIL-PCR (High Efficient Thermal Asymmetric Interlaced PCR) method, we amplified and obtained a 450 bp left flanking sequence of the exogenous gene insertion site in transgenic rice BPL9K-2. Comparison with rice reference genome data revealed that its left border was inserted after the 1,037,765th nucleotide residue on the short arm of chromosome 10 of the rice genome. Based on the rice reference genome sequence and the right border sequence of the exogenous gene, primers were designed to amplify a specific fragment of 485 bp. Database comparison revealed that its right border was inserted before the 1,037,825th nucleotide residue on the short arm of chromosome 10 of the rice genome. Due to the insertion of the exogenous gene and abnormal recombination, 59 nucleotides were deleted from the rice genome. Based on the left and right flanking sequences, an event-specific qualitative PCR detection method for transgenic rice BPL9K-2 was established, which could amplify specific bands of 449 bp and 485 bp, respectively. This method exhibited good specificity and high sensitivity, capable of detecting transgenic components in templates where the relative content of BPL9K-2 genomic DNA was 0.1%. Based on the flanking sequences, a three-primer PCR detection method was established for rapid identification of the exogenous genotype in transgenic progeny plants. The establishment of these methods provides technical support for the application and detection of transgenic rice BPL9K-2.

Full Text

Event-Specific Detection Methods for Genetically Modified Rice BPL9K-2

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Abstract

The hiTAIL-PCR (high-efficiency thermal asymmetric interlaced PCR) method was employed to amplify the 450 bp left flanking sequence of the exogenous gene insertion site in transgenic rice BPL9K-2. Comparison with the rice reference genome revealed that the left border inserted after nucleotide residue 1,037,765 on the short arm of chromosome 10. Based on the rice reference genome sequence and the exogenous gene right border sequence, primers were designed to amplify a 485 bp specific fragment, which through database comparison was found to insert before nucleotide residue 1,037,825 on the short arm of chromosome 10. Due to exogenous gene insertion and abnormal recombination, 59 nucleotides were deleted from the rice genome. Event-specific qualitative PCR detection methods for transgenic rice BPL9K-2 were established based on the left and right flanking sequences, amplifying specific bands of 449 bp and 485 bp, respectively. This method exhibited good specificity and high sensitivity, capable of detecting transgenic components in templates with a relative content of 0.1% BPL9K-2 genomic DNA. A three-primer PCR method was also developed for rapid identification of exogenous gene genotypes in transgenic progeny plants based on the flanking sequences. These established methods provide technical support for the application and detection of transgenic rice BPL9K-2.

Keywords: transgenic rice, flanking sequence, event-specific detection, genotyping

Introduction

Flanking sequences refer to the DNA sequences on both sides of a specific locus in a chromosome, and in transgenic organisms they denote the DNA sequences flanking the exogenous gene expression cassette. Flanking sequences of exogenous genes not only significantly influence the normal transcription, translation, and regulation of the transgene but also serve as important identity information for genetically modified (GM) crops. They represent the unique identifier distinguishing different transformation events and are crucial for studying the genetic stability of GM crops as well as for intellectual property protection, detection, and regulation, garnering widespread attention in the industry.

Due to the random nature of exogenous gene insertion during genetic transformation, the probability of an exogenous gene inserting at a specific position in the rice genome is extremely low (approximately 1 in 466 trillion), with

great uncertainty. The novel sequence formed by the splicing of exogenous and genomic sequences is almost impossible to replicate, making it unique. This uniqueness forms the material basis for identity authentication of transgenic rice and provides essential technical data for establishing event-specific detection methods. Event-specific detection methods based on flanking sequences are efficient and accurate for distinguishing different transformation events, and many such methods have been patented. For example, Wang et al. [1] established an event-specific PCR detection method for glyphosate-resistant transgenic soybean GTS40-3-2. Zhai et al. [2] obtained the 5' flanking sequence between the exogenous gene and maize genome in transgenic maize LY038 using modified adaptor-ligation PCR, and subsequently established an event-specific qualitative detection method for LY038. In rice, Wang et al. [3] used TAIL-PCR to determine the insertion site information of Cry1Ab transgenic rice and established corresponding qualitative and quantitative PCR detection methods. Su et al. [4] determined the full sequence of the exogenous DNA insertion in the Cry1Ab/Cry1Ac fusion gene rice line "Bt Shanyou 63" and established a real-time fluorescent quantitative PCR method. Jiang, Wei, Guo, and colleagues respectively obtained flanking sequences of transgenic rice B2A68, EB7001S, and BarKasalath-01 using hiTAIL-PCR and established event-specific PCR detection methods based on these sequences [5-7].

After obtaining positive transformants, rapid screening for homozygous lines in transgenic crop progeny is not only a necessary step for functional validation of exogenous genes but also an important component of transgenic crop breeding. Traditional screening methods involve multi-generation selfing of transgenic crops, analyzing trait segregation in selfed progeny or using conventional PCR to identify exogenous gene segregation to determine whether the gene is homozygous. This approach requires continuous tracking and detection of transgenic materials for at least two generations starting from the T1 generation (transgenic generation), with individual plant seed collection each generation, making it time-consuming, requiring large sample sizes, and involving cumbersome procedures that are not conducive to rapid acquisition of homozygotes. Real-time fluorescent quantitative PCR offers good specificity and high sensitivity for identifying homozygous lines but involves complex operations, high equipment and consumable costs, and compromised detection accuracy in cases of multiple copies. Du et al. [8] designed three primers based on flanking sequence information for multiplex PCR, which can amplify multiple target fragments in a single PCR reaction. Genotypes can be determined based on fragment size and number, providing a convenient and rapid method to accurately identify homozygotes in a single segregation generation (T2), thereby improving the efficiency of genotyping transgenic materials. Due to its accuracy and convenience, this method has been applied to homozygosity testing of multiple transgenic rice varieties [5-7, 9, 10].

This study employed hiTAIL-PCR technology to determine the left and right flanking sequences of the exogenous gene in transgenic rice BPL9K-2, and subsequently established event-specific PCR detection methods for this transforma-

tion event and a three-primer method for detecting exogenous gene genotypes, providing technical support for future application of this rice line.

Materials and Methods

1.1 Plant Materials

The single-copy transgenic rice BPL9K-2 (*Oryza sativa* L.) is a new transgenic rice line developed by our research group, containing the phosphinothricin resistance gene *Bar* driven by the CaMV35S promoter and a phytase-antimicrobial peptide fusion gene *PhyLf* driven by the rice glutelin promoter Gt13aP. The *PhyLf* gene was optimized for rice codon preference by fusing the *Escherichia coli* phytase gene *AppA2* with the bovine lactoferricin gene *LfcinB* [11].

Non-transgenic rice materials 9K19-1, 9K19-5, 11C2277, 11C2292, and Ke108A are new indica rice lines developed by our research group, while 7001S, Guangzhan63S, and R106 are introduced and propagated varieties. Transgenic rice materials BPL9K-1, BPL9K-4, EB7001S-5, B1C893, EB185BS, B2A68, BarKasalath, and Bar9K29-9-2 are new transgenic rice lines developed by our research group.

1.2 Experimental Reagents

The TransTaq DNA Polymerase HiFi Fidelity (HiFi) kit was purchased from TransGen Biotech (Beijing), dNTPs from Takara Bio (Beijing), and 2×Taq MasterMix (Dye) from CoWin Biosciences.

1.3 Rice Genomic DNA Extraction

Genomic DNA was extracted from fresh rice leaves using the CTAB method [12]. DNA concentration and purity were measured using a Thermo Scientific NanoDrop 2000 micro-volume UV spectrophotometer with 2 µL samples, while DNA integrity was assessed by 1.0% agarose gel electrophoresis with another 2 µL sample. Other routine reagents were purchased from Sinopharm Chemical Reagent Co.

1.4 Identification of Left Flanking Sequence

The left flanking sequence of transgenic rice BPL9K-2 was amplified using the hiTAIL-PCR (high-efficiency thermal asymmetric interlaced PCR) method according to Liu et al. [13]. Four long arbitrary degenerate primers (LAD1, LAD2, LAD3, LAD4) were obtained from literature, while specific nested primers LB-0a, LB-1a, and LB-2a were designed based on the T-DNA left border sequence of vector pCB-Gt13aP-PhyLf. Primer sequences are listed in .

Using the recipient rice material 9K19-5 as a control, DNA concentration was adjusted to 20-30 ng/µL. The first round of amplification was performed using long arbitrary primers LAD1-LAD4 individually combined with specific primer

LB-0a in a 20 µL reaction system. The first-round products were diluted 40-fold as template for the second round using primer pair AC1/LB-1a in a 25 µL system. Second-round products were diluted 10-fold as template for the third round using primer pair AC1/LB-2a in a 25 µL system. Second- and third-round amplification products were separated by 1.0% agarose gel electrophoresis, and specific bands differing by 45 bp between rounds were selected for recovery and sequencing. Sequencing results were compared with the rice reference genome to determine the left flanking sequence of the exogenous gene expression cassette. Primer synthesis was completed by BGI Tech (Shenzhen), and sequencing was performed by Tsingke Biotechnology (Hunan).

1.5 Identification of Right Flanking Sequence

The left flanking sequence obtained above was compared against the rice genome sequence database on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to predict the exogenous gene insertion site in the rice genome. Forward primer RB-F (5' -CCAGCTCGAATTTCCCCGAT-3') was designed based on the T-DNA right border sequence, and reverse primer RB-R (5' -AGCACAAATGTGGACGCTCA-3') was designed based on the downstream rice genome sequence at the insertion site. Using recipient material 9K19-5 as a control, PCR amplification was performed with these primers, and products were analyzed by 1.0% agarose gel electrophoresis. Specific bands were recovered and sequenced to obtain the right flanking sequence of transgenic rice BPL9K-2. The 25 µL PCR reaction system contained 2 µL of 2 mM dNTPs, 2.5 µL of 10×Buffer, 0.25 µL of 5 U/µL HiFi Taq, 1 µL each of 10 µM forward and reverse primers, 1 µL of 100 ng/µL DNA template, and water to 25 µL. The PCR program was: 95°C for 5 min; 35 cycles of (95°C for 30 s, 56.4°C for 30 s, 72°C for 30 s); and 72°C for 10 min.

1.6 Establishment of Event-Specific PCR Detection Methods

Forward primer LB-F and reverse primer LB-R were designed based on the rice genome sequence and T-DNA sequence in the left flanking sequence to establish an event-specific PCR detection method using the left flanking sequence. Similarly, forward primer RB-F and reverse primer RB-R were designed based on the T-DNA sequence and rice genome sequence in the right flanking sequence to establish a detection method using the right flanking sequence. Primer sequences for both pairs are listed in . DNA from different transgenic and non-transgenic rice varieties was used as template to test the reliability of the event-specific PCR methods. The 25 µL PCR reaction system contained 10 µL of 2×Taq MasterMix, 1 µL each of 10 µM forward and reverse primers, 1 µL of 100 ng/µL DNA template, and water to 25 µL. The PCR program was 95°C for 5 min; 35 cycles of (95°C for 45 s left/30 s right, 57.4°C for 30 s, 72°C for 30 s); and 72°C for 10 min. Products were analyzed by 1.0% agarose gel electrophoresis.

1.7 Sensitivity Analysis of Event-Specific PCR Detection Methods

Genomic DNA from transgenic rice BPL9K-2 and non-transgenic rice 9K19-5 was adjusted to 100 ng/μL and mixed at different volume ratios to achieve relative contents of 100%, 20%, 5%, 2%, 1%, 0.5%, and 0.1% BPL9K-2 DNA. One microliter of each mixed DNA sample was used as template for PCR amplification with primer pairs RBG-F/RBG-R and LB-F/LB-R using the same system and program as in section 1.6, with three replicates per sample. Five microliters of PCR product were analyzed by 1.0% agarose gel electrophoresis, and the gel images were quantitatively analyzed using Bio-Rad Image Lab 3.0. Using the 750 bp band of the DL 2000 molecular weight marker as a reference (containing 150 ng DNA when 5 μL is loaded), the relative brightness of target bands in different lanes was measured to calculate the relative DNA content of amplified bands.

1.8 Three-Primer Method for Identifying Exogenous Gene Homozygosity/Heterozygosity

Reverse primer GB-R (5' -GGATTTGACGAGCGAGCGAT-3') was designed based on the downstream rice genome sequence at the insertion site and combined with the event-specific detection primer pair RB-F/RB-R for PCR amplification of genomic DNA from different transgenic lines and their progeny. The 20 μL PCR system contained 10 μL of 10×Taq MasterMix, 1 μL each of 10 μM primers, 1 μL of 100 ng/μL DNA template, and water to volume. The PCR program was 95°C for 5 min; 35 cycles of (95°C for 45 s, 56.4°C for 30 s, 72°C for 30 s); and 72°C for 10 min. PCR products were analyzed by 1.0% agarose gel electrophoresis, and exogenous gene homozygosity/heterozygosity was determined based on band number and size.

Results and Analysis

2.1 hiTAIL-PCR Amplification Results and Characteristics of Left Flanking Sequence

Second- and third-round hiTAIL-PCR products were separated by 1.0% agarose gel electrophoresis. Specific bands were identified as those present in the third round that were approximately 45 bp smaller than in the second round and absent in the control group. The amplification results using four arbitrary primers combined with specific nested primers in the second and third rounds are shown in [Figure 1: see original paper].

The third-round product using arbitrary primer LAD3 was selected for sequencing, yielding a 450 bp sequence. Comparison with the rice genome database revealed that nucleotides 1-234 matched nucleotides 1,037,532-1,037,765 on chromosome 10 of rice cultivar Nipponbare (NCBI accession AP014966.1), while nucleotides 235-450 matched the exogenous gene sequence on the left border side. The exogenous gene was truncated at the 49th nucleotide residue within

the T-DNA left border, with 49 bp of the T-DNA left border sequence deleted (5'-TGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAAT-3') ([Figure 2: see original paper]).

2.2 Right Flanking Sequence Detection and Characteristics

Using transgenic rice BPL9K-2 DNA as template, PCR amplification with primer pair RB-F/RB-R yielded a specific fragment of 485 bp. Sequencing revealed that nucleotides 1-309 were identical to the vector right border sequence, while nucleotides 310-485 matched nucleotides 1,037,825-1,037,986 on chromosome 10 of rice cultivar Nipponbare (NCBI accession AP014966.1). The exogenous gene was truncated at the 32nd nucleotide residue within the T-DNA right border, with 32 bp of the T-DNA right border sequence deleted (5' -TGGCGGGTAAACCTAAGAGAAAAGAGCGTTTA-3') ([Figure 3: see original paper]).

Based on the left and right flanking sequence information and comparison with the rice genome database, the exogenous gene inserted in a non-coding region of the short arm of rice chromosome 10 (NCBI accession AP014966.1), with the left border inserting after nucleotide residue 1,037,765 and the right border inserting before nucleotide residue 1,037,825. The insertion resulted in deletion of 49 nucleotides from the T-DNA left border and 32 nucleotides from the right border, with 59 nucleotides deleted from rice chromosome 10 (positions 1,037,766 to 1,037,824, NCBI accession AP014966.1). A gene coding region and an ncRNA coding region were located 170 bp upstream and 977 bp downstream of the insertion site, respectively ([Figure 4: see original paper]).

2.3 Establishment of Event-Specific PCR Detection Methods

Using primer pairs LB1-F/LB1-R and RBG-F/RBG-R designed based on left and right flanking sequence information, transgenic rice BPL9K-2 was detected with other transgenic and non-transgenic rice varieties as controls. Results showed that only transgenic rice BPL9K-2 produced specific bands of 449 bp and 485 bp, while non-transgenic materials (9K19-1, 9K19-5, 11C2277, 11C2292, 7001S, Guangzhan63S, Ke108A, R106) and transgenic materials (BPL9K-1, BPL9K-4, EB7001S-5, B1C893, EB185BS, B2A68, BarKasalath-01, Bar9K29-9-2) yielded no corresponding fragments ([Figure 5: see original paper]), demonstrating excellent specificity of the detection method.

2.4 Sensitivity Analysis of Event-Specific PCR Detection

Mixed samples were analyzed by PCR using primers LB1-F/LB1-R and RBG-F/RBG-R based on left and right flanking sequences, with electrophoresis results shown in [Figure 6: see original paper]. Using the 750 bp band of the DL 2000 marker as a reference, target band brightness was measured to calculate corresponding DNA content. Results indicated that when the relative content of BPL9K-2 genomic DNA in the template was 0.1% (approximately 0.1 ng),

the DNA content in the corresponding lane was similar to that of the similarly-sized molecular weight marker band (500 bp) ([Figure 7: see original paper]). This demonstrates that the established event-specific PCR detection method has high sensitivity, capable of detecting transgenic components at a relative content of 0.1%.

2.5 Rapid Genotyping of Exogenous Genes by Three-Primer Method

The three-primer combination RBG-F/RBG-R/GEB-R was used for PCR detection of individual plants from different BPL9K-2 lines. In homozygous exogenous gene plants, the long inserted fragment prevented amplification by primer pair RB-F/GB-R, yielding only a 325 bp product from RB-F/RB-R. In non-transgenic rice 9K19-5 lacking the exogenous gene, RB-F/GB-R amplified the rice genome sequence to produce a 485 bp band. Heterozygotes, containing the exogenous gene on one chromosome but not the other, yielded both 325 bp and 485 bp bands simultaneously ([Figure 8: see original paper]). This study validated the accuracy of this method through PCR detection of different BPL9K-2 lines and their progeny. This approach shortens the time required to obtain transgenic homozygotes and has practical value for accelerating the breeding application of transgenic rice BPL9K-2.

Discussion

This study successfully obtained the left and right flanking sequences of transgenic rice BPL9K-2 using hiTAIL-PCR and other methods. The exogenous gene inserted after nucleotide residue 1,037,765 and before nucleotide residue 1,037,825 on the short arm of rice chromosome 10. The insertion caused deletion of 49 bp from the T-DNA left border, 32 bp from the right border, and 59 bp from rice chromosome 10. Based on the left and right flanking sequence information, we established an event-specific qualitative PCR detection method for transgenic rice BPL9K-2 and a three-primer PCR method for detecting exogenous gene homozygosity/heterozygosity.

Different transformation events involve insertion of exogenous genes at different genomic positions in the recipient, resulting in different adjacent genomic sequences that can be used to distinguish transformation events [14]. Event-specific PCR detection identifies GM organisms containing the same exogenous fragment by amplifying the specific junction region between the recipient genome and insertion fragment [15]. This method is widely applied in detecting various GM crops and their products, including rapeseed, soybean, wheat, maize, cotton, and rice, due to its simplicity, speed, strong specificity, and high sensitivity. This study identified the flanking sequences of the exogenous gene in transgenic rice BPL9K-2 and established its event-specific PCR detection method, which exhibits good specificity and high sensitivity, providing a technical foundation for the application and regulation of transgenic rice BPL9K-2.

Agrobacterium-mediated T-DNA insertion constitutes illegitimate recombina-

tion, typically causing deletion or duplication of host genome sequences at integration sites and deletion of T-DNA sequences [16, 17]. Tzfira et al. [18] proposed that double-strand break repair mechanisms represent the primary pathway for T-DNA insertion and integration into the host genome. Rare site-specific restriction endonucleases from *Agrobacterium* are expressed in the host, creating double-strand breaks in host genomic DNA and digesting T-DNA in vivo, leading to partial base deletions in T-DNA left and right borders and adjacent rice genome sequences. In transgenic rice BPL9K-2, partial base degradation occurred at T-DNA borders during integration into the rice genome. Microhomology of 4 bases (ACTT) and 3 bases (ATT) existed at the left border breakpoint and rice genome breakpoint, respectively ([Figure 2: see original paper]), while 3-base microhomology (ATT) existed at the right border breakpoint and rice genome breakpoint ([Figure 3: see original paper]). The left border of the vector lost 49 bp, the right border lost 32 bp, and 59 bp were deleted from the rice chromosome, with the exogenous gene in BPL9K-2 entering host cells via microhomology-mediated transfer.

Exogenous fragment insertion may adversely affect the expression and regulation of endogenous genes, causing functional deficiencies and leading to undesirable effects such as reduced or lost fertility, dwarfism, decreased tiller number, and extended growth duration. For example, in Bt transgenic rice T51-1, the exogenous Bt gene inserted in the promoter of the rice endogenous disease resistance-like gene *Os10g0183000*, resulting in significantly reduced seed setting rate, withered leaf tips, and yellowing leaves compared to its non-transgenic recipient Minghui63 [19]. The insect-resistant and herbicide-resistant transgenic rice B1C893 double-copy transformant was completely sterile, while the single-copy transformant had its exogenous gene inserted in a non-coding region near the telomere on the short arm of rice chromosome 8, with an ncRNA coding region only 140 bp upstream. The special chromatin structure of the subtelomeric region likely caused undesirable effects from exogenous gene insertion, including reduced plant height, increased effective panicles, shorter panicle length, fewer grains per panicle, decreased seed setting rate, reduced pollen fertility, lower thousand-grain weight, and extended growth duration [20]. In transgenic rice EB7001S, the exogenous gene integrated into a non-coding region of the rice genome without insertion of other vector backbone sequences, with upstream and downstream gene coding regions located far from the insertion site (4,050 bp and 5,576 bp, respectively), and the insertion did not cause significant changes in agronomic traits [21]. Similarly, transgenic rice B2A68 contains a single-copy exogenous gene inserted at nucleotide residue 36,170 on rice chromosome 3 in a non-coding region, with no changes in appearance or agronomic traits compared to the recipient material [5]. Therefore, although the mechanism by which exogenous gene insertion affects rice traits remains unclear, it can be inferred to relate to exogenous gene copy number, insertion site, and distance to upstream and downstream gene coding regions. If the exogenous gene has multiple copies, inserts within an endogenous gene or special chromosomal region (such as near telomeres), or the insertion site is too close to upstream/downstream gene or

ncRNA coding regions, the insertion will likely alter original rice traits. The single-copy transgenic rice BPL9K-2 has its exogenous gene inserted in a non-coding region of rice chromosome 10, with the nearest gene coding region 170 bp upstream and the nearest ncRNA coding region 977 bp downstream. At the molecular level, the exogenous fragment caused minimal changes to the original genome without disrupting original gene structure, thus having minimal impact on original gene expression and possessing practical application value. Agronomic trait evaluation confirmed this conclusion: compared with control 9K19-5, transgenic rice BPL9K-2 showed no significant changes in plant height, panicle length, effective tiller number per plant, seed setting rate, thousand-grain weight, or yield per plant, except for slightly extended growth duration.

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Note: Figure translations are in progress. See original paper for figures.