Kor. J. Hort. Sci. Technol. 29(2):123-129, 2011

Rapid and Unequivocal Identification Method for Event-specific Detection of Transgene Zygosity in Genetically Modified Chili Pepper

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Abstract. To identify unintended vertical gene-transfer rates from the developed transgenic plants, rapid and unequivocal techniques are needed to identify event-specific markers based on flanking sequences around the transgene and to distinguish zygosity such as homo- and hetero-zygosity. To facilitate evaluation of zygosity, a polymerase chain reaction technique was used to analyze a transgenic pepper line B20 (homozygote), P915 wild type (null zygote), and their F1 hybrids, which were used as transgene contaminated plants. First, we sequenced the 3'-flanking region of the T-DNA (1,277 bp) in the transgenic pepper event B20. Based on sequence information for the 3'- and 5'-flanking region of T-DNA provided in a previous study, a primer pair was designed to amplify full length T-DNA in B20. We successfully amplified the full length T-DNA containing 986 bp from the flanking regions of B20. In addition, a 1,040 bp PCR product, which was where the T-DNA was inserted, was amplified from P915. Finally, both full length T-DNA and the 1,040 bp fragment were simultaneously amplified in the F1 hybrids; P915 × B20, Pungchon × B20, Gumtap × B20. In the present study, we were able to identify zygosity among homozygous transgenic event B20, its wild type P915, and hemizygous F1 hybrids. Therefore, this novel zygosity identification technique, which is based on PCR, can be effectively used to examine gene flow for transgenic pepper event B20.

***Additional key words*:** border sequence, genetically modified organism (GMO), polymerase chain reaction, selection marker

Introduction

Over the last decade, various transgenic plants with new traits have been developed and commercialized. Researches on such plants have also become an active part of the agricultural industry. Development of genetically modified (GM) plants, mainly maize, soybean, and cotton, has focused on optimizing herbicide tolerance and/or insect resistance. In regards to plants produced worldwide, 77% of soybean, 26% of maize, 49% of cotton, and 21% of canola are GM plants. The number of GM plants worldwide has increased during the last 13 years and was up to 134 million ha in 2009. These GM plants are produced in 25 countries and by 14 million farmers (James, 2009).

Together with the rapid increase in the development of GM plants, there has been an increase of interest in understanding the gene flow with respect to the environmental risk assessment (Hill and Sendashonga, 2003). In this regard, there has been an increasing demand for knowledge and information about the environmental risk assessment in terms of gene flow from GM plants to wild plants. Unintended gene flow from GM plants to related species in the field is a highly important factor not only because of spatial spread of genetic information causing resistant biotypes but also unlimited release of GM plants to the wild. Hybrid plants created by gene flow can be screened preliminarily through its morphology, sterility, bioassays such as herbicide treatment and marker genes, protein assays, etc. However, rapid and unequivocal techniques are needed to distinguish heterozygous created by unintended gene transfer from homozygous transgenic plants, since a large amount of samples should be evaluated in environmental risk assessments.

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※ Received 1 February 2011; Accepted 3 March 2011. This research was supported by the Chung-Ang University Research Scholarship Grants in 2008.

Detection of introduced gene elements at the DNA level has been accomplished using PCR. PCR is a widely used and powerful technique to identify genetically modified organisms. Various PCR techniques such as quantitative real time PCR, reverse transcription PCR, multiplex PCR, and nested PCR methods have been used to detect and/or quantify transgenes (Hernández et al., 2003; James et al., 2003; Seo et al., 2009; Zhu et al., 2010; Zimmermann et al., 1998).

The chili pepper (*Capsicum annuum* L.) is the most widely cultivated vegetable crops (44,584 ha in 2010) and is often used as a food additive and in fresh fruits in Korea. Because most chili peppers are produced in an open field culture, they are constantly exposed to various diseases and can be easily infected, which results in a loss of productivity. Infection with the cucumber mosaic virus pathotype P0 (CMVP0) has been the major factor in the reduction of production in Korea (Lee et al., 2006). Recently, a new CMVP0 resistance-breaking virus, CMVP1, was reported and identified by Lee et al. (2006). Because it has been difficult to develop a resistant cultivar by conventional breeding, transgenic pepper event B20 was developed by genetic transformation of a coat protein (CP) gene from Fny-CMV to allow the chili pepper (P915 line) to have a high tolerance against both CMVP0 and CMVP1 (Lee et al., 2009). This event contains a transgene cassette carrying a 35S CaMV promoter, the hsp70 intron, and the CMV0-CP gene of Fny-CMV strain, which encodes a coat protein and *nptII* gene for kanamycin selection (Lee et al., 2009).

This study describes the development of a simple procedure, which involves amplifying full length T-DNA using PCR, to detect zygosity among wild type pepper, GM event, and their hybrids and to evaluate unintended vertical gene transfer.

Materials and Methods

Plant Materials and DNA Isolation

Seeds of non-transgenic (P915) and transgenic pepper (B20) were obtained from Biotechnology Institute of Nongwoo Bio (Yeoju, Korea). Transgenic pepper event B20, its wild type P915 and two commercial cultivars Pungchon, Gumtap, and F1 hybrid lines (P915 × B20, Pungchon × B20, Gumtap × B20) were used in this study. By allowing gene transfer from GM pepper B20 to its relatives, we obtained a heterozygous F1 hybrid with Pungchon, Gumtap, and P915 using B20 as a paternal parent. Pollens of B20 were collected and pollinated on two commercial cultivars and P915. After pollination and fertilization, the developed fruits were harvested and seeds were collected. Collected seeds were sterilized with 70% ethanol for 2 minutes, washed 2-3 times with dH_2O, soaked into 2% of hypochlorite (Yuhanclorox, Korea) for 15 minutes and finally washed 5 times with dH_2O. Sterilized seeds were sown on horticulture nursery media (Punong, Korea) and grown in a growth room at 25℃.

DNA Extraction

Genomic DNA was isolated from leaf tissues of the wild type P915, transgenic line B20, and three F1 hybrids (P915 × B20, Pungchon × B20, Gumtap × B20) as previously described by Kim and Hamada (2005). Genomic DNA concentration was determined by measuring ultraviolet (UV) absorption at 260 nm and purity was ascertained by measuring the absorbance due to proteins at 280 nm and 230 nm, and calculating the A260/A280 and A260/A230 ratios. All measurements were conducted using a Beckman DU 700 UV/vis spectrophotometer (Beckman Instruments, Fullerton, CA).

PCR-based Genome Walking and Identification of Flanking Region

Genome walking was performed using the GenomeWalker Universal kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions. Genomic DNA was digested with *Alu*I, *Dra*I, *Eco*RV, *Hpa*I, *Pvu*II and *Stu*I, and was ligated with the GenomeWalker adaptor (Clontech Laboratories). PCR was carried out using each enzyme-digested DNA library, 10 pmoles AP1 primer, 10 pmoles *NPT*II gene specific reverse primer 1 (GSP1), 0.2 mM dNTP mixture, 2.5 U of EX Taq polymerase (TaKaRa, Shiga, Japan) and 10X EX Taq buffer (TaKaRa) in a 50 μL reaction volume using a xp thermal cycler (Bioer Technology, Tokyo, Japan). The cycling condition were as follows: 5 cycles at 94℃ for 25 s and 72℃ for 3 min followed by 20 cycles at 94℃ for 25 s and 67℃ for 3 min, with a final cycle at 67℃ for 7 min. Nested PCR was performed under the same conditions using 1/10 of the primary PCR product as DNA template and using the AP2 primer and GSP2 primer.

The amplified PCR products were separated by electrophoresis on a 0.8% agarose gel. Each PCR fragment was ligated into a T&A cloning vector (Real Biotech, Taipei, Taiwan) and the constructed plasmids were transformed into the competent *Escherichia coli* strain, DH5α (HIT Competent Cells, Real Biotech, Taipei, Taiwan). The plasmid containing the insert DNA was isolated from transformed *E. coli* and then sequenced. The obtained sequence was aligned with vector and/or flanking region sequence with the CLC Sequence Viewer program (CLC bio, Katrinebjerg, Denmark).

PCR Condition and Enzyme Mapping of PCR Products

DNA samples with a A260/280 ratio over 1.8 of were selected and used for PCR. Genomic DNAs were diluted to 100 ng·μL^{-1} and 1 μL of the diluted DNA solution was

used as a template for PCR amplification. A reaction mixture containing 100 ng DNA, 1x reaction buffer, 20 mM dNTPs (1.6 μL, 250 mM), 0.5 pmole of each primer, and 1.25 units of Ex Taq DNA Polymerase (5units·μL^{-1}, TaKaRa, Japan) was prepared in a total volume of 20 μL. The PCR conditions were as follows: 1 cycle of 5 minute initial denaturation at 94℃, 40 cycles of 30 seconds denaturation at 94℃, 30 seconds of annealing at 60℃ and 90 seconds for primer extension at 72℃ using a thermocycler (Applied biosystems, USA). An additional extension step was added for 2 min at 72℃. Amplified products were subjected to electrophoresis in 1.0% agarose gels for 25 minutes and then stained with ethidium bromide (0.5μg·mL^{-1}). Stained gels were visualized under a UV illuminator (Bio-Rad Laboratories, Inc.). To identify full-length T-DNA fragments amplified from B20 and F1 hybrids, enzyme mapping was conducted using the *Xba*I restriction enzyme, which divides full-length T-DNA into two DNA fragments. Fifteen micro liter of each PCR product, 2 μL of 10X Ex Taq buffer (TaKaRa, Japan), 0.25 μL of *Xba*I (15 units·μL^{-1}, TaKaRa Bio., Japan) and 20 μL of ddH_2O were mixed into a 1.5 mL tube. Mixes were then incubated for 3 hours at 37℃ in an incubator. Finally, digested products were subjected to electrophoresis in 1.0% agarose gels and then visualized under a UV illuminator after ethidium bromide staining.

Results

Determination of 3'–flanking Region of the Transgene

The PCR based genome walking method was used to identify the 3'-flanking sequence of the transgene in the B20 transgenic event (Fig. 1A). Approximately 1,717 bp of amplified PCR product was generated from a *Dra*I digested DNA library through GenomeWalker PCR. The specific fragment was purified and cloned into the T&A cloning vector and sequenced. The obtained sequence (1,717 bp) was aligned with vector sequence using the CLC Sequence Viewer program (CLC bio, Denmark) and the 1,277 bp of the 3'-flanking region sequence and 440 bp of the vector region was identified (Fig. 2).

Using information previously reported on the sequence of the 3'-flanking region and 5'-flanking region (Seo et al.,

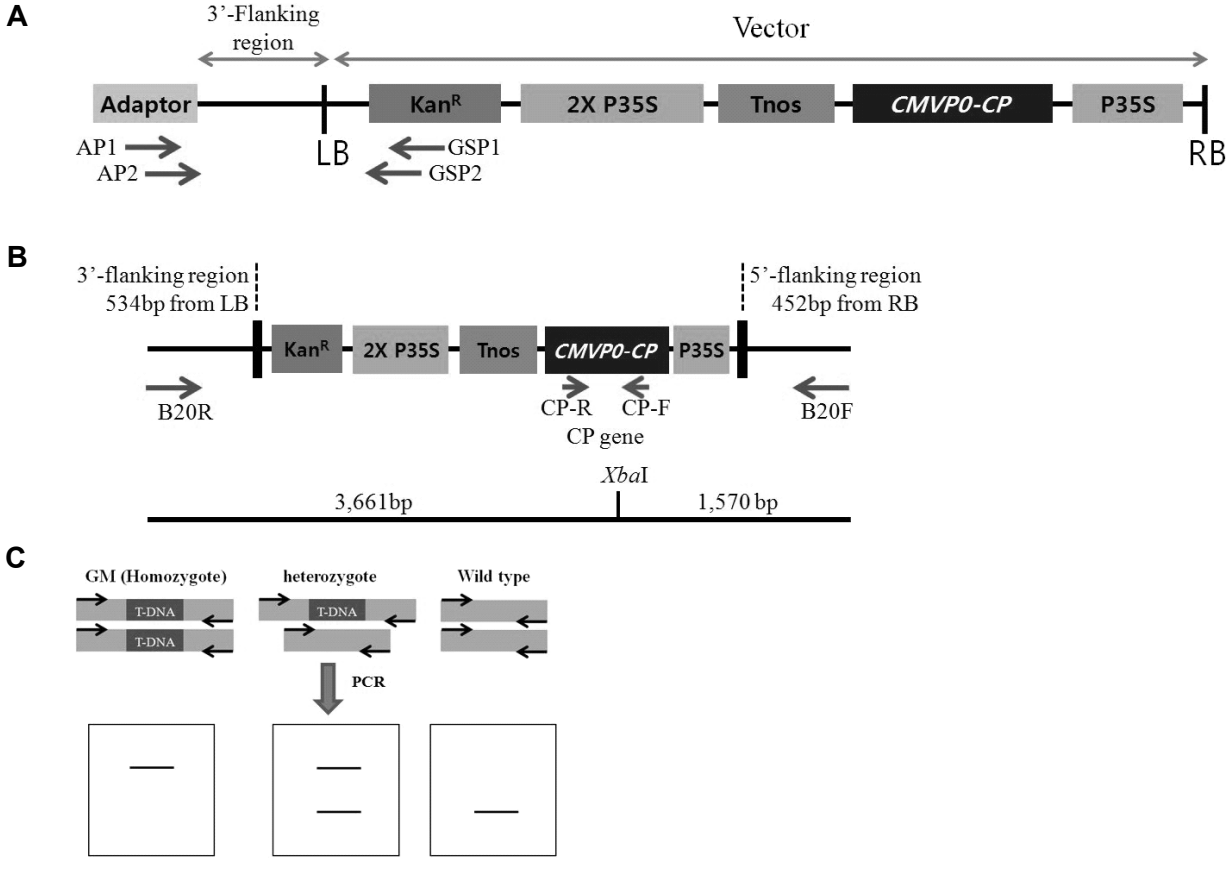

Fig. 1. Amplification strategies for identifying the 3'-flanking region of GM pepper B20 (A) and zygosity detection of wild type, GM, and hybrids (B and C). A: AP1 and AP2, adaptor specific primers; GSP1 and GSP2, gene specific primers; *Kan*^{R}, kanamycin resistance gene; P35S, promoter from cauliflower mosaic virus; T*nos*, nos terminator gene, *CMVP0-CP*, coat protein gene; LB and RB, left and right border respectively, C: B20F and B20R, flanking region specific primers, CP-F and CP-R, coat protein gene specific primers.

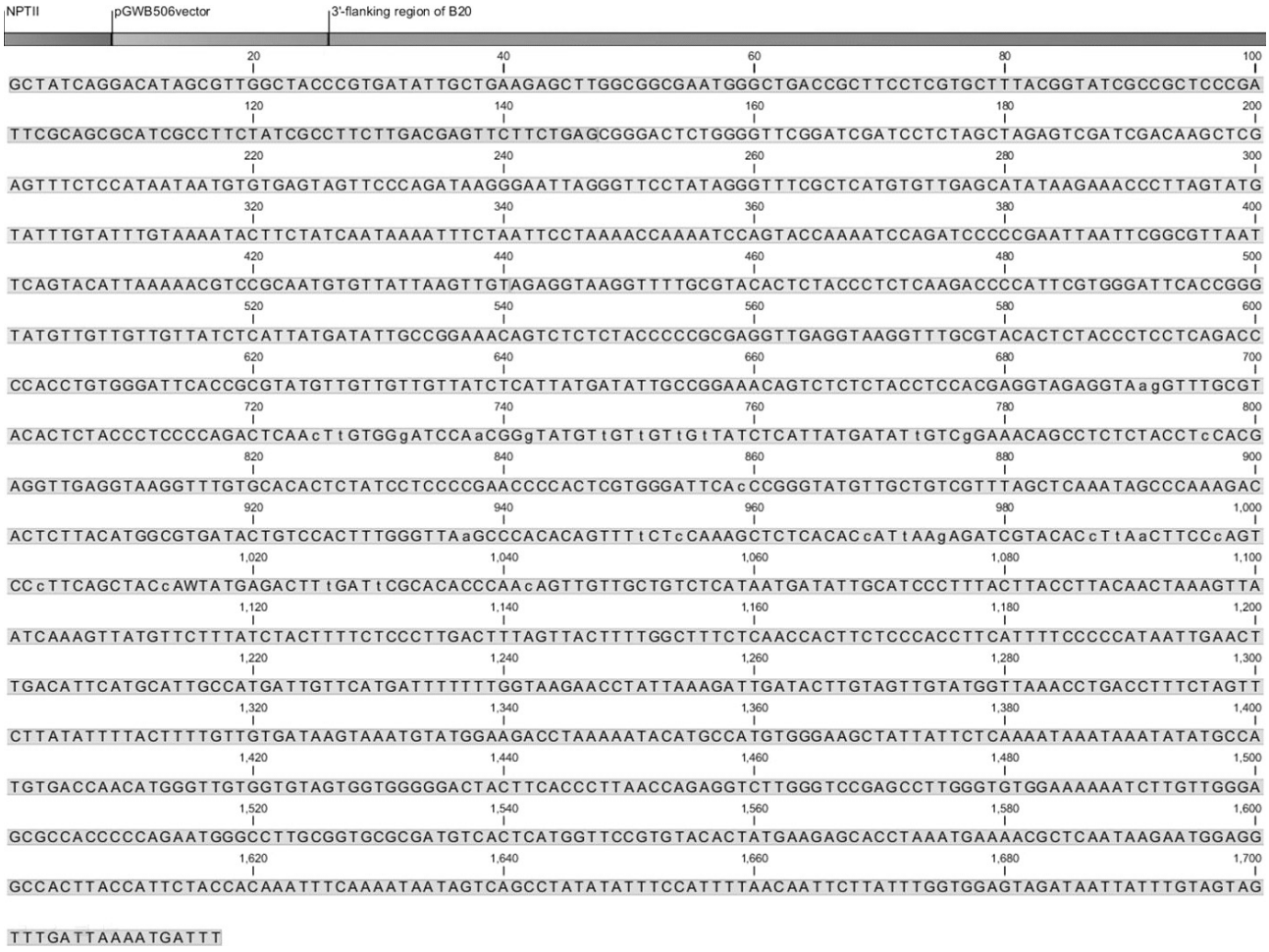

Fig. 2. Sequence of the 3'-flanking region of GM pepper B20 using PCR based genome walking.

Table 1. Primer pairs used for PCR amplification.

Primer	Name	Sequence
Adaptor primer	AP1	5'-GTAATACGACTCACTATAGGGC-3'
	AP2	5'-ACTATAGGGCACGCGTGGT-3'
Gene specific primer	GSP1	5'-CGAATATCATGGTGGAAAATGG-3'
	GSP2	5'-GCTATCAGGACATAGCGTTGG-3'
Coat protein	CP-F	5'-GTAGGGAGTGAACGCTGTAGACC-3'
	CP-R	5'-GAAGTACTAGCTCGTCCGTCTCG-3'
T-DNA amplification	B20F	5'-GACAACACGCAGCTAAAGATTG-3'
	B20R	5'-TTAATGGTGTGAGAGCTTTGGA-3'

2009), a pair of event specific primers was prepared to amplify full length T-DNA (Table 1). The forward primer (B20F, 5'-GACAACACGCAGCTAAAGATTG-3') was designed from the sequence of the 5'-flanking region and the reverse primer (B20R, 5'-TTAATGGTGTGAGAGCTTTGGA-3') was designed from the sequence of the 3'-flanking region obtained in this experiment. PCR was conducted using the primer pair to identify proper amplification of the target site for both B20 and P915 (Fig. 3). Full length T-DNA containing a 986 bp flanking region was successfully amplified from B20 genomic DNA. In addition, a PCR product that was about 1,040 bp was obtained from wild type P915. This sequence was larger in size than both the 5'- and 3'-flanking region (totally 986 bp), indicating that some of the DNA was deleted when T-DNA was incorporated into the genome.

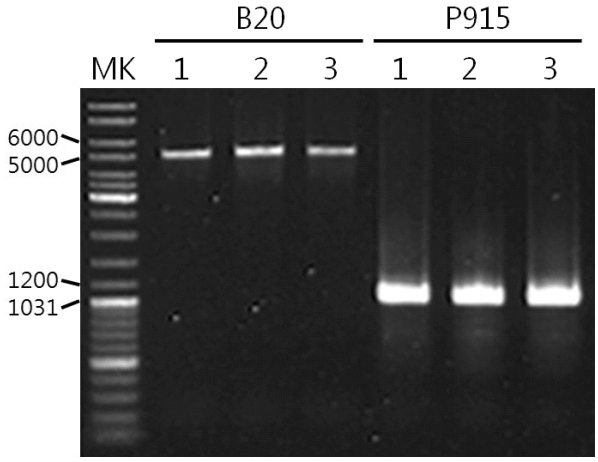

Fig. 3. PCR amplification and identification of target products in GM pepper and non GM pepper using B20F and B20R primers. MK, size marker; B20, GM pepper event; P915, wild type; 1, 2, and 3 means sample numbers, respectively.

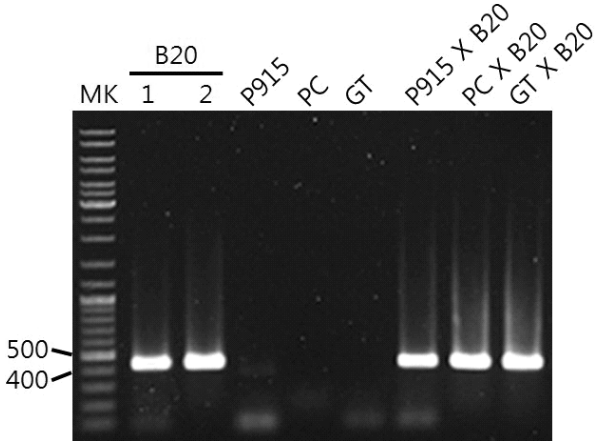

Fig. 4. PCR amplification and identification of the coat protein gene (430 bp) in GM, non-GM (P915), commercial cultivars, and F1 Hybrids for identification transgene inheritance. MK, size marker; 1 and 2, sample number of GM pepper event; PC, Pungchon; GT, Gumtap.

Amplification and Identification of Transgene from GM and Hybrids

To identify vertical gene transfer to the F1 hybrids during artificial pollination, the coat protein (430 bp) gene was amplified using PCR (Fig. 4). Genomic DNA of B20 was used as a positive control. The coat protein genes (430 bp) were successfully amplified in all of the F1 hybrids. However, no PCR product was amplified in non GM lines; P915, Pungchon, and Gumtap. Therefore, artificial pollination was successfully accomplished and this hybrid material could be used as a source of zygosity identification by PCR assuming transgene contamination.

Identification of Zygosity of GM, Wild Type, and F1 Hybrids

Assuming that vertical gene transfer occurred from GM to non GM plants, we attempted to simultaneously amplify T-DNA and the inserted region by PCR using a primer set (B20F and B20R) that was designed based on sequence information of the flanking regions. Only one PCR product should be amplified from gDNA of homozygous GM pepper and non GM lines (Fig. 1C). In these experiments, a PCR product of 5,231 bp was amplified from GM including T-DNA, both flanking regions and the 1,040 bp product from P915. If plants acquired transgenes due to unintended gene flow from GM plants, both PCR products should be simultaneously amplified because those plants should be hemizygote. We also examined artificially pollinated F1 lines gene transferred plants; P915 × B20, Pungchon × B20, and Gumtap × B20. In these experiments, two PCR products were simultaneously amplified from the F1 hybrids (Fig. 5A). In addition, the PCR products were digested directly by *Xba*I for enzyme mapping (Fig. 5B). Band patterns obtained by enzyme mapping were identical between the B20 and F1 hybrids,

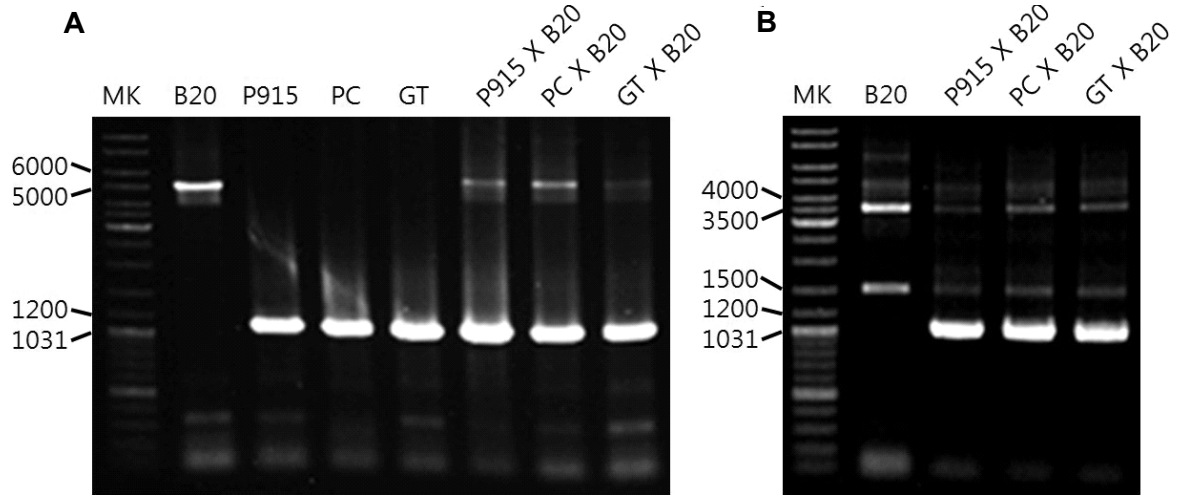

Fig. 5. Identification of zyogosity using PCR amplification of full length T-DNA containing flanking regions (A) and band patterns of PCR products by enzyme mapping (B). MK, size marker; B20, GM pepper event; P915, wild type; PC, Pungchon; GT, Gumtap.

indicating that the 5,231 bp PCR products of GM and hybrids were full length T-DNA containing both flanking regions.

Discussion

In the present study, we determined the sequence of the 3'-flanking region of transgene from transgenic pepper event B20 via the PCR based genome walking technique. Using this approach, the 1,277 bp of the 3'-flanking region was obtained. Using the sequence information on the 3'-flanking region obtained in this study and the 5'-flanking region reported in a previous study (Seo et al., 2009), we designed specific primers and successfully amplified the 5,231 bp corresponding to full length T-DNA containing the flanking regions and the 1,040 bp PCR product from P915. In addition, both products were amplified from the F1 hybrids. This result indicates that the PCR method using an event specific primer set can identify GM pepper event B20 as well as the zygosity between GM and non-GM plants.

The vector used for transformation of the GM pepper event investigated in this study contains a 35S CaMV promoter, *npt*II as a selection marker, a gene of interest *CMVP0-CP* for the Fny-CMV strain, and nos terminator. For this event, a primer pair starting from a region in the 35S promoter region to the end of the *CMVP0-CP* gene was used as a selection marker for transgenic T0 plants (Lee et al., 2009) and the 35S promoter and *nos*II terminator gene were detected using a specific primer from leaf samples buried in the soil to evaluate the persistence of DNA (Lee et al., 2007). In addition, a primer to detect the gene from the 5'-flanking region to the 35S promoter region was used as an event specific marker for multiplex PCR (Seo et al., 2009). These primers can be reliable markers for detecting transgenic pepper event. Because the 35S promoter gene and/or *nos*II terminator gene occurs naturally (Wolf et al., 2000), the amplification of such genes with these primer pairs cannot be used as representative markers for GM pepper event B20. However, the event-specific PCR using a primer pair based on the flanking regions can be used to detect transgenic lines as well as possible F1 hybrids by transgene contamination due to unintended gene flow in terms of zygosity identification. Holst-Jensen et al. (2003) categorized PCR-based assays of GMO derivatives corresponding to the level of specificity of target. Category 1 correspond to screening methods that target sequences such as the Beta lactamase gene for ampicillin resistance in prokaryotes, 35S promoter, 35S terminator, nopline synthase terminator of *Agrobacterium tumefaciens*, and neomycine-3'-phosphotransferase II gene coding for neomycin or kanamycin resistance. Thus, it can only be used to screen GMOs with limited sequence information. The second category corresponds to gene-specific methods and the phosphinotricin acetyltransferase (*bar*) gene and CryIA (b) belong to this group. Category 3 involves methods that target junctions and these methods amplify specific regions of only the T-DNA. Category 4 corresponds to event-specific methods that detect junctions between the inserted DNA and host genome DNA. Our primer may belong to category 4 because it can amplify such junctions as well as whole T-DNA elements, suggesting it has high specificity for targeted transgenes of the GM pepper B20.

In the case of transgenic maize events, La Paz et al. (2010) found that no significant mutation rate in endogenous genes occurred in endogenous genes. This result reflects the stability of transgene in transgenic maize. Although six SNPs were found in over 500 bp upstream of the 5'-flanking region, such single nucleotide mutations do not affect detection using PCR based approaches. It has been known that foreign genes, once incorporated, are inherited stably (Delannay et al., 1989; Duan et al., 1996; Fearing et al., 1997; Müller et al., 1987; Padgette et al., 1995; Widmer et al., 1997). In this study, homogeneous transgenic event B20 was observed in the T_4 generation and the transgene could be reliably detected using a PCR based approach. Presuming that the mutation rate is very low and the transgene can be easily detected by the event specific PCR method described here, PCR products amplified by our event specific primer can be used as a powerful selection maker not only for monitoring GM pepper B20, but also for assessing unintended gene flow in the field or market place once it is commercialized.

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