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A Multiplex PCR Method for the Detection of Genetically Modified Alfalfa (*Medicago sativa* L.) and Analysis of Feral Alfalfa in South Korea

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ABSTRACT

Methods for detecting the presence of genetically modified (GM) crops are evolving to comply with legislation and to enhance monitoring by biotechnology companies and regulators. In order to cover a broad range of detection methods for a new GM crop, conventional multiplex PCR methods are required. Based on the genetic information on three GM alfalfa varieties (J101, J163, and KK179), which were recently approved in South Korea, we developed a fast, reliable, and highly specific multiplex polymerase chain reaction (PCR) method with basic PCR equipment and inexpensive reagents. To validate and verify the newly developed multiplex PCR method, we applied a limit of detection assay and random reference material analysis. We also monitored the unintentional environmental release of GM alfalfa in South Korea by performing the multiplex PCR analysis with 91 feral alfalfa specimens collected from 2000 to 2018. Our methodology is a sensitive, simple, quick, and inexpensive tool for detecting and identifying three GM alfalfa varieties.

Keywords: Alfalfa, Genetically modified organism, Genetically modified organism detection, Medicago sativa, Multiplex polymerase chain reaction

Introduction

Since the first commercialization of genetically modified (GM) crops which known as living modified organism in 1996, the area dedicated to cultivation of GM crops has steadily increased to 189.8 million hectares worldwide (ISAAA, 2017). Major GM crops include maize, soybean, cotton, rapeseed, and more recently, alfalfa (*Medicago sativa L.*), which has emerged as an important perennial forage crop. Alfalfa, a member of the family Fabaceae, is the most important perennial forage crop for dairy cattle, and is also used for improving rhizosphere fertility (Tesfaye *et al*., 2005).

Intensive research into genetically engineering of alfalfa has been conducted to improve its agronomic performance, forage quality, and industrial attributes (Kumar, 2011). Most research has focused on applying transgenic methods to improve forage quality (Guo *et al*., 2001; Le *et al*., 2017; Reddy *et al*., 2005), increase resistance to abiotic stressors such as salt and drought (Bao *et al*., 2009; Jiang *et al*., 2009; Jin *et al*., 2010; Zhang *et al*., 2012), increase herbicide resistance (D`Halluin *et al*., 1990), and

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to induce the production of novel compounds (Lee *et al*., 2008; Peréz Aguirreburualde *et al*., 2013; Saruul *et al*., 2002; Vlahova *et al*., 2005). To date, all GM alfalfa have been developed by Monsanto Corporation, with the incorporation of herbicide resistance (called Roundup Ready alfalfa) and/or lignin biosynthesis inhibition (called reduced lignin alfalfa); these varieties have been approved in many countries, including South Korea. The varieties J101 and J163 include the transgene *CP4 epsps*, which provides resistance to the herbicide glyphosate under the control of the FMV promoter. KK179 was developed with the RNA interference (RNAi) technique to suppress the endogenous caffeoyl-CoA-3-O-methyltransferase (CCOMT) gene, a key enzyme in S lignin production (Barros *et al*., 2019). These three single traits (J101, J163, and KK179) and two stack traits (J101×J163 and KK179×J101) are authorized for food and feed production, and have been deregulated for cultivation in the USA, Canada, Japan, Mexico, and Argentina.

Detection and identification of GM alfalfa is currently performed using real-time polymerase chain reaction (qPCR) methods (Guertler *et al*., 2019). For regulatory purposes, we developed the event-specific qPCR method using plasmid standards due to the lack of available reference materials (RMs). We synthesized plasmids after searching patents for available data and validated the detection method in-house. We designed the event-specific primer and probes with FAM, ZEN, and IBFQ for qPCR, and the amplification sizes were 102 bp (J101), 118 bp (J163), and 178 bp (KK179). The establishment of a reliable, rapid, and cost-effective identification method for GM alfalfa is crucial for the regulation of transboundary movement. Quantitative PCR is highly sensitive and does not require gel electrophoresis, but conventional multiplex PCR is suitable for qualitative analysis and can be implemented in under-equipped laboratories.

The aim of this study was to establish an event-specific multiplex PCR detection method for three GM alfalfa traits (J101, J163, and KK179) based on the available genetic information. To validate the methods according to the general genetically modified organism (GMO) testing method, we performed limit of detection (LOD) assays and random RM DNA analysis. To assess the broad applicability of our multiplex PCR method, we applied it to analysis of feral alfalfa specimens from South Korea. Based on these results, we suggest that the multiplex PCR method is suitable for the detection and identification of three GM alfalfa in samples.

Materials and Methods

Reference materials and feral alfalfa specimens

RMs for GM alfalfa (J101, J163, and KK179) were obtained from the National Institute of Food and Drug Safety Evaluation (NIFDS, Cheongju, Korea). A total of 91 feral alfalfa specimens collected from 2000 to 2018 in South Korea were obtained from the National Institute of Biological Resources (NIBR, Incheon, Korea). Dried alfalfa leaf tissues were stored at −80°C until DNA extraction.

DNA extraction

Plant genomic DNA was extracted from alfalfa RMs and from the leaf tissue of feral alfalfa using a Nucleic Acid Extractor (NP986; Tianlong, Xi'an, China) and Nucleic Acid Extraction kit (T085H, Tianlong, China), following the manufacturer's protocol. Total DNA amounts were measured by the spectrophotometer ND-2000 (Thermo Fisher Scientific, Wilmington, DE, USA), and the final concentration was adjusted to 50 ng/μL for PCR. All extracted DNA was stored at −20°C until use.

PCR analysis

Genetic information for the three GM alfalfas was obtained from patents and from a previous study (Guertler *et al*., 2019). Event specific primers were designed for establishing the multiplex PCR, and β-actin (GenBank accession no. EU664318) was used for PCR control. The primers were synthesized by Macrogen Inc. (Seoul, Korea), and were diluted in nuclease-free water (Qiagen, Hilden, Germany) to 100 μM. For the PCR analysis, we used the 2X EF-Taq PCR Pre-Mix (Solgent, Daejeon, Korea) with each batch of genomic DNA (50 ng) and event specific primers (0.2 μM) in 30 μL total reaction volume. A Proplex PCR system (Applied Biosystems, Waltham, MA, USA) was applied for establishment and identification of GM alfalfa according to the following steps: pre-denaturation at 95°C for 5 minutes; 35 cycles consisting of denatur-

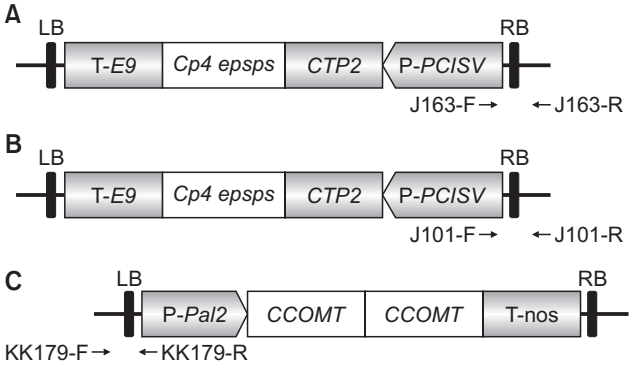

Figure 1. Schematic diagrams of PCR primer positions for the three LM alfalfa events (A, J163; B, J101; C, KK179). The locations and the primers are indicated by arrows. The vertical bold lines represent the flanking region of alfalfa genome (RB, right border; LB, left border; F, forward primer; R, reverse primer; gray pentagon, Promoter; open square, coding gene; gray square, 3' terminator).

ation at 95°C for 0.5 minutes, annealing at 59°C for 0.5 minutes, and extension at 72°C for 0.5 minutes; and 1 cycle of final extension at 72°C for 10 minutes. A 10 μL aliquot of each PCR product was resolved using gel electrophoresis on 2.5% (w/v) agarose gel at constant voltage (135 V) for 25 minutes, and the images were captured by Chemi-Doc XRS$^+$ (Bio-Rad, Hercules, CA, USA).

Sensitivity and application of multiplex PCR

To verity the efficiency of the GM alfalfa multiplex PCR method, we performed LOD analysis using serial dilution of RM DNA of the three GM alfalfas, multiplex PCR with randomly mixed RM DNA templates, and feral alfalfa sample analysis. The three RM DNA mixture was serially diluted with non-GM alfalfa genomic DNA for LOD assay (100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2, 0 ng/μL). Random mixed RM DNA samples were used to test the specificity of the multiplex PCR method. To test the practical application of the multiplex PCR method for the analysis of feral alfalfa samples, dried leaf samples of 91 specimens from NIBR were analyzed.

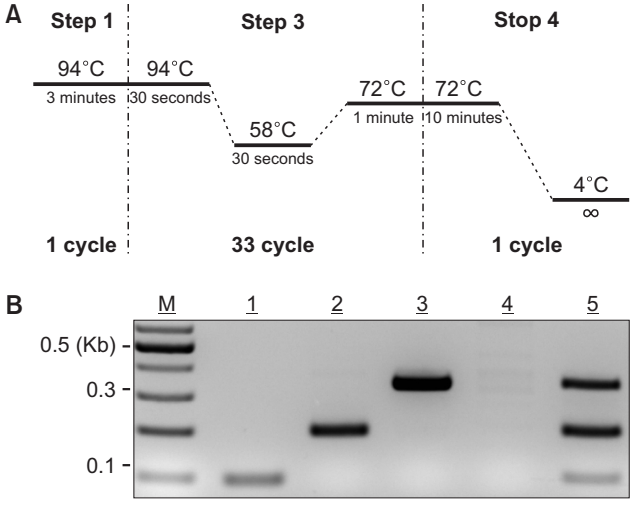

Figure 2. Establishment of multiplex polymerase chain reaction (PCR). (A) Schematic diagram of the optimal multiplex PCR condition. (B) Agarose gel image using multiplex PCR method for alfalfa genomic DNA. lane 1, J163; lane 2, J101; lane 3, KK179; lane 4, Non-LM alfalfa; lane 5, mixed reference materias; M, bp marker.

Results

Establishment of multiplex PCR

To develop the alfalfa multiplex PCR method, we acquired genetic information for three GM alfalfa events (Fig. 1) and designed event-specific PCR primers (Table 1). Each specific primer for flanking the alfalfa genome sequence and each transgene cassette were validated, and the primers without non-specific amplification were selected. As a result, all PCR primers showed event specific amplification for each GM alfalfa event (Fig. 2). These results indicate that the GM alfalfa multiplex PCR is capable of simultaneously detecting each event with high

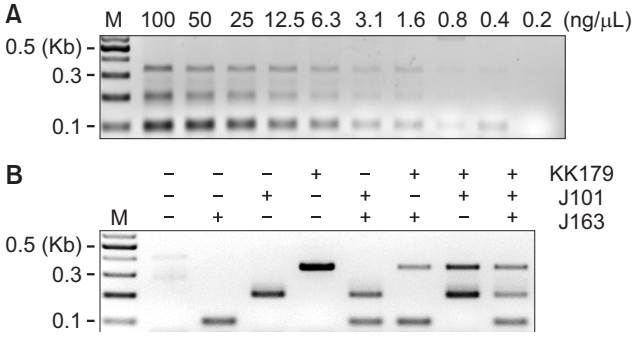

Figure 3. Sensitivity of multiplex PCR. (A) LOD of multiplex PCR for three alfalfa events with serial diluted mixed DNA template. (B) Efficiency of multiplex PCR using a random mixture of genomic DNA of alfalfa RMs. PCR, polymerase chain reaction; LOD, limit of detection; RMs, reference materials; M, 100 bp marker.

Table 1. Oligonucleotide primers used for multiplex PCR method to detect three LM alfalfa events

Event	Primer name	Sequence (5′-3′)	Product size (bp)
J163	J163-F	GGACTGAGAATTAGCTTCCA	98
	J163-R	ACAAGGTCATCCAAACTGAA	
J101	J101-F	GGACTGAGAATTAGCTTCCA	202
	J101-R	ATCTTTACAGTGACAATGTATATGGA	
KK179	KK179-F	GTCTTCAAAATACAAGTCAAACAC	347
	Kk179-R	CTTTCATTTTATAATAACGCTGCG	
β-actin	β-actin-F	GTCTCTCACGATTTCGCGCT	147
	β-actin-R	GTTCCTATCTATGAAGGATATGCCC	

F, forward primer; R, reverse primer.

specificity.

Sensitivity of multiplex PCR

The genomic DNA amounts of GMO volunteers or of processed food yield low quality DNA for GMO identification. The minimum level of quality at which sample genomic DNA can be successfully used for multiplex PCR is therefore essential to define (Choi *et al*., 2018). The LOD was tested using a serially diluted three GM alfalfa genomic DNA mixture (Fig. 3A). The multiplex PCR band was detectable at the 12.5 ng/μL concentration of the DNA mixture, but the recommended minimum DNA amount to effectively perform the analysis is 12.5 ng/μL.

The random mixed alfalfa RM DNA was used to confirm the efficiency and sensitivity of the alfalfa multiplex PCR method. The alfalfa multiplex PCR method was able to effectively identify the constituents of all random RM DNA mixtures (Fig. 3B). These results indicate that the multiplex PCR method is sufficient to identify all GM alfalfa single and stacked events, including the two GM alfalfa stack events (J101×J163 and KK179×J101) that are currently approved in South Korea.

Table 2. Information of feral alfalfa specimens examined in this study

No.	Specimen No.	No.	Specimen No.	No.	Specimen No.
S04	NIBRVP0000501595	S05	NIBRVP0000292518	S06	NIBRVP0000292523
S07	NIBRVP0000292517	S08	NIBRVP0000441696	S09	NIBRVP0000292533
S10	NIBRVP0000292513	S11	NIBRVP0000292514	S12	NIBRVP0000292516
S13	NIBRVP0000436383	S14	NIBRVP0000354456	S15	NIBRVP0000375266
S16	NIBRVP0000398052	S17	NIBRVP0000385357	S18	NIBRVP0000292529
S19	NIBRVP0000292525	S20	NIBRVP0000292524	S21	NIBRVP0000292522
S22	NIBRVP0000292521	S23	NIBRVP0000292520	S24	NIBRVP0000209546
S25	NIBRVP0000308143	S26	NIBRVP0000303892	S27	NIBRVP0000303891
S28	NIBRVP0000305367	S29	NIBRVP0000317390	S30	NIBRVP0000317395
S31	NIBRVP0000130307	S32	NIBRVP0000428425	S33	NIBRVP0000430227
S34	NIBRVP0000130306	S35	NIBRVP0000130305	S36	NIBRVP0000292527
S37	NIBRVP0000292520	S38	NIBRVP0000489731	S39	NIBRVP0000489213
S40	NIBRVP0000487003	S41	NIBRVP0000555041	S42	NIBRVP0000584727
S43	NIBRVP0000584033	S44	NIBRVP0000595744	S45	NIBRVP0000587621
S46	NIBRVP0000548652	S47	NIBRVP0000350272	S48	NIBRVP0000350674
S49	NIBRVP0000439717	S50	NIBRVP0000575558	S51	NIBRVP0000587695
S52	NIBRVP0000592403	S53	NIBRVP0000575852	S54	NIBRVP0000575853
S55	NIBRVP0000587027	S56	NIBRVP0000606411	S57	NIBRVP0000348540
S58	NIBRVP0000180475	S59	NIBRVP0000230317	S60	NIBRVP0000139629
S61	NIBRVP0000240944	S62	NIBRVP0000217170	S63	NIBRVP0000119898
S64	NIBRVP0000120825	S65	NIBRVP0000120826	S66	NIBRVP0000207750
S67	NIBRVP0000112397	S68	NIBRVP0000456918	S69	NIBRVP0000292526
S70	NIBRVP0000386967	S71	NIBRVP0000305407	S72	NIBRVP0000308144
S73	NIBRVP0000429882	S74	NIBRVP0000308142	S75	NIBRVP0000209991
S76	NIBRVP0000480382	S77	NIBRVP0000477472	S78	NIBRVP0000292519
S79	NIBRVP0000357281	S80	NIBRVP0000292515	S81	NIBRVP0000397803
S82	NIBRVP0000397448	S83	NIBRVP0000375414	S84	NIBRVP0000375030
S85	NIBRVP0000672868	S86	NIBRVP0000638956	S87	NIBRVP0000539399
S88	NIBRVP0000601290	S89	NIBRVP0000603483	S90	NIBRVP0000452750
S89	NIBRVP0000603483	S90	NIBRVP0000452750	S91	NIBRVP0000620900
S90	NIBRVP0000452750	S91	NIBRVP0000620900		

Application of the multiplex PCR

The multiplex PCR method for GMO identification has been applied for detection of GM volunteers, which were collected from GMO monitoring (Choi *et al*., 2018; Eum *et al*., 2019; Jo *et al*., 2016; Shin *et al*., 2016). To apply the multiplex PCR method for alfalfa sample analysis, we performed PCR with feral alfalfa specimens from NIBR in South Korea (Table 2). The 91 alfalfa specimens collected from 2000 to 2018 were analyzed using the newly developed alfalfa multiplex to identify unintentionally released GM alfalfa in the natural environment (Fig. 4). No GM alfalfa were detected using the multiplex PCR, and these results were confirmed by simplex PCR performed with each event-specific PCR primer (data not shown).

Discussion

Over 500 GM events, including stacked events, have been authorized worldwide (ISAAA, 2017). Implementation of management policy is typically based on the presence or absence of GM DNA or protein in tested samples. Because GM crop companies are continuously developing new GM-events, detection and identification methods for these events must be established in order for management to be successful. As the variety of commercially available GM crops has increased, the use of multiple GMO detection systems has become routine. A multiplex detection system using conventional PCR would be a powerful tool for the detection and quantification of transgenic elements.

Alfalfa has been used as livestock feed for decades because of its high forage quality. Alfalfa is also used for various non-agricultural purposes such as rehabilitation of rangelands, erosion control and reduction in forests and mined soils, and in revegetation of damaged land (Sullivan, 1992). In South Korea, alfalfa seed has been used for erosion protection on road cut slopes via seed spray for many years, possibly leading to unintentional release of GM alfalfa into the natural environment. To monitor the release of GM alfalfa into nature, it is necessary to establish time and cost effective detection methods. The GMO environmental monitoring program in the Ministry of Environment (MOE) and the National Institute of Ecology (NIE) in South Korea has searched for volunteers of GM maize, soybean, canola, and cotton since 2009 (Eum *et al*., 2019), and we have recently added alfalfa for GMO monitoring due to the steady increase in alfalfa imports and the increasing proliferation of wild alfalfa (NIE, 2018). Reliable detection methods for GM alfalfa will enable the identification of volunteers and the informed management of GMOs released in nature.

Event-specific detection methods for three GM alfal-

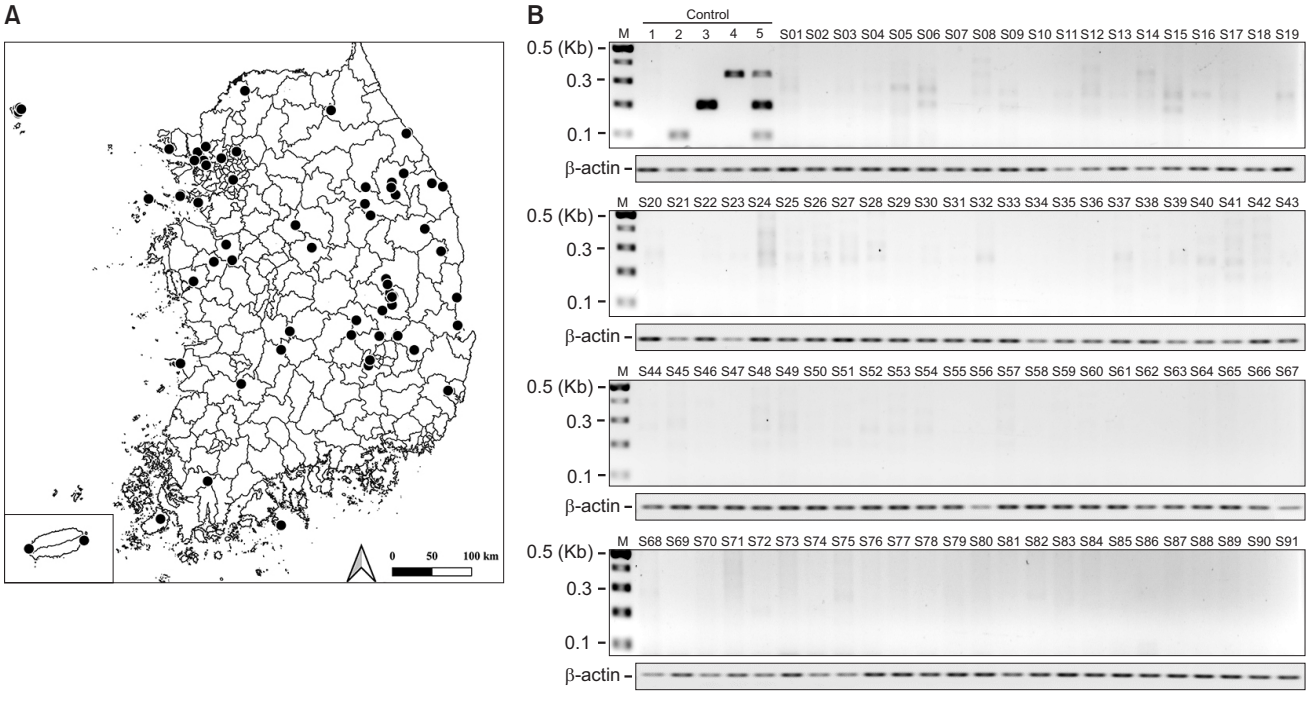

Figure 4. Application of the multiplex polymerase chain reaction (PCR) method for analysis of the 91 feral alfalfa specimens. (A) Locations of alfalfa sample collection sites. Filled circles exhibit the location of a feral alfalfa specimen collection site. (B) Analysis of multiplex PCR using 91 samples (lane 1, non-GMO; lane 2, J163; lane 3, J101; lane 4, KK179; lane 5, mixed three reference materials; S01-S91; number of specimen samples; M, 100 bp marker).

fas by real-time PCR have been developed (Guertler *et al*., 2019). The present study established specific qPCR detection methods for alfalfa events J101, J163, and KK179 and validated the methods according to the EU guidelines for GMO testing. For in-house validation, the research group performed LOD and robustness tests with thermal cyclers. An inter-laboratory comparison study was also performed by seven laboratories organized by the Federal Office of Consumer Protection and Food Safety (BVL). These methods would provide a powerful tool for the qualitative and quantitative detection of GM alfalfa, but many under-equipped laboratories in developing countries lack the expensive equipment and materials, including probes and reagents that are necessary for the application of this system. To overcome these limitations, we developed a fast and cost effective multiplex PCR method using event-specific primers. To successfully detect GM alfalfa using multiplex PCR, the size of the PCR products should be easily separated by gel electrophoresis. In this study, we designed specific primers for J163, J101, and KK179 (Table 1). J101 and J163 were developed with the same transgene cassette to exhibit herbicide resistance (Fig. 1), and primers specific to the transgene and plant genome were therefore necessary to identify J101 and J163. Moreover, to reduce primer interference in the multiplex PCR, we applied the same transgene binding primer (J163-F and J101-F) for J101 and J163 (Table 1). The amplicon length of PCR for each amplification is crucial for the successful development of the multiplex PCR method (Mathuoka *et al*., 2001). We performed the event-specific multiplex PCR using primers for 98 bp (J163), 202 bp (J101), and 347 bp (KK179) to separate PCR products in 2.5% agarose gel (Fig. 2). The PCR amplification yielded variable sizes of separation in the agarose gel without the long run time of electrophoresis. These results indicate that the newly developed multiplex PCR method is suitable for identifying the three GM alfalfas in one reaction, possibly reducing the time and cost necessary to perform the analysis.

To evaluate the sensitivity of the multiplex PCR method, we conducted an LOD assay and random RM DNA mixture analysis. Serially diluted alfalfa gDNA mixtures (100–0.2 ng/μL) were used for multiplex PCR, and the amplification strength was decreased by DNA concentration dependently. The minimum concentration at which the multiplex PCR band was detectable was 12.5 ng/μL, but we recommended that 12.5 ng/μL should be used for qualitative analysis. The majority of GM volunteers detected by GMO monitoring have been homozygotes or heterozygotes, indicating that the absolute quantity of genomic DNA is critical for GMO identification (in press). In a comparison of single event and stacked event PCR (Fig. 3B), the amplification strength of single event PCR was greater than that of three stacked event PCR. These results indicate that the minimum concentration of genomic DNA from volunteer plants needed to detect GM alfalfa.

Feral alfalfa is commonly observed on roadsides and natural habitats from East Asia to Europe, and in South Africa, Australia, and North and South America (Michaud *et al*., 1988). In South Korea, alfalfa plants and their seeds are used for forage and to prevent soil erosion of cut slopes. Because of low self-sufficiency of alfalfa, the majority of alfalfa plants and seeds are imported. Moreover, according to the Act on Transboundary Movement of GMOs in South Korea, a 3% labeling threshold for GM seeds could be allowed for crop trade. For these reasons, there is a high likelihood of the release of GM alfalfa seed into the natural environment. To monitor the unintentional release of GM alfalfa to the natural environment, it is necessary to establish a detection system for all GM alfalfas currently approved in South Korea. In this study, we used 91 feral alfalfa specimens collected from natural habitats between 2000 and 2018 to screen for unintentionally released GM alfalfa. The feral alfalfa samples were collected from natural environmental sites nearby open area and roadside in the Korean peninsula, including Baengnyeong Island and Jeju Island (Fig. 4A). The results of our feral alfalfa specimen analysis by alfalfa multiplex PCR indicated that no GM alfalfa has yet been released in South Korea (Fig. 4B). However, there is still an increased risk of environmental release of GM alfalfa, and the management of GM alfalfa seeds must be enforced. In conclusion, our newly developed GM alfalfa detection method using conventional multiplex PCR is suitable for single and stack event analysis and applicable for the analysis and identification of GM events in feral alfalfa.

Author Contributions

JRL and WC conceived of and designed the experiments. IRK and HSL performed all experiments and collected the plant samples. WC and IRK wrote the paper. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgments

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