J. Korean Soc. Appl. Biol. Chem. **53**(5), 639-646 (2010)

Article

Development of Detection System Using Multiplex PCR and Liquid Beadarray for Stacked Genetically Modified Rice Event (LS28×Cry1Ac)

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Received April 27, 2010; Accepted June 9, 2010

A multiplex system was developed to assess detection of stacked genetically modified (GM) rice (LS28 × Cry1Ac) based on multiplex polymerase chain reaction (PCR) and liquid beadarray, and the accuracy of the system was analyzed. Standard and specific bulging specific (SBS) primers with standard primers were used to simultaneously detect multiple targets in stacked events of rice. Five sets of primers for the stacked events were applied to amplify their targets, and were separated distinctly in agarose gel. A liquid beadarray assay for the stacked GM rice was performed using the multiplex PCR products, followed by target biotinylation and hybridization between biotinylated-tagged target and anti-tagged bead. Fluorescent signals of the hybridized target sequences were detected by the Luminex system. The signaling patterns were analyzed by their mean fluorescent intensity (MFI) value. Results showed that liquid beadarrays with standard and SBS primers were in complete agreement with the PCR data, and detection of the different target elements was found to be very specific with no cross reaction among samples. Therefore, our detection system developed for stacked GM crop using multiplex PCR and liquid beadarray can be a useful and efficient system for screening and analyzing multiple transgenes in a single tube for qualitative analysis.

Key words: detection, liquid beadarray, multiplex PCR, SBS primer, stacked GM crop

Stacked genetically modified organisms (GMOs) are those containing more than one gene genetically engineered into a crop [Akiyama *et al*., 2005]. There is a considerable difference between genetically modified (GM) hybrid and stacked GMO. Stacked GM crops have been created by conventional cross-breeding of parental GM lines to impart multiple practical traits by gene stacking or pyramiding, whereas GM hybrids originate from crossing of a parental GM line and a non-GM inbred line [De Schrijver *et al*., 2007]. Stacked GM crops such as maize, cotton, and canola are becoming an increasing part of GM crops due to their easier and faster breeding practice as well as preference by farmers for cultivation. A number of new stacked GM events as well as single events have been authorized for use as food and feed [Halpin, 2005; Taverniers, 2008]. Several stacked GM crops including ten cotton events, two soybean events, four rapeseed events, and over twenty maize events are available or under steps for final evaluation of risk assessment for commercialization [www.gmo-compass.org]. The continuing increase of GMO events to be detected and identified has become a challenge faced by many major GMO-importing countries including Korea. Effective detection methods for stacked GM crops may be recommended strongly before their regulatory approval including risk assessment of the crop. Unfortunately, information is very limited as to how stacked GM events should be assessed and detected [De Schrijver *et al*., 2007]. To assess stacked GM crops, sensitive and specific detection tools have to be developed and validated for risk management as well as identification of each GM events.

Microarray or PCR assays, which are presently preferred, have been used mainly as a tool for GMO detection to identify introduced transgenes, due to their

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doi:10.3839/jksabc.2010.097

sensitivity and specificity [Xu *et al*., 2007; Holst-Jensen, 2009]. These tools focus on the amplification of the event specific sequence in the GMO [Taverniers *et al*., 2005; Hamels *et al*., 2009]. However, for stacked GMOs, little information is available on their detection or identification approach.

To screen introduced foreign genes specific for their corresponding crop events, many researchers have used PCR-based methods, which could amplify target regions in the transgenic construct. Such tests have been based mainly on screening target elements such as the cauliflower mosaic virus 35S promoter (P35S), *Agrobacterium tumefaciens* 3' nos terminator (t-NOS), and the left and right borders. Therefore, multiplex PCR has been applied especially for simultaneous detection of introduced genes in various crop events [Matsuoka *et al*., 2001; Onishi *et al*., 2005; Schmidt *et al*., 2008; Shrestha *et al*., 2008; Kim *et al*., 2009]. Multiplex PCR developed by Chamberlain *et al.* [1988] has been used for the detection of point mutations, single nucleotide polymorphisms (SNPs), large deletions, and quantitative analysis of gene expression in disease diagnosis. Several studies have used multiplex PCR for detection of GM maize, canola, and rice; however, these references used multiple GM events with their corresponding event-specific primers in mixed GM samples such as grains or processed material for food or feed [Vollenhofer *et al*., 1999; Chiueh *et al*., 2002; Kim *et al*., 2007; Akiyama *et al*., 2008; Quirasco *et al*., 2008]. An approach to detect stacked GM rice carrying lepidopteran insect and rice blast resistances in a rice cultivar was also reported [Shin *et al*., 2009].

In the present report, we demonstrated a detection system for stacked GM rice event based on multiplex PCR using specific SBS primers [Kang *et al*., 2008]. These primers were approximately 30-40 nucleotides in length, and comprised three regions: a 12-16-mer 5'-oligonucleotide, a homopolymer of four adenosines, and a 15-20-mer 3'-oligonucleotide. The sequence of the SBS primer was complementary to its target sequence with the exception of the four adenines, which created a bulge over two nucleotides in the template DNA. The key feature of the SBS primer was that the central bulging region structurally separated the 3'-and 5'-end segments. Because the four consecutive adenosines could correspond to any two nucleotides in the target sequence, a solid bulge structure is formed at the center of the SBS primer when it is fully annealed to the target sequence. Due to this central bulge region, the SBS primers have the advantage of a broad range of annealing temperatures as well as primer specificity in multiplex PCR. Therefore, we evaluated the result from multiplex PCR with SBS primers and standard primers.

In the present study, a liquid beadarray assay was used for detecting stacked genes in GM rice event. Liquid beadarray, also referred to as suspension or fluid microbead array, has been known for its precise analysis due to effective elimination of the optical error of the researchers [Wilson *et al*., 2005; Dunbar, 2006]. Compared to microarray, liquid beadarray has been reported as a more effective tool due to its hybridization in solution and a strong signal intensity, as well as high sensitivity [Oh *et al*., 2009]. However, this method has not yet been utilized for GMO detection. Thus, in the present study, liquid bead array was challenged to detect stacked GM rice event.

The objective of the present study was to validate multiplex PCR system as well as liquid beadarray for detection of stacked GM event. We introduced a multiplex amplification method using SBS and standard combination primers to detect five target genes in a single tube simultaneously and developed a multiplex PCR-coupled liquid beadarray assay system. This approach could combine the specificity and sensitivity of the PCR assay with the multiplexed and high throughput detection capabilities of the Luminex system.

Materials and Methods

Stacked GM rice event. *Oryza sativa* L. *japonica* cv. Nagdong was used as a non-GM control plant. Two individual lines from stacked GM rice (LS28×Cry1Ac) event were used. These stacked events were progenies by a crossing between LS28 transgenic rice as the maternal parent and Cry1Ac transgenic rice as the paternal parent. The LS28 event, as GM rice showing rice blast resistance, was transformed with *OsCK1* (choline kinase) [Shin *et al*., 2009]. The Cry1Ac event, as GM rice showing lepidopteran pest-resistance, was a transgenic rice with one of insecticidal toxin genes from *Bacillus thuringiensis* [Cheng *et al*., 1998; Lee *et al*., 2009]. All these events were kindly provided by National Academy of Agricultural Science (Rural Development Administration, Suwon, Korea).

Genomic DNA extraction from rice event. Fresh leaf samples (500 mg) were ground in liquid nitrogen using a mortar and pestle, and then treated with the DNeasy Plant Kit (Qiagen, Hilden, Germany). Isolated genomic DNA from various rice events were resuspended in triple-distilled water and quantified by a UV spectrophotometer. Appropriate concentration of each DNA was determined and applied to PCR.

Primer design for simplex or multiplex PCR. Primers for multiplex PCR as well as simplex PCR were designed according to the nucleotide sequence database

NCBI (National Institutes of Health, Bethesda, MD). The nucleotide sequences of the primer sets are shown in Table 1. For detecting a rice endogenous gene as an internal positive control, sucrose phosphate synthase (SPS) gene was chosen and its nucleotide sequences were used for primer design [Ding *et al*., 2004]. Transformation construct-specific primers were designed based on the P35S and t-NOS nucleotide sequences. The stacked genes of the rice events were Cry1Ac and LS28; their detection primers were designed from the respective nucleotide sequences in the NCBI database. The GenBank accession numbers of nucleotide sequences for primer design are presented in Table 1.

Multiplex PCR amplification. Each simplex PCR was carried out before multiplex PCR to verify the proper amplification with the respective primers. The conditions of each PCR component for simplex or multiplex PCR were optimized. Genomic DNA from rice samples was used as the template for the PCR. The PCR conditions for all primer sets were optimized. The reaction mixture for PCR contained 25 μL of 2×Max Taq Hot start master mix (BioQuest, Seoul, Korea), 10 pmole of each primer pairs, 20 ng of template, and distilled water at a final volume of 50 μL. PCR was performed in a thermal cycler (MyCycler, BioRad, Hercules, CA). Pre-treatment was executed at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min 30 s. Further elongation was performed at 72°C for 10 min. PCR amplicons were separated in 2.5% MetaPhor agarose gel (CAMBREX, East Rutherford, NJ) at 100 V for 30 min and then visualized by ethidium bromide staining. As a reference, a 100-bp DNA ladder (Fermentas, Burlington, Ontario) was used for size comparison.

Primer design of target-specific primer extension (TSPE) with a probe and biotin labeling. After multiplex amplification with each primer pair, primer extension of the amplicons was performed using a probe that was complementary to sequences of the PCR amplicons, with 24-mer, 5' end-tagged sequences (Table 2). Five probe sequences corresponding to the five detected PCR amplicons (SPS, P35S, t-NOS, Cry1Ac, and LS28) are presented in Table 2. For labeling, biotinylated dCTP was used in the PCR mixture. PCR mixture contained 0.5 μM of each probe, 50 μM of each dATP, dGTP, and dTTP, 20 μM dCTP, 20 μM biotin-dCTP (Invitrogen), 75 mM Tris HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 1 U *Taq* polymerase (Ultratools, Barcelona, Spain), and a 5-μL aliquot of PCR product in a total volume of 20 μL for the linear amplification. Pretreatment at 94°C for 5 min, and 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min were performed, followed by further elongation at 72°C for 5 min. Single-stranded biotinylated targets called TSPE products were obtained for further hybridization.

Bead preparation by coupling. Tag sequences incorporated at the 5' end of the corresponding probe could bind to the complementary 24-mer anti-tag sequences of microsphere beads (FlexMAP beads, Tm Bioscience, Toronto, Ontario) during hybridization. Anti-tag having amine residue was attached to carboxylated microsphere beads having a unique spectral address, by covalent linkage called the coupling process. Coupling was carried out as follows. Ethylcarbodiimide hydrochloride (EDC) (25 μg) was added to a mixture of 0.2 nmole anti-tag and 5×10^5 beads in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 4.5) and incubated in the dark with agitation at room temperature for 30 min. After addition of 25 μg EDC, the mixture was incubated under the same conditions as mentioned above. The mixture containing beads was rinsed with 1 mL of 0.02% Tween-20 by vortexing, and centrifuged at 12,000×g for 1 min. The supernatant was discarded, and the beads were resuspended in 1 mL of 0.1% sodium dodecyl sulfate (SDS). After one additional centrifugation step under the same conditions, the anti-tagged beads were stored in 200 μL TE (pH 8.0) in the dark at 4°C.

Hybridization between TSPE product and anti-tagged beads. TSPE products (20 μL) originally amplified from each genomic DNA were hybridized with the corresponding anti-tagged beads. About 1,000 beads per reaction were used; thus, approximately 5,000 beads were present in the bead mixture. After adjusting the total volume to 50 μL with dH_2O, denaturation at 95°C for 5 min was done on the PCR machine. After boiling, hybridization was done at 37°C for 30 min on the PCR machine. Hybridized samples were transferred into each well of 96-well, 2-micron filter plates (Millipore, Bedford, Ohio). The hybridized sample in each well was washed using a vacuum manifold, and the sample was resuspended in 1×TM buffer (0.2 M NaCl, 0.1 M Tris, 0.16% Triton X-100, pH 8.0). This washing step was performed twice. Washed beads in the filter plates were resuspended in 1×TM buffer containing 2 μg/mL streptavidin-R-phycoerythrin (Sigma-Aldrich, St. Louis, MO), and were incubated at room temperature for 15 min in the dark with agitation.

Luminex operation and signal analysis. Fluorescence emitted from microspheres was measured using a Luminex 200 cytometer processor (Luminex Corp., Austin, TX). Following incubation, the beads were passed through a fine column, which were then scanned by two lasers to classify each bead showing unique spectral signal, and to quantify the hybridized target by detecting the phycoerythrin

signal. MasterPlex CT (MiraiBio, San Francisco, CA) software was used to operate the Luminex and to analyze the data. MFI values from the samples in 96-well plate were read and analyzed.

Results and Discussion

Multiplex PCR optimization. Each primer was tested with multiplex PCR as well as simplex PCR to analyze the specificity and sensitivity. Primers used in the present study were five sets which could detect genus specific gene (SPS), transformational construct specific genes (P35S, t-NOS), and introduce transgenes (Cry1Ac and LS28). As an internal positive control, the SPS gene which is taxon-specific was selected. SPS PCR products from Cry1Ac transgenic rice, LS28 transgenic rice, and two stacked lines of LS28×Cry1Ac rice, as well as non-GM rice 'Nagdong', were all amplified with the predicted amplicon sizes (Fig 1B; Table 1). Detection primers with the optimized quantity of 10 pmole, were used in the PCR for transformational construct-specific genes such as P35S and t-NOS. Simplex PCR products treated with these primer pairs were observed only in transgenic rice samples (Fig. 1A). Samples from the negative control and non-GM rice did not react with these primer pairs. The P35S::bar construct-specific detection method originally developed by Bayer CropScience (Monheim am Rhein, Germany) to detect LL rice event series (Querci *et al.*, 2009), was applied to detect rice events in the present study. As the elements assayed are common to many

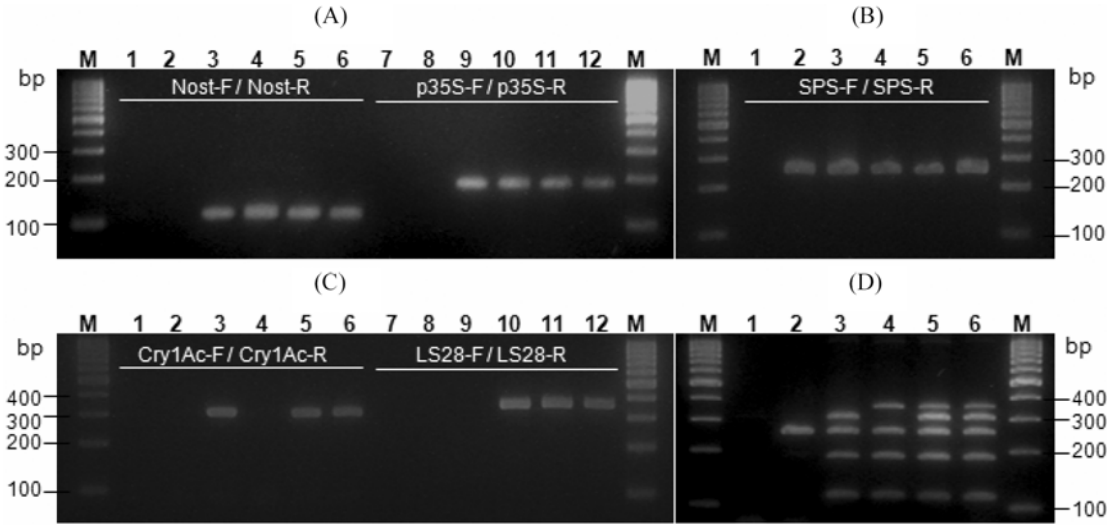

Fig. 1. PCR products amplified from various rice samples with construct-specific or gene-specific primers. Simplex PCR results obtained using construct-specific primers (A) internal gene-specific primers, (B) transgene-specific primers, (C) multiplex PCR result obtained using five primer sets, (D) M, 100 bp DNA ladder; lanes 1 and 7, dH_2O; lanes 2 and 8, non-GM rice; lanes 3 and 9, Cry1Ac rice; lanes 4 and 10, LS28 rice; lanes 5 and 11, LS28×Cry1Ac-stacked GM rice line 1; lanes 6 and 12, LS28×Cry1Ac-stacked GM rice line 2.

Table 1. List of primers for multiplex PCR detection of stacked GM rice

Primer name	Sequences (5' to 3')	Target	Amplicon (bp)	Specificity	Reference Genbank No.
Nost-F	CTGTTGCCGGTCTTGCGATG	t-NOS	185	Standard	FJ905223
Nost-R	GCGCGATAATTTATCCTAGTTTG				
p35-F	GACAGTGGTCCCAAAGATGGAC	CaMV P35S	115	Standard	AB303064
p35-R	CCCTTACGTCAGTGGAGATATC				
SPS-F	AGCAACAGTCCAGT*AAAA*AGAGAGCCCCGAAC	SPS	251	SBS formed	U33175
SPS-R	GAGAGGAAAGGG*AAAA*AAGCGTCACGTACCA				
Cry1Ac-F	CAGATCATGGCCTC*AAAA*CAGTTGGATTCTCCGG	Cry1Ac	311	SBS formed	AY126450
Cry1Ac-R	GGCACATTGTTGTT*AAAA*GTGGTGGGATTTCGT				
LS28-F	GACCGACTGAAAAACT*AAAA*CTCAAAACTGCAAGGA	Choline kinase	354	SBS formed	AY256847
LS28-R	ACAAACGCTTCTGC*AAAA*ATCAGTGTCTGGATATT				

Italicized and underlined letters indicate the added adenosine residues to create bulge between two regions in the primer.

Table 2. Probes for multiplex detection using liquid beadarrays of stacked GM rice. The tag and anti-tag sequences are patented by Luminex™

Probes	Tag sequences (appended to primer) (5' to 3')	Primer sequences after the tag	Tag and anti-tag set no.
T- t-NOS	TCAACAATCTTTTACAATCAAATC	TGATTAGAGTCCCGCAAT	6
T-P35S	CTTTAATCTCAATCAATACAAATC	AATCCCACTATCCTTCGC	1
T- SPS	TCAAAATCTCAAATACTCAAATCA	GCCACGGACTCCTCTAAT	18
T-Cry1Ac	CTTTTACAATACTTCAATACAATC	AGGTAAACTCAGGTCCGG	20
T-LS28	CTACAAACAAACAAACATTATCAA	TCAATCATGATGTTGCCA	28

events, and some GMO do not bear these elements, it may not be appropriate for specific GMO identification. However, it is still efficient for screening or detecting GMOs in single or mixed samples as seen in this result.

Cry1Ac is an insecticidal protein showing toxicity in insect midgut upon absorption, and may cause synergistic target effects or cross resistance to receptor plants. To detect the insecticidal transgene of Cry1Ac in various rice samples, a pair of primers was tested. This primer pair could discriminately amplify Cry1Ac in both the single Cry1Ac event and the LS28×Cry1Ac-stacked events (Fig. 1C). For rice blast-resistant LS28 transgenic rice, detection primers were synthesized and applied to various rice samples (Fig. 1C). The LS28 gene was detected successfully in the LS28 single event and in the LS28× Cry1Ac-stacked events with the predicted amplicon size. TA cloning of the amplified PCR products from simplex PCR (Fig. 1A, 1B, and 1C) was carried out to confirm the nucleotide sequences. Each PCR amplicon from the positive sample was ligated with T-vector and propagated by cloning, and the inserted sequences were determined. All sequences analyzed from these clones were found to contain the expected corresponding sequences based on the primer design (data not shown).

The present study confirmed that each detection primer pair could efficiently amplify the corresponding gene. Multiplex PCR was carried out with various GM rice samples including non-GM rice as templates to determine whether all of the different genes could be simultaneously detected in a single reaction tube. When a mixture of five different kinds of primer sets was used in the PCR, non-specific bands were not present at the annealing temperature (60) under the optimized PCR conditions (Fig. 1D). Except for the negative control, the SPS internal gene was amplified in all the rice samples with a size of 251 bp (Fig. 1D), whereas P35S or t-NOS was amplified in all rice event samples, except for non-GM rice Nagdong. Transgene-specific primers for Cry1Ac could amplify the target of the Cry1Ac rice event (Fig. 1D, lane 3), but not of the LS28 transgenic rice event (Fig. 1D, lane 4). LS28-specific primers amplified distinct target gene in the LS28 transgenic rice (Fig. 1D, lane 4), but not in Cry1Ac rice event (Fig. 1D). Two individual lines from the LS28× Cry1Ac-stacked rice event showed all target genes of Cry1Ac, LS28, P35S, t-NOS, and SPS separately as well as simultaneously. The intensity of individual bands in the same reaction by multiplex PCR appeared to be lower than those of the simplex PCR, except for the amplicon of the SPS gene (Fig. 1D, lane 2). Relatively thinner band in multiplex PCR could be explained by the PCR efficiency. When PCR products are amplified together, they could also compete for the PCR reagents as well as the template. An increased number of binding primer pairs could result in lower binding efficiency. However, all bands were distinctively amplified to be seen in each lane regardless of the size. Even the smallest (115 bp t-NOS) could be detected as a distinctive band in multiplex PCR (Fig. 1D). Thus, the product ratio problem in multiplex PCR and the experimental limitations of small amplicon size could be overcome by using combined SBS and standard primers.

Consequently, all amplicons from the multiplex PCR under the optimized condition showed that they were distinguished from each other. Moreover, we found that the result of multiplex PCR was in complete agreement with that of individual simplex PCR. A previous study by Hamels *et al*. [2009] reported that gel analysis for multiplex PCR was limited due to either non-specific amplification, which could interfere with gel analysis, or the inability to discriminate the slight differences in the length of amplicons. Contrary to the report of Hamels *et al*. [2009], we were able to demonstrate that multiplex PCR detection using a combination of standard and SBS primers is an adequate tool for producing multiple targets in various regions of the genomic DNA extracted from events. Therefore, multiplex PCR evaluated in the present study seemed to be an efficient tool for detecting combined stacking traits in GM rice events, and it could be applied to the detection of other stacked GM crop events containing the same element detected here.

Liquid beadarray assay for multiplex detection. To analyze multiplex amplification using fluorescent beadarray,

Table 3. Mean value of MFI units expressed from multiplex liquid beadarrays of stacked GM rice

	t-NOS	P35S	SPS	Cry1Ac	LS28
Negative	29	44	23	24	20
non-GM rice	61	58	5124	17	55
Cry1Ac rice	2299	3541	4298	1685	45
LS28 rice	2605	4594	3799	15	1308
LS28×Cry1Ac line1	3055	6201	3659	1927	1567
LS28×Cry1Ac line2	2596	5408	3772	1559	1495

Left column represents rice event samples, and first row, the names of the detected genes. Each value represents the mean value calculated from repeats.

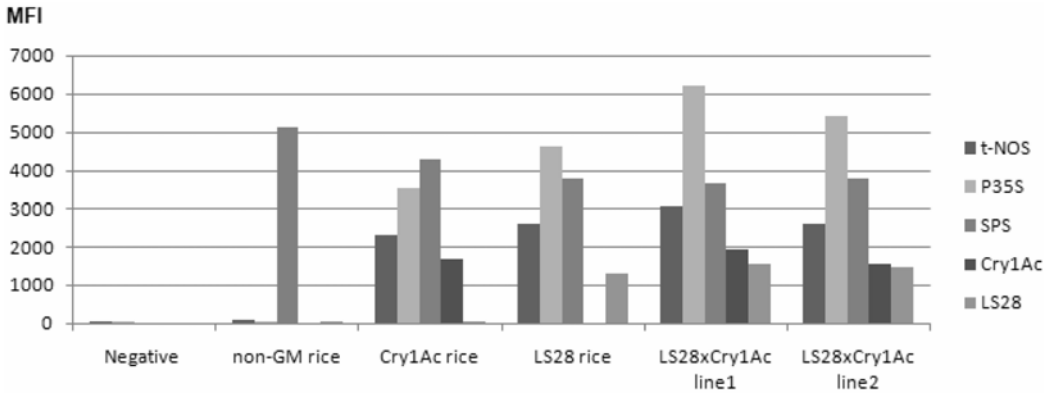

Fig. 2. Multiplex liquid microarray using target gene-specific probes based on multiplex PCR products. MFI indicates the extent of the hybridized reaction between bead and TSPE product.

MFI from hybridized bead samples were read and analyzed. The beadarray experiments subsequent to multiplex PCR were repeated three times to confirm that the pattern of MFI values is reproducible. MFI units were detected and counted for the streptavidin phycoerythrin signal on fluorescent beads, and the mean value was calculated based on the results of repeated tests (Table 3). The cut-off value of MFI was set as 150 for minimal signal intensity. A total of five different beadarrays capturing corresponding amplicons from multiplex PCR products were used in this assay.

The background signals for all beads were lower than the cut-off (MFI 150), whereas the positive signals were in the range of 1,308 MFI units (between LS28 beads and LS28 rice) to 6,201 MFI units (between P35S beads and LS28×Cry1Ac rice) (Table 3). According to Mahony *et al*. [2007], low background levels on all of the remaining beads indicate the absence of cross-hybridization talk or signal interference on other beads. The negative control (added with distilled water instead of DNA template) did not produce significant signals in the hybridized bead reaction (Table 3 and Fig. 2). Samples from non-GM rice showed high signals only for the SPS endogenous gene. Samples from the Cry1Ac event showed positive signals over 1,600 MFI in four corresponding genes except for the LS28 gene, and the LS28 rice sample showed positive signals in four genes except for the Cry1Ac gene. Two independent lines of the LS28×Cry1Ac rice event showed similar patterns of fluorescent signaling, with five different kinds of genes showing distinct signals in the hybridized reaction (Table 3 and Fig. 2). Interestingly, the P35S signals were recorded to be much higher than the other signals in some reactions (Table 2). The t-NOS signals were lower than P35S signals in all cases, although they were simultaneously present in the tissue sample. As they were construct-specific genes for the expression of the selection marker bar in transgenic rice, stacked rice events have twice the amount of these elements, because stacked events in the present study have the same genetic element of P35S and t-NOS. However, signals from t-NOS did not appear to be equally expressed as that from P35S. This phenomenon might be due to the difference between primer affinities or the differences in the quantity of the first PCR products. The MFI value represents the presence of a gene. This value could not be explained fully quantitatively, because MFI values came from the final end point products. However, these results could be used for semi-quantitative analysis only with the controls, whose the absolute copy numbers of genes are known. Thus, they

can be compared to each other relatively, and it is possible to determine the relative amount from the samples.

The pattern from the liquid beadarray assay in two stacked events appeared to be very similar to that of multiplex PCR. This result showed that multiple targets contained in a rice event could be amplified, differentiated, and even identified properly in a single liquid reaction according to the high specificity of hybridization between tag and anti-tag.

The advantage of the liquid beadarray assay is that PCR products can be detected by their signal even with minute amount of PCR products, which cannot be detected easily in gel-based analyses. Our results from liquid beadarray were categorized also in post-PCR identification using amplicons for the microarray detection system by GMOchips that was developed for GMO screening [Leimanis *et al*., 2006]. Since Leimanis *et al*. [2006] introduced the GMOchip and succeeded in making the technology of microarray work in tandem with PCR, we also could see the possibility of using liquid beadarray for GMO detection. The specificity for the detection of the different GM elements relies on the fact that the method exploits two independent specific steps: PCR-specific assay conducted with specific primers and hybridization of the PCR products with specific probes [Hamels *et al*., 2009]. However, when the amplified solution is transferred to plates, this step may lead to contamination that can result in a false positive. As this can be a limitation of the method compared to the one-step detection method such as real-time PCR, dealing with samples in liquid beadarrays requires extra care and caution. As for the multiplex system, real-time PCR generally can simultaneously assess four targets at maximum, including the positive control in one tube; however, the liquid beadarray theoretically can increase the numbers of targets to one hundred [Wilson *et al*., 2005].

For efficient multiplex PCR detection of stacked GM crops, improvements in the number of target genes, the specificity of the primers, the prevention of primer dimerization, as well as optimization of the PCR conditions need to be considered. Furthermore, researchers need to discriminate between combined presence of two or more single trait GMOs and stacked GMOs [Akiyama *et al*., 2005; Xu *et al*., 2009]. Thus, elaborate methods equipped with multiplexing and quantification, but which are still specific for diverse objectives, should be developed in the future.

We showed that the multiplex PCR detection system used in the present study under optimized condition could be an appropriate tool, without primer interfer ence or dimerization, for the detection of stacked GM rice events. Combining multiplex PCR and the liquid beadarray assay for assessment of stacked GM rice allowed a simultaneous detection of multiple targets in a single reaction with high specificity. Here, we present results that will be useful for GM crop assessment in detecting multiple targets simultaneously with multiplex PCR-based methods for various purposes, such as a part of the approval process or post-market monitoring.

Acknowledgment. Authors thank the National Academy of Agricultural Science for providing GM rice material. This research was supported by the Biogreen 21 project (20080401034032008020) from the RDA in Korea.

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