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Detection of Recombinant DNA Segments Introduced to Genetically Modified Maize (*Zea mays*)

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Polymerase Chain Reaction (PCR) techniques are increasingly used for the detection of genetically modified (GM) crops in foods. In this paper, recombinant DNAs introduced into the seven lines of GM maize, such as Event 176, Bt11, T25, MON810, GA21, DLL25, and MON802, are sequenced. On the basis of the obtained sequence, 14 primer pairs for the detection of the segments, such as promoter, terminator regions, and construct genes, were designed. To confirm the specificities of the designed primer pairs, PCR was performed on genomic DNAs extracted from GM and non-GM maize, GM and non-GM soy, and other cereal crops. Because the presence of the corresponding DNA segments was specifically detected in GM crops by the designed primer pairs, it was concluded that this method is useful for fast and easy screening of GM crops including unauthorized ones.

KEYWORDS: ***Zea mays*; genetically modified; PCR; detection technique; unauthorized GM crops**

INTRODUCTION

Recently, numerous advances have been made in the development of new varieties of crop plants including soy, maize, rapeseed, cotton, and potatoes, and many of the world's governments have authorized the marketing of genetically modified organisms (GMOs) as food or feed. The general public, however, has shown anxiety over this new technology. Pressure from consumer groups and public demand has led several countries, including Japan, to require labeling for the presence of GMOs in foods. In conjunction with these labeling policies, the Polymerase Chain Reaction (PCR) method is one of the most widely used systems for identifying the presence of GMOs.

We have previously reported PCR methods for the detection of r-DNAs in Roundup Ready soybeans, five lines of genetically modified (GM) maize, and the Sunup papaya (*1*−*3*). These methods were specific to the individual GM lines and were not applicable to the detection of r-DNAs in other GM crops. In the present study, we examined a DNA segment specific screening system for GM maize and other GM crops including unauthorized ones. Many foreign DNA segments including construct genes, promoter and terminator regions, and intron sequences are introduced to confer new traits to crops. Therefore, we here designed 14 primer pairs for the detection of the individual segments derived from exogenous organisms and evaluated the specificity of their detection to each of the introduced DNA segments. The segments concerned in this paper are as follows: the coding regions of the genes for phosphonothricin acetyl transferase (*pat* or *bar*), CP-4 enolpyruvylshikimate-3-phosphate synthase (*cp4-epsps*), maize enolpyruvylshikimate-3-phosphate synthase (*m-epsps*), glyphosate oxidoreductase (*gox*), insecticidal CryIA(b) protein [*cryIA(b)*] derived from *Bacillus thuringiensis*, and neomycin phosphotransferase II (*nptII*); the promoter region of rice actin (Pr-act); the 35S promoter and 35S terminator regions derived from the cauliflower mosaic virus (P-35S and T-35S, respectively); and the terminator regions of nopaline synthase (NOS-ter) derived from *Agrobacterium tumefaciens*.

MATERIALS AND METHODS

Maize and Other Cereal Samples. Seeds of five lines of GM maize, namely, the progenies of insect-resistant Bt11 and Event 176 developed by Novartis Seeds Inc. (Greensboro, NC), insect-resistant MON810 and glyphosate-tolerant GA21 developed by Monsanto Co. (St. Louis, MO), and glufosinate-tolerant T25 developed by Aventis CropScience (Lyon, France), were directly imported from the United States. A ground sample of insect-resistant and glyphosate-tolerant MON802 and genomic DNA extracted from glufosinate-tolerant DLL25 were kindly provided by Monsanto Co. As a conventional nongenetically modified (non-GM) maize, Hybrid 1412 dried seeds (Dairyland Seed Co., West Bend, WI; directly imported from the United States) were used. Seeds of glyphosate-tolerant Roundup Ready (RR) soy developed by Monsanto Co. were directly imported from the United States. As a

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conventional non-GM soy, dried grain produced in the U.S. state of Ohio in 1998 was directly imported from the United States. The rice (*Oryza sativa*) variety Kinuhikari, the wheat (*Triticum aestivum*) variety Haruyutaka, and the barley (*Hordeum vulgare*) variety Harrington were used to study the specificity of the designed primer pairs.

Extraction of Genomic DNA. Seeds were ground in an electric mill (Yu Chi Machinery Co., Ltd.,Chang Hua, Taiwan, model DM-6). Ground samples (500 mg) were taken for DNA extraction. Genomic DNA was extracted using the DNeasy Plant Maxi kit (Qiagen) according to the manufacturer's instructions with slight modifications. The incubation time was extended from 10 min to 1 h after the addition of buffer AP1 and RNase A solution to ground samples in the first extraction step, and genomic DNA was eluted with water from a silica spin column. The DNA concentration of solutions was determined by measuring the UV absorption at 260 nm. The purity of the extracted DNA was evaluated by agarose gel electrophoresis using UV absorption ratios of 260/280 nm and 260/230 nm; in the majority of maize lines studied, the absorption ratio at 260/230 nm was >1.7, and that at 260/280 nm was between 1.7 and 2.0. These DNA samples were used for the subsequent PCR analysis.

Oligonucleotide Primers. Oligonucleotide primers were synthesized by Greiner Japan Co., Ltd. (Tokyo, Japan), purified on a reversed-phase column, then diluted with an appropriate volume of water to a final concentration of 50 μmol/L, and stored at −20 °C until use.

To confirm the feasibility of PCR amplification of the extracted DNA, the following five primer pairs were synthesized for maize, soy, rice, wheat, and barley, respectively: ZE01/ZE02 (ZE01, TGC TTG CAT TGT TCG CTC TCC TAG; ZE02, GTC GCA GTG ACA TTG TGG CAT), Le1 01-5′/Le1 01-3′(Le1 01-5′, GGC TGA TAA CAC ACT CTA TTA TTG T; Le1 01-3′, TGA TGG ATC TGA TAG AAT TGA CGT T), rAct pro 2-5′/rAct pro 1-3′ (**Table 2n**), Gli1-5′/Gli1-3′ (Gli1-5′, GCC ACA AGA GCA AGT TCC ATT GGT A; Gli1-3′, AAC TTG TGA GCT TGC ATG CGC TAT, 403 bp), and HvLD1-5′/HvLD1-3′(HvLD1-5′, GTG TGC TTC TTT GAT GGT CCA G; HvLD1-3′, TGC GCC TTA GTT GGA TGA TAC A, 149 bp). The primer pairs ZE01/ZE02 and Le1 01-5′/Le1 01-3′ were previously reported (*1*, *4*). The primer pair rAct pro 2-5′/rAct pro 1-3′ was designed as McElroy reported (*5*) (GenBank Accession No. S44221) with minor modifications. The primer pairs Gli1-5′/Gli1-3′ and HvLD1-5′/HvLD1-3′ were designed by referring to GenBank Accession No. U51303 and AF022725, respectively.

The expression cassettes used to insert the trait gene into Event 176, Bt11, T25, MON810, GA21, DLL25, and MON802 are shown in **Figure 1**. To examine the DNA sequences inserted into Bt11, Event 176, MON810, T25, DLL25, GA21, and MON802, 13 5′-primers (adh1 1-5′, CM01, HS01, PE01, CD01, cryIA 3-5′, CTP2 1-5′, gox 1-5′, rAct pro 1-5′, cryIA 2-5′, P35S 1-5′, T25 1-5′, and Tr7 ter 1-5′) and 14 3′-primers (CR02, PA01, NOS-ter 2-3′, NOS-ter-3′, NOS-3, T35S 1-3′, T35S 3-3′, T35S 4-3′, bar 1-3′, cryIA 2-3′, epsps 2-3′, gox 1-3′, P35S 2-3′, and Tr7 ter 1-3′) were synthesized (**Table 1**). Primers of cryIA 2-5′ and adh1 1-5′ were designed by referring to our previous DNA sequence results for Event 176, Bt11, and MON810 (*1*, *2*). Primers of Tr7 ter 1-5′ and Tr7 ter 1-3′ were designed by referring to a previous publication (*6*) and the DNA sequence information described in the safety assessment document of DLL25, GenBank Accession No. V00090. The primers of gox 1-5′ and gox 1-3′ primers were designed by referring to the DNA sequence information of *gox* described in the feed and environmental assessment documents of MON809 and Sequence 8 from U.S. Patent 5776760 (*7*) (GenBank Accession No. AR016595). The primers of T35S 3-3′, T35S 4-3′, and P35S 2-3′ were designed by referring to GenBank Accession No. V00141 and to previous studies on P-35S (*8*) and T-35S (*8*−*10*). The primer of bar 1-3′ was designed by referring to GenBank Accession No. X05822. The primer of NOS-ter 2-3′ was designed by referring to previous papers (*11*, *12*) and GenBank Accession No. V00087. The cryIA 2-3′ primer was synthesized by referring to sequence 10 from U.S. Patent 5625136 (*13*) (GenBank Accession No. I41424) and previous reports (*1*, *2*). The epsps 2-3′ primer was designed by referring to the DNA sequence results for Roundup Ready soy, as obtained in our previous paper (*4*) and U.S. Patent 5804425 (*14*). The primer of NOS-3 was synthesized by referring to a previous publication (*11*). The primers of cryIA 3-5′ and CTP2 1-5′ were designed by referring to sequencing results of amplification products, which we amplified by using the primer pairs N or P, as shown in **Figure 1vii**. Other primers, PE01, CD01, CM01, P35S 1-5′, T25 1-5′, rAct pro 1-5′, HS01, CR02, PA01, T35S 1-3′, and NOSter 1-3′, were synthesized by referring to our previous papers (*1*, *2*).

Polymerase Chain Reaction and DNA Sequencings. Amplifiction reactions were carried out in a 25 μL total volume on a PTC-200 thermal cycler (MJ Research, Watertown, MA). End concentrations of PCR components were as follows: PCR buffer II (Applied Biosystems), ×1; $MgCl_2$, 1.50 mmol/L; genomic DNA, 25 ng; primers, 0.5 μmol/L; dNTPs, 200 μmol/L; AmpliTaq Gold Polymerase (Applied Biosystems, Foster City, CA), 0.625 unit/reaction. Amplification for sequencing was performed in the thermal cycler according to the following PCR step-cycle program: preincubation at 95 °C for 10 min; 45 cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 2 min; followed by a final extension at 72 °C for 7 min. The direct DNA sequencings of PCR products were performed by Nippon Flour Mills Co., Ltd. (Tokyo, Japan). Cycling condition for detection of rDNA segments was performed according to the following PCR conditions in all cases: preincubation at 95 °C for 10 min; 40 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 7 min; each experiment was repeated at least three times.

Agarose Gel Electrophoresis. Agarose gel electrophoresis was carried out according to our previous reports (*1*, *2*). After completion of PCR, 5 μL of PCR mixture was electrophoresed at constant voltage (100 V) on a 3% agarose gel that had been supplemented with 0.5 μg/mL ethidium bromide in TAE [40 mmol/L Tris-HCl, 40 mmol/L acetic acid, and 1 mmol/L EDTA (pH 8.0)] buffer solution. A 100 bp ladder (New England Biolabs Inc., Beverly, MA) was used for size control of amplified fragments. The gel was photographed with a Molecular Imager FX system (Bio-Rad Laboratories, Inc., Hercules, CA).

RESULTS AND DISCUSSION

In recent years many countries have adopted new labeling systems focused on information for the general public. An essential element of this process is the availability of reliable detection methods for monitoring the labeling system. PCR has been shown to be a promising technique, and various PCR systems to detect GM soy and GM maize have been reported. The methods, however, are limited to PCR conditions for screening of GMOs by using of P-35S and NOS-ter regions or for specific detection of GM soy and some GM maize. A large number of genes and DNA sequences are used for functional segments in GMO, and some of the DNAs are frequently introduced. We consider that PCR systems able to detect the segments will be useful and informative in the monitoring of the labeling system. Therefore, we analyzed the sequences of expression cassettes introduced in GMOs and developed many PCR systems to detect the DNA segments.

To design the oligonucleotide primers for PCR detection of individual DNA segments introduced into the GM crops, we obtained information on the genetic construction and the amino acid sequences and/or the sequences of DNA segments from food safety assessment documents of the Japan Food Hygienic Association and from environmental safety documents of the MAFF. However, these documents do not fully describe the inserted whole DNA sequences including the spacer regions between DNA segments. In addition, we could not obtain any official documents on MON802 because the GM maize line was not approved in Japan. We therefore amplified the previously reported r-DNA regions (*1*, *2*) (broken lines in **Figure 1**) and the r-DNA regions determined in the present report (bold lines in **Figure 1**) by PCR with appropriate primer pairs A−S

Table 1. PCR Primers Used for DNA Sequencings

Name of Primer Pair	Name	Sequence (5'→3')	Specificity		Length	Reference
A	PE01	AGA TTC TTC ACT CCG ATG CAG CCT A	PEPC pro /sense	Event176	0.9 kbp	(*1*)
	CR02	CTC TCG GCG TAG ATT TGG TAC A	*cryIA(b)* /anti-sense			(*1*)
B	CD01	CGG ATG GTC CTT ATG CAA TTT TGT C	CDPK-pro /anti-sense	Event176	0.9 kbp	(*1*)
	CR02	CTC TCG GCG TAG ATT TGG TAC A	*cryIA(b)* /anti-sense			(*1*)
C	cryIA 2-5'	TGG ACA ACA ACC CAA ACA TCA A	*cryIA(b)* / sense	Event176	2.1 kbp	-
	T35S 3-3'	TGG ATT TTG GTT TTA GGA ATT AGA AA	CMV/anti-sense			-
D	CM01	CAC TAC AAA TGC CAT CAT TGC GAT A	CMV /sense	Event176	0.6 kbp	(*1*)
	bar 1-3'	GAT AGC GCT CCC GCA GAC	*bar* /anti-sense			(*1*)
E	CM01	CAC TAC AAA TGC CAT CAT TGC GAT A	CMV /sense	Bt11 and MON810	1.2 kbp (Bt11)	(*1*)
	CR02	CTC TCG GCG TAG ATT TGG TAC A	*cryIA(b)* /anti-sense		1.4 kbp (MON810)	(*1*)
F	adh1 1-5'	GCA CTG AAT TTG TGA ACC C	*adh1*-1S /sense	Bt11	2.1 kbp	-
	NOS ter 2-3'	CTA TAT TTT GTT TTC TAT CGC	NOS ter /anti-sense			-
G	CM01	CAC TAC AAA TGC CAT CAT TGC GAT A	CMV /sense	Bt11and T25	0.7 kbp (Bt11)	(*1*)
	PA01	AGA TCA TCA ATC CAC TCT TGT GGT G	*pat* /anti-sense		0.4 kbp (T25)	(*1*)
H	P35S 1-5'	ATT GAT GTG ATA TCT CCA CTG ACG T	CMV /sense	Bt11	1.1 kbp	(2)
	NOS-3	TTA TCC TAG TTT GCG CGC TA	NOS ter /anti-sense			(*11*)
I	P35S 1-5'	ATT GAT GTG ATA TCT CCA CTG ACG T	P35S /sense	T25	0.8 kbp	(*2*)
	T35S 1-3'	ACT AAG GGT TTC TTA TAT GCT CAA CA	CMV /anti-sense			-
J	T25 1-5'	GCC AGT TAG GCC AGT TAC CCA	*pat*/sense	T25	0.2 kbp	(*2*)
	T35S 4-3'	TTT TAG TAC TGG ATT TTG GTT TTA GGA ATT AG	T35S/anti-sense			-
K	rAct pro 1-5'	ATC TTT GGC CTT GGT AGT TTG	Pr-act/sense	GA21	2.3 kbp	-
	NOS ter 1-3'	ATT GCG GGA CTC TAA TCA TAA	NOS ter /anti-sense			(*2*)
L	cTr7 ter 1-5'	TGG TAC ATT GCC GTA GAT GAA AG	complement Tr7/sense	DLL25	0.8 kbp	-
	P35S 2-3'	CCT CTC CAA ATG AAA TGA ACT TCC T	P35S/anti-sense			-
M	P35S 1-5'	ATT GAT GTG ATA TCT CCA CTG ACG T	CMV /sense	DLL25	1.1 kbp	(*2*)
	Tr7 ter 1-3'	GGR TCG ATC CTA CGT AAG GAT A	Tr7/anti-sense			-
N	HS01	AGT TTC CTT TTT GTT GCT CTC CT	hsp70 01-5'	MON802	2.0 kbp	(*1*)
	cryIA 2-3'	GAC GTG AAG AGC TCA TTG ACA G	*cryIA(b)* /anti-sense			-
O	cryIA 3-5'	TGT ACA TCG ACC GCA TCG AGT	*cryIA(b)* / sense	MON802	1.8 kbp	-
	NOS-3	TTA TCC TAG TTT GCG CGC TA	NOS ter /anti-sense			(*11*)
P	HS01	AGT TTC CTT TTT GTT GCT CTC CT	hsp70 01-5'	MON802	0.6 kbp	(*1*)
	epsps 2-3'	GAC TTG TCG CCG GGA ATG	*cp4-epsps* /anti-sense			-
Q	CTP2 1-5'	CTT CGT CCT CTT AAG GTC ATG TCT TCT	CTP2 from *A. thaliana* /sense	MON802	1.6 kbp	-
	NOS-3	TTA TCC TAG TTT GCG CGC TA	NOS ter /anti-sense			(*11*)
R	HS01	AGT TTC CTT TTT GTT GCT CTC CT	hsp70/ intoron	MON802	0.9 kbp	(*1*)
	gox 1-3'	GTT GAG ACG ACG AAG TTC CCA	*gox* /anti-sense			-
S	gox 1-5'	ATG CTA GCC ACC TTA TCC GTC A	*gox* /sense	MON802	1.1 kbp	-
	NOS-3	TTA TCC TAG TTT GCG CGC TA	NOS ter /anti-sense			(*11*)

Table 2. PCR Primers for Detection of r-DNA Segments

	Name	Primer Sequence	sense/antisense	rDNA segment	Length
(a)	pat1-5'	AAG AGT GGA TTG ATG ATC TAG AGA GGT	sense	*pat*	161 bp
	pat1-3'	ATG CCT ATG TGA CAC GTA AAC AGT ACT	anti-sense		
(b)	bar 2-5'	ACT GGG CTC CAC GCT CTA CA	sense	*bar*	186 bp
	bar 2-3'	AAA CCC ACG TCA TGC CAG TTC	anti-sense		
(c)	bar 3-5'	CAT CGT CAA CCA CTA CAT CGA GA	sense	*bar*	104 bp
	bar 1-3'	GAT AGC GCT CCC GCA GAC	anti-sense		
(d)	cryIA 4-5'	GGA CAA CAA CCC MAA CAT CAA C	sense	*cryIA(b)*	107 bp
	cryIA 3-3'	CGA TGG GGG TGT AAC CGG T	anti-sense		
(e)	cryIA 4-5'	GGA CAA CAA CCC MAA CAT CAA C	sense	*cryIA(b)*	152 bp
	cryIA 4-3'	GCA CGA ACT CGC TSA GCA G	anti-sense		
(f)	m-eps 1-5'	GTC GAA GCG GAC AAA GCT G	sense	point mutated *m-epsps*	193 bp
	m-eps 1-5'	CCC TCA TTC TTG GTA CTC CAT CA	anti-sense	native *m-epsps*	290 bp
(g)	P35S 1-5'	ATT GAT GTG ATA TCT CCA CTG ACG T	sense	P35S	101 bp
	P35S 2-3'	CCT CTC CAA ATG AAA TGA ACT TCC T	anti-sense		
(h)	T35S 1-5'	GAA ACC CTT AGT ATG TAT TTG TAT TTG TAA AAT ACT TC	sense	T35S	84 bp
	T35S 4-3'	TTT TAG TAC TGG ATT TTG GTT TTA GGA ATT AG	anti-sense		
(i)	NOS-1	GAA TCC TGT TGC CGG TCT TG	sense	NOS ter	180 bp
	NOS-3	TTA TCC TAG TTT GCG CGC TA	anti-sense		
(j)	NOS ter 3-5'	GTC TTG CGA TGA TTA TCA TAT AAT TTC TG	sense	NOS ter	151 bp
	NOS ter 3-3'	CGC TAT ATT TTG TTT TCT ATC GCG T	anti-sense		
(k)	epsps 1-5'	GCC TCG TGT CGG AAA ACC CT	sense	*cp4-epsps*	118 bp
	epsps 3-3'	TTC GTA TCG GAG AGT TCG ATC TTC	anti-sense		
(l)	gox 2-5'	TGC CAG GAA ACT TGA CTA GCG	sense	*gox*	103 bp
	gox 2-3'	CGA ATC AAC CAA GGC ATG ATG	anti-sense		
(m)	npt 1-5'	GAC AGG TCG GTC TTG ACA AAA AG	sense	*nptII*	155 bp
	npt 1-3'	GAA CAA GAT GGA TTG CAC GC	anti-sense		
(n)	rAct pro 2-5'	CGT TGC AGC GAT GGG TAT	sense	Pr-act	121 bp
	rAct pro 1-3'	GGG CTT GCT ATG GAT CGT G	anti-sense		

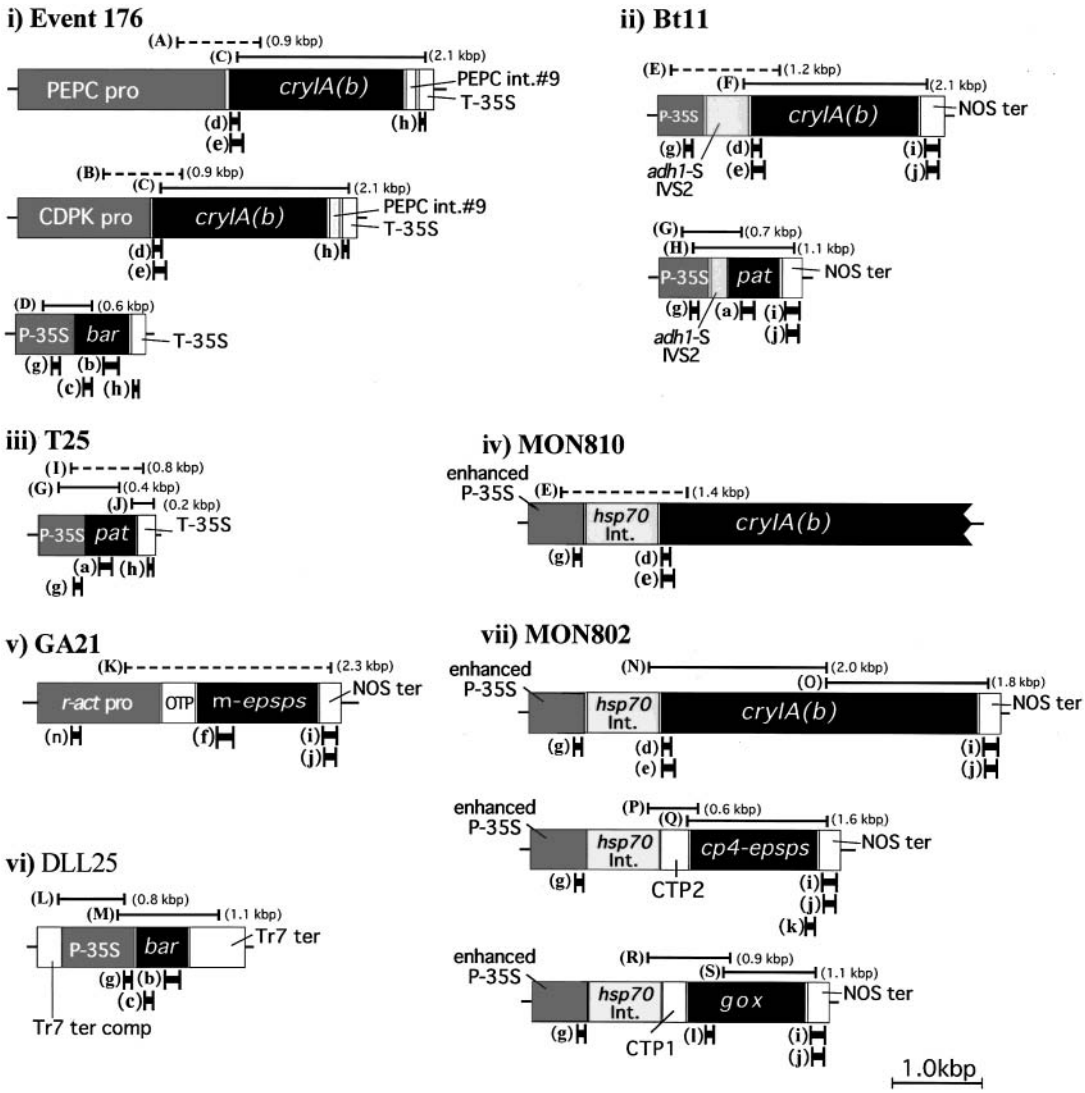

Figure 1. Schematic diagrams of sequenced regions and amplified regions in relation to the r-DNA segments introduced into the five lines of GM maize (i–vii). The sequenced regions in this paper and the regions reported previously (*1*, *2*) are shown as solid lines and broken lines, respectively. The primer pairs used for sequencing are listed alphabetically by a capital letter at the left side of each line, and the nucleotide sequences are shown in **Table 1**. The expected lengths of PCR products are indicated in parentheses at the right side of the lines. (i) Event 176: Event 176 has three expression cassettes. Two cassettes including an insect-resistant trait consist of [maize phosphoenol pyruvate carboxylase promoter (PEPC pro)]-[synthetic *crylA(b)* gene derived from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1]–[DNA fragment containing the no. 9 intron sequence (PEPC int. 9) from maize phosphoenol pyruvate carboxylase]–[35S terminator (T-35S) derived from cauliflower mosaic virus (CMV)] and of [maize calcium-dependent protein kinase (CDPK pro)]–[synthetic *crylA(b)*]-[PEPC int. 9]–[T-35S]. The cassette including a herbicide-tolerant trait as a selectable marker consists of [35S promoter derived from CMV (P-35S)]–[*bar* gene derived from *Streptomyces hygroscopicus*]–[T-35S]. In the safety assessment documents, which are available to the public from the Japan Food Hygienic Association, the two constructs for the *crylA(b)* gene controlled by [PEPC pro] and [CDPK pro] are identical. (ii) Bt11: Bt11 has two expression cassettes. The cassette including an insect-resistant trait consists of [P-35S]–[DNA fragment containing the no. 6 intron sequence (IVS6) from maize alcohol dehydrogenase 1 gene (*adh1*-S)]–[synthetic *crylA(b)* gene]–[nopaline synthase terminator (NOS ter) derived from *Agrobacterium tumefaciens*]. Another cassette including a herbicide-tolerant trait as a selectable marker consists of [P-35S]–[DNA fragment containing the no. 2 intron sequence (IVS2) from *adh1*-S]–[synthetic *pat* gene derived from *Streptomyces viridochromogenes*]–[NOS-ter]. (iii) T25: The expression cassette including a herbicide-tolerant trait consists of [P-35S]–[*pat* gene]–[T-35S]. (iv) MON810: The cassette including an insect-resistant trait consists of [P-35S with the duplicated enhancer regions (enhanced P-35S pro)]–[fragment of DNA (*hsp70* int.) containing the no. 1 intron sequence from maize the *hsp70* gene (heat-shock protein)]–[synthetic *crylA(b)* gene]. (v) GA21: The cassette including a herbicide-tolerant trait consists of [rice actin promoter containing the no. 1 intron (*r-act pro.*)]–[optimized transit peptide sequence (OTP); DNA sequence for chloroplast transit peptide (CTP) synthesized from data on the peptide sequences of the N-terminal upstream region of ribulose-1,5-bisphosphate carboxylase (RuBisCO) derived from maize and sunflower]–[point-mutated 5-enolpyruvylshikimate-3-phosphate synthase gene (*epsps*) derived from maize (*m-epsps*)]–[NOS-ter]. (vi) DLL25: The cassette including a herbicide-tolerant trait consists of [P-35S]–[*bar* gene]–[T-DNA transcript no. 7 (Tr7) terminator derived from *A. tumefaciens*]. In addition, a DNA sequnece complementary to Tr7 is partially inserted upstream of P-35S. (vii) MON802: MON802 has three expression cassettes. The cassette including an insect-resistant trait consists of [enhanced P-35S]–[*hsp70* int.]–[synthetic *crylA(b)* gene]-[NOS-ter]. The two expression cassettes for herbicide-tolerant traits consist of [enhanced P-35S]–[*hsp70* int.]–[DNA sequence for CTP derived from *Arabidopsis thaliana* (CTP2)]–[synthetic *epsps* derived from *Agrobacterium* sp. strain CP4 (cp4-*epsps*)]–[NOS-ter] and [enhanced P-35S]–[*hsp70* int.]–[DNA sequence for CTP isolated from the small subunit gene of the RuBisCO gene derived from *A. thaliana* (CTP1)]–[glyphosate oxidoreductase gene (*gox*) derived from *Ochrobactrum anthropi* strain LBAA, which encodes the glyphosate metabolizing enzyme]–[NOS-ter].

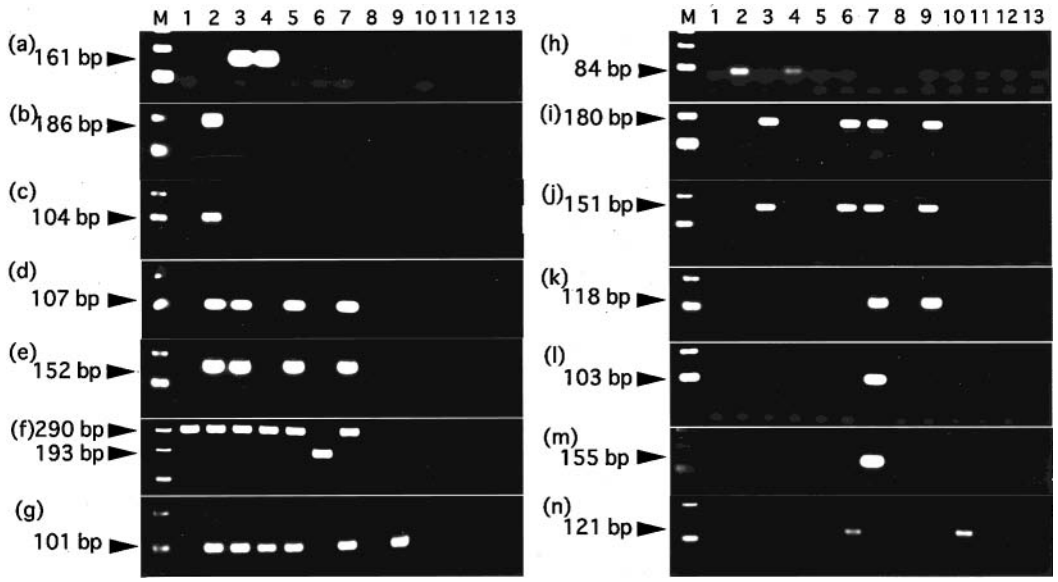

Figure 2. Agarose gel electrophoresis of PCR products amplified from maize or other cereal genomic DNAs. Arrowheads indicate the expected PCR amplification products. The primer pairs for detection of *pat* (a), *bar* (b and c), *cryIA(b)* (d and e), *m-epsps* (f), P-35S (g), T-35S (h), NOS-ter (i and j), *cp4-epsps* (k), *gox* (l), *nptII* (m), and Pr-actin (n), respectively, were used. Lanes 1–7: amplification of maize DNAs from non-GM maize, Event 176, Bt11, T25, MON810, GA21, and MON802, respectively. Lanes 8–12: amplification of non-GM soy, GM soy, rice, wheat, and barley, respectively. Lane 13: negative control (no template DNA); M, 100 bp ladder size standard.

designed from known sequence information (**Table 1**). In the case of MON802, sequencing between the intron and terminator was divided into two regions for each trait, such as *cryIA(b)*, *cp4-epsps*, and *gox* genes.

The 14 regions in the introduced genes of the approved GM maize lines (Event176, Bt11, T25, Mon810, GA21, and DLL25) were amplified by using the specific primers A−M shown in **Table 1** and **Figure 1** and were sequenced. In the case of MON802, we first amplified and sequenced the region between the *hsp70* intron and the *cryIA(b)*, *cp4-epsps*, or *gox* gene by using the primer pairs N, P, and R, respectively (**Table 1**). Following the design of the cryIA 3-5′, CTP2 1-5′, and gox 1-5′ primers by referring to the results of the above DNA sequences, we amplified and sequenced the regions between these three genes and NOS-ter, as shown in **Figure 1vii**.

On the basis of the obtained sequence, we newly designed about 100 primer pairs for the detection of each functional r-DNA segment derived from one exogenous organism, that is, *pat*, *bar*, *cp4-epsps*, *gox*, *m-epsps*, *cryIA(b)*, P-35S, NOS-ter, and T-35S. PCR was carried out with each primer pair on the genomic DNAs extracted from non-GM and GM maize (Event 176, Bt11, T25, MON810, GA21, and MON802), non-GM and GM soy, and major cereal crops such as rice, barley, and wheat to evaluate its sensitivity and specificity. Because of an insufficient amount of the provided DNA of DLL25, it was impossible to examine whether all of the designed primers amplify any unexpected products for DLL25 genomic DNA or not. However, the primer pairs of b, c, and g specifically amplified the products for the genomic DNA of DLL25 with expected length (data not shown).

Finally, we selected the most appropriate primer pairs for detection of each functional DNA segment; these primer pairs are listed alphabetically in **Table 2** as pairs a−n. The sequence information and PCR results for each of the DNA segments introduced into more than one GM line are as follows.

***pat* and *bar* Genes.** A herbicide-tolerant gene *pat*, encoding the phosphinothricin acetyltransferase, is introduced into T25 and Bt11 lines. The nucleotide sequence of the synthetic *pat* gene introduced into Bt11 was identical to that of T25 and was 70% homologous to the sequence of the original *pat* gene derived from *Streptomyces viridochromogenes* (GenBank Accession No. M22827). The *bar* gene also encodes the same enzyme of *pat* and is introduced into Event 176 and DLL25 lines. The DNA sequence of the *bar* gene introduced into Event 176 and DLL25 was identical to that of the original *bar* gene derived from *Streptomyces hygroscopicus* (GenBank Accession No. X05822) except for the substitution at the first codon (guanine instead of adenine). The DNA sequences of synthetic *pat* and *bar* genes are ∼65% homologous. As shown in **Figure 2a**−**c**, PCR amplifications using a primer pair a for the *pat* gene and two primer pairs b and c for the *bar* gene that we designed (**Table 2**) were specific each other.

***cryIA(b)* Gene.** An insect-resistant gene *cryIA(b)* derived from *B. thuringiensis* subsp. *kurstaki* is introduced into Event 176, Bt11, MON810, and MON802; however, some differences in the DNA sequences among the above GM lines were found by our sequencing. The DNA sequences of the *cryIA(b)* gene introduced into Bt11, Event 176, and MON810/MON802 differed with respect to codon usage and length at the nucleotide level, but our sequenced regions of the *cryIA(b)* gene were identical between MON810 and MON802. The DNA sequences of the *cryIA(b)* gene introduced into Bt11 were identical to sequence 22 from U.S. Patent 5880275 (15), and that introduced into Event 176 was identical to sequence 3 from U.S. Patent 5625136 (*13*) (GenBank Accession No. I41419). We designed two primer pairs, d and e, having identical nucleotide sequences in the 5′-terminal region of *cryIA(b)* genes introduced into those four lines, as shown in **Figure 3**. We obtained the expected 107 and 152 bp (**Figure 2d**,**e**) fragments from the four lines of GM maize tested with primer pairs d and e, respectively. All maize lines into which the *cryIA(b)* gene had been introduced could be specifically detected using these primer pairs. The resulting amino acid sequences of the *cryIA(b)* genes introduced into three insect-resistant maize lines (Bt11, Event 176, and MON802) were identical to N-terminal 648, 615, and whole 1156 residues, respectively, of the natural CryIA(b) protein derived from *B. thuringiensis* subsp. *kurstaki* strain HD-1 (PIR Accession No. A29125). The 13th nucleotide in the primer cryIA

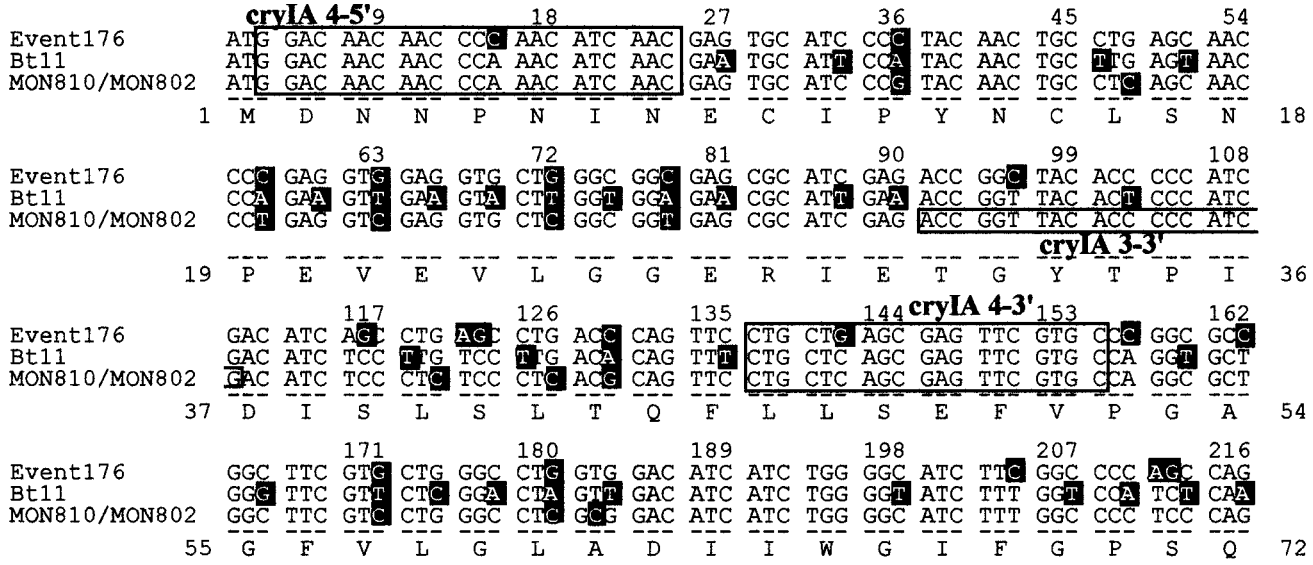

Figure 3. N-Terminal 216 bp sequences and the resulting amino acid sequence of the *crylA(b)* gene introduced into Event 176, Bt11, and MON810/MON802, respectively. Differences in DNA sequences from others are shown by outline type. The DNA sequences of the primer region for detection of the *crylA(b)* gene are boxed. The region of 3–24 bp was used as a 5′-primer (cryIA 4-5′), and the regions of 91–109 and 136–154 bp were used as 3′-primers (cryIA 3-3′ and cryIA 4-3′).

4-5′ and the 14th nucleotide in the primer cryIA 4-3′, where are found differences in the sequences, were designated M and S in synthesis, respectively.

***m-epsps* Gene.** A hebicide-tolerant gene *m-epsps*, which is originally derived from *Z. mays*, is modified at the eight bases from the sequence of the native one, as described in a previous paper (*2*). Therefore, we thought that the recombinant *m-epsps* gene would show PCR results identical to those of the native one. As shown in **Figure 2f**, however, use of the primer pair f for detection of *m-epsps* resulted in not only the expected 193 bp band but also an unexpected 300 bp band. We then sequenced both of the amplification products. The results suggested the latter (290 bp) band derived from the native *m-epsps* gene of maize included an intron of 97 nucleotides. Hence, we were able to distinguish between the point-mutated *m-epsps* gene and the native *m-epsps* gene by using primer pair f.

P-35S and T-35S. The P-35S sequence is widely used in many transgenic crops for the promoter region to strongly express the trait genes. **Figure 4** shows the oligonucleotide comparisons of the P-35S region from the cauliflower mosaic virus (CMV) genome (GenBank Accession No. V00141 and J02046) with the P-35S regions sequenced from Event 176, Bt11, T25, MON810, and DLL25. Some differences among the oligonucleotides are observed. The T-35S sequences introduced into Event 176 and T25 were also somewhat different from that of the CMV 35S genome. Therefore, we designed the primers for P-35S and T-35S in the oligonucleotides common to each region (**Figures 4** and **5**). The PCR results for the detection of P-35S are shown in **Figure 2g**. We can find strong PCR bands in the expected lanes. The P-35S sequence was also detected in the RR soy, because it was used as the promoter region for the *cp4-epsps* gene (**Figure 2g**) described as in the previous paper (*4*). On the other hand, the PCR bands obtained by primer pair h for T-35S were weak (data not shown), although they appeared in the expected lane. Therefore, the PCR conditions for primer pair h were optimized by changing to 3.0 mmol/L $MgCl_2$ and an annealing temperature of 55 °C. The results of the PCR amplification under the new conditions are shown in **Figure 2h**.

NOS-ter. The NOS-ter sequence is widely used in transgenic crops for the termination of transcript of trait genes. The sequences used in GM maize (Bt11, GA21 and MON802) and that of the gene encoding NOS (GenBank Accession No. V00087) of *A. tumefaciens* are highly conserved without the 5′-end region. The specificities of PCR using two primer pairs i and j are shown in **Figure 2i**,**j**. The PCR amplifications of NOS-ter were found in Bt11, GA21, and MON802 GM maize. The NOS-ter was detected in RR soy, because it was also used as the terminator region for the *cp4-epsps* gene (**Figure 2i**,**j**) described in the previous paper (*4*). The results suggested that the amplification region of NOS-ter designed in this study was not introduced into MON810.

Others. Other DNA segments, such as *cp4-epsps*, *gox*, and *nptII*, were used in MON802 maize and in other crops. Our DNA sequence data for *cp4-epsps* and *gox* were consistent with the safety assessment documents for MON809 and the sequence information described in U.S. patent documents (*7*, *14*). Two primer pairs for the detection of these segments were designed as shown in **Table 2k**,**l**, and the results of electrophoresis of PCR products are shown in **Figure 2k**,**l**, respectively. The *cp4-epsps* was detected in MON802 and RR soy, because the gene was introduced in both GM crops as shown in **Figure 1** and the previous paper (*4*). The DNA sequence data for *nptII* and Pr-act were also consist with the safety assessment documents.

Two primer pairs for the detection of *nptII* and Pr-act were also designed, and the PCR amplification gave specific bands with the expected length (**Figure 2m**,**n**). To verify the specificities of primer pair m, PCR amplifications were carried out by using pCR2.1 (Invitrogen Co., Carlsbad, CA) as template DNA. The PCR products from the amplification gave the same length in electrophoresis (data not shown). Pr-act is derived from the promoter sequence of rice actin gene, and the PCR product from rice genomic DNA is shown (**Figure 2n**).

To roughly investigate the sensitivities of each PCR amplification using the primer pair designed in this study, the DNAs were extracted from two kinds of GM maize mixture samples including 0.1 and 0.5% of Event176, Bt11, T25, GA21, and MON810 in non-GM maize and GM soy samples including 0.1% RR soy in non-GM soy. The sequences of *pat*, *bar*, *cryIA-(b)*, P-35S, and NOS-ter were detected from the genomic DNA extracted from a 0.1% mixture of GM maize by using the designed PCR primer pairs (data not shown). Furthermore, the

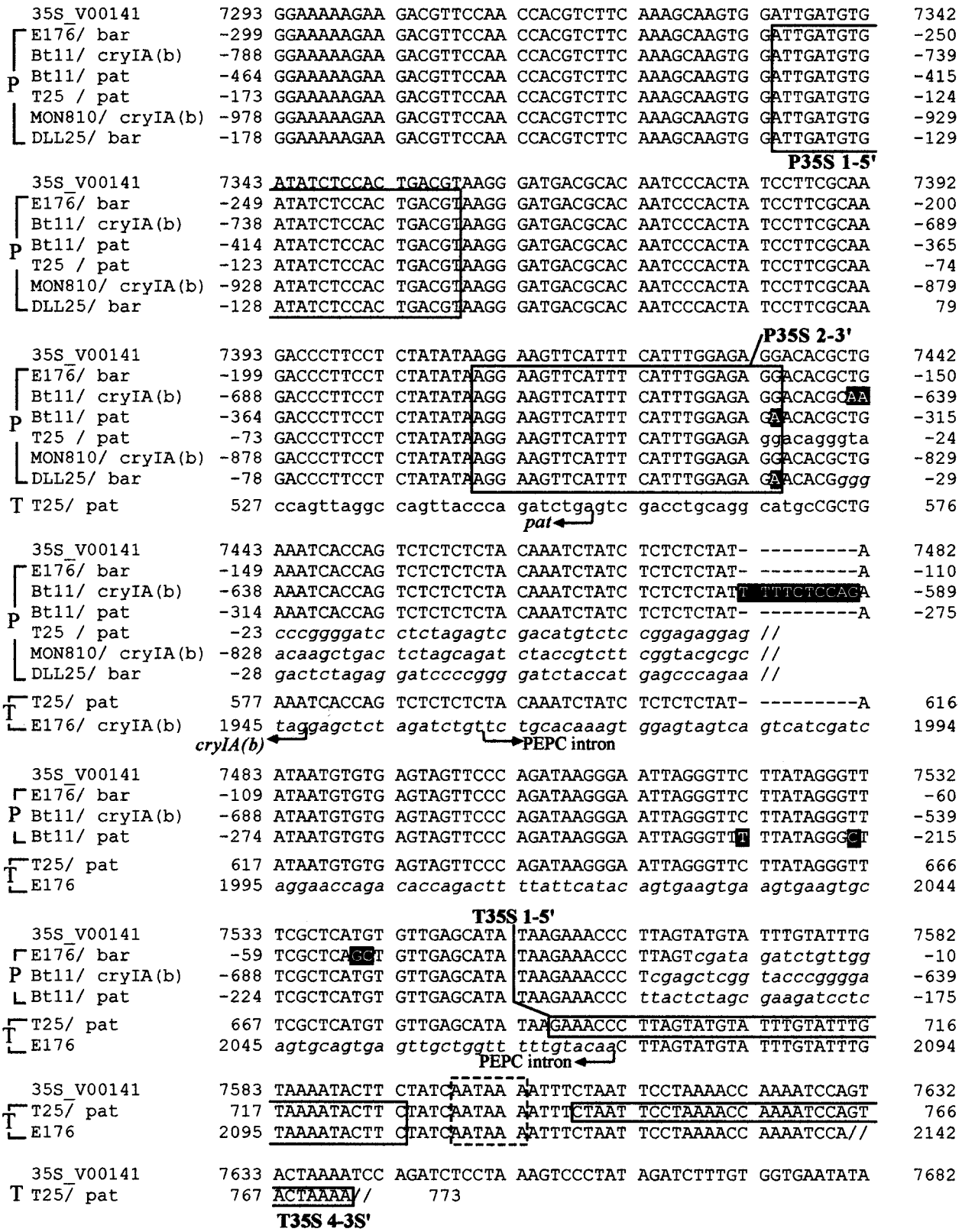

Figure 4. DNA sequences of the P-35S and T-35S regions introduced into Event 176, Bt11, T25, MON810, and DLL25 and of the CMV genome (Accession No. V00141), respectively. DNAs are numbered from the adenine of the initiation codon (ATG) of the trait gene as +1. Differences in DNA sequences from the CMV genome are shown by outline type. The ends of sequencing of introduced DNA are shown by double slash. The DNA sequences of the primers for P-35S and T-35S detection are boxed. Box with broken line indicates polyadenylation signal sequence. The sequences not having significant homology with CMV genome are indicated by small letters. P, promoter region; T, terminator region on left side.

sequences of P-35S, NOS-ter, and *epsps* were also detected from a 0.1% mixture of GM soy (data not shown). In the case of m-epsps, T-35S, and Pr-actin, however, the sensitivities were 0.5% (data not shown). These sensitivities would be acceptable to secure the verification of non-GMO materials and to monitor the reliability of the labeling system in each country.

The profiles of PCR amplifications using the primer pairs described above [*pat*, *bar*, *cp4-epsps*, *gox*, *m-epsps*, *cryIA(b)*,

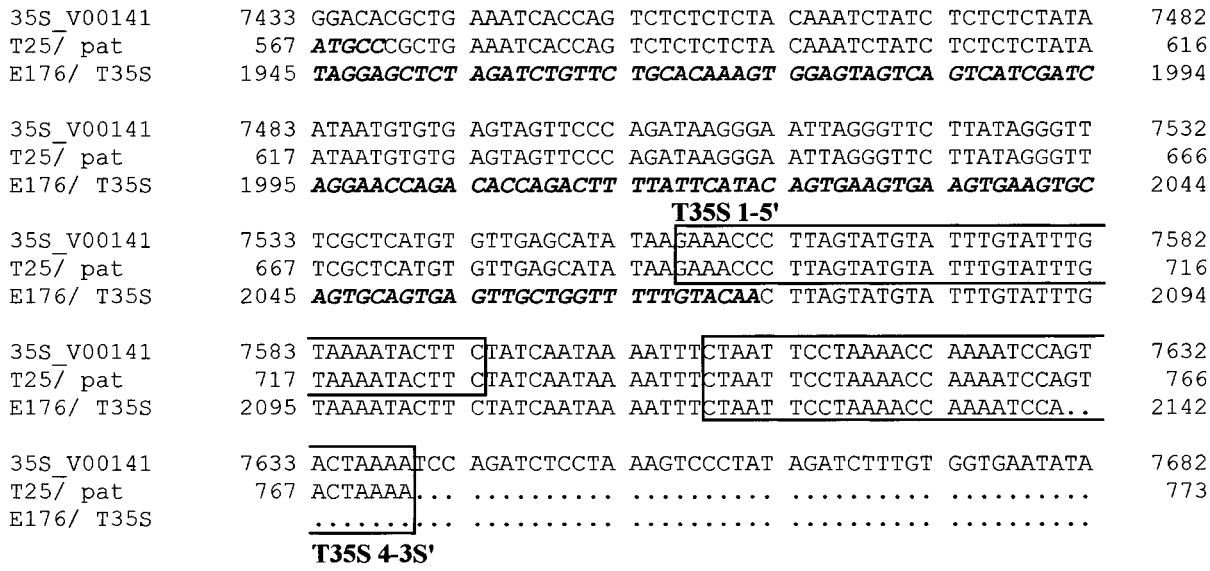

Figure 5. Schematic diagram of T-35S region inserted into Event 176 and T25 and of the CMV genome (Accession No. V00141), respectively. The DNA sequences of the primers for T35S detection are boxed. Italic indicates difference in DNA sequence from the CMV genome.

Table 3. Profile of PCR Amplifications on Each GM Maize by Using the Primer Pairs for Detection of the r-DNA Segment

target:	*pat*	*bar*	*crylA(b)*	*m-epsps*	P-35S	T-35S	NOS-ter	*cp4-epsps*	*gox*	*nptII*	Pr-actin
primer pair:	(a)	(c)	(e)	(f)	(g)	(h)	(j)	(k)	(l)	(m)	(n)
amplicon:	161 bp	104 bp	152 bp	193 bp	101bp	84 bp	151 bp	118 bp	103 bp	155 bp	121 bp
non-GM maize	−	−	−	−	−	−	−	−	−	−	−
Event176	−	+	+	−	+	+	−	−	−	−	−
Bt11	+	−	+	−	+	−	+	−	−	−	−
T25	+	−	−	−	+	+	−	−	−	−	−
MON810	−	−	+	−	+	−	−	−	−	−	−
GA21	−	−	−	+	−	−	+	−	−	−	+
MON802	−	−	+	−	+	−	+	+	+	+	−
non-GM soy	−	−	−	−	−	−	−	−	−	−	−
GM soy	−	−	−	−	+	−	+	+	−	−	−
rice	−	−	−	−	−	−	−	−	−	−	+
wheat	−	−	−	−	−	−	−	−	−	−	−
barley	−	−	−	−	−	−	−	−	−	−	−

Pr-actin, P-35S, NOS-ter, T-35S, and *nptII*] are shown in **Table 3**. The profiles suggested that the combination of detection on each DNA segment was effective to distinguish these lines of nonprocessed GMOs. Furthermore, it might be possible to distinguish the GMO line contained in processed foods on the basis of the profile of PCR amplifications. However, it was difficult to distinguish precisely what type of GMO line was contained in the samples, because the profile results could be interpreted in any of the following five ways: (1) the sample contains a specific GM line; (2) the sample contains more than one kind of GMO from different crops; (3) the sample contains a small amount of the original sequence as a foreign gene introduced through contamination of the original organism; (4) the sample contains a small amount of other crops having a DNA segment identical to that introduced in the suspected GM-crop; and (5) in the case of processed foods, PCR could not be performed using certain primers pairs because the degradation of DNAs derived from different materials could be different.

Despite the above-mentioned problems, this method provides useful information for the existence of GMOs including nonapproved ones because most GM crops utilize one or more segments to construct r-DNA. Therefore, we conclude that this method can be effectively used for fast and easy screening of GM crops.

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