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papaya in Japan. A qualitative detection method for line 55-1, using a PCR test and a histochemical assay, has been developed (Akiyama et al., 2002; Goda, Asano, Shibuya, Hino, & Toyoda, 2001; Wakui et al., 2004; Yamaguchi et al., 2006). A method for the extraction and purification of genomic DNA from fresh papaya, by a simple operation in a short time, has been established (Ohmori et al., 2008). However, there are no suitable methods for the extraction and purification of DNA from processed papaya products, which could be used for the detection of GM papaya. Hence, a method to extract and purify DNA from processed papaya products is required.

In the present study, we developed a method for the detection of GM papaya, using an ion-exchange resin type kit to extract and purify DNA from processed papaya products, such as dried papaya, canned papaya and papaya jam.

2. Materials and methods

2.1. Samples

Dried papaya (sample G; unbleached and infused with sugar), canned papaya (sample B; a product containing papaya, pineapple, *nata-de-coco*, sugar and citric acid) and papaya jam (sample C; a product containing papaya, pectin, sugar and lemon juice) were used for the experiments. All processed papaya products in Table 2 were purchased through the internet. Dried papaya and canned papaya were washed with abundant water, and homogenized with the same weight of water as the sample. Papaya jam was used directly for the extraction and purification of genomic DNA.

2.2. Extraction and purification of genomic DNA by IER-100G

DNA was extracted and purified from 10 g of each of the homogenized samples (dried papaya, canned papaya and papaya jam), using an ion-exchange resin type kit (Genomic-tip 100G, IER-100G; Qiagen, Hilden, Germany). The procedure was as follows: 30 mL of buffer G2, 20 μL of RNase (100 mg/mL; Qiagen) and 500 μL of cellulase (Sigma–Aldrich, St. Louis, MO) were added to the homogenized dried papaya or canned papaya samples and vortexed thoroughly, followed by incubation at 50 °C for 1 h. For the homogenized papaya jam sample, 30 mL of buffer G2, 20 μL of RNase, 500 μL of cellulase and 20 μL of α-amylase were added, and vortexed, followed by incubation at 50 °C for 1 h. Then, 200 μL of proteinase K (20 mg/mL; Promega, Madison, WI) were added and the cocktail was incubated at 50 °C for another 1 h. During incubation, samples were mixed several times by inverting the tubes. The incubated cocktail was centrifuged at 3000 × g for 20 min at 4 °C. The supernatant was applied to an IER-100G column, which was pre-equilibrated with 4 mL of buffer QBT. The IER-100G column was washed three times with 7.5 mL of buffer QC, followed by the application of 1 mL of pre-warmed buffer QF (50 °C). The IER-100G column was transferred to a new centrifuge tube, and 2 mL of pre-warmed buffer QF (50 °C) were added to elute the DNA. The eluate containing DNA and an equal volume of isopropyl alcohol were mixed thoroughly. DNA was collected by centrifugation at 12,000 × g for 15 min at 4 °C. The pellet was rinsed with 1 mL 70% (v/v) ethanol and centrifuged at 12,000 × g for 3 min. The supernatant was discarded and the precipitate was dried. The DNA was dissolved in 20 μL water prior to analysis.

2.3. Extraction and purification of genomic DNA by IER-20G

DNA was extracted and purified from 1 g of the homogenized dried papaya and canned papaya, using an ion-exchange resin type kit (Genomic-tip 20G, Qiagen), according to the official standard methods adopted by the Ministry of Health, Labour and Welfare of Japan (2002). The procedure was as follows: 15 mL of buffer G2, 20 μL of RNase and 200 μL of proteinase K were added and the cocktail was incubated at 50 °C for 2 h. During incubation, samples were mixed several times by inverting the tubes. The incubated cocktail was centrifuged at 3000 × g for 15 min at 4 °C. The supernatant was applied to an IER-20G column, which was pre-equilibrated with 1 mL of buffer QBT. The IER-20G column was washed three times with 2 mL of buffer QC. The IER-20G column was transferred to a new centrifuge tube, and 1 mL of pre-warmed buffer QF (50 °C) was added twice to elute the DNA. The eluate containing DNA and an equal volume of isopropyl alcohol were mixed thoroughly, and the subsequent operations were carried out according to the procedure using IER-100G.

2.4. Extraction and purification of genomic DNA by QIAamp DNA Stool Mini Kit

DNA was extracted and purified from 0.4 g of the homogenized dried papaya, using a silica membrane-type kit (QIAamp DNA Stool Mini Kit, Qiagen), according to the manufacturer's instructions (Qiagen, 2010).

2.5. Extraction and purification of genomic DNA by DNeasy Plant Maxi Kit

DNA was extracted and purified from 1 g of homogenized dried papaya, using a silica membrane-type kit (DNeasy Plant Maxi Kit; Qiagen), according to the official standard methods adopted by the Ministry of Agriculture, Forestry and Fisheries of Japan (2002). The procedure was as follows: 10 mL of pre-warmed AP1 buffer (65 °C) and 20 μL of RNase were added and the cocktail was incubated at 65 °C for 1 h. During incubation, samples were mixed three times by inverting the tubes. The incubated cocktail was centrifuged at 3000 × g for 10 min at room temperature; 7 mL of supernatant

Table 1
Comparison of the DNA yield and purity obtained with several DNA extraction methods.

Sample	Kit	Amount of homogenated sample used (g)	Volume of extracted DNA solution (μL)	Concentration of extracted DNA solution (ng/μL)	Amount of extracted DNA (ng)	Purification efficiency (ng/g)[a]	A_{260}/A_{280}	A_{260}/A_{230}	Number of copies of the *chymopapain* gene
Dried papaya	QIAamp DNA Stool Mini	0.4	50	24.0 ± 0.9	1198 ± 45	5895 ± 250	2.17 ± 0.03	0.57 ± 0.14	206,331 ± 25,000
	GM Quicker 3	1.0	50	9.3 ± 0.8	464 ± 40	887 ± 92	1.82 ± 0.18	8.48 ± 18.00	1,234,050 ± 207,664
	Wizard Cleanup Resin	0.5	50	18.7 ± 0.6	933 ± 30	3696 ± 94	1.78 ± 0.10	0.07 ± 0.03	209,238 ± 70,696
	DNeasy Plant Maxi	1.0	100	6.7 ± 1.1	665 ± 110	1282 ± 205	2.29 ± 0.26	0.41 ± 0.23	688 ± 634
	IER-20G	1.0	20	79.2 ± 8.2	1584 ± 163	3104 ± 293	1.90 ± 0.02	1.82 ± 0.01	6,444,303 ± 468,189
	IER-100G	10.0	20	718.3 ± 42.8	14,367 ± 856	2859 ± 168	1.94 ± 0.01	2.44 ± 0.01	5,900,680 ± 530,558
Canned papaya	IER-20G	1.0	20	3.9 ± 0.5	77 ± 9	151 ± 18	1.28 ± 0.10	1.29 ± 0.10	646,755 ± 232,426
	IER-100G	10.0	20	66.3 ± 5.2	1327 ± 105	238 ± 17	1.90 ± 0.02	2.14 ± 0.08	4,264,498 ± 187,356

[a] Purification efficiency represents the amount of purified DNA per gram of starting material.

Table 2
Extraction and purification of DNA from processed papaya products.

Product		Sample ID	Ct[a]	Purification efficiency (ng/g)[b]	A_{260}/A_{280}	A_{260}/A_{230}	Ingredients listed on the packaging
Dried papaya	Plain	A	23.7	26,588.1	1.87	1.88	Papaya
		B	22.1	13,130.3	1.87	2.11	Papaya
		C	25.5	82,719.8	1.78	1.75	Papaya
		D	22.5	3013.0	1.84	2.29	Papaya
		E	24.6	2132.7	1.84	2.14	Papaya
	Candied, unbleached	G	24.2	2859.4	1.94	2.44	Papaya, sugar, preservative (sodium metabisulfite)
	Candied, bleached	H	ND	4.4	2.18	0.36	Banana, papaya, raisin, prune, sugar, plant oil, banana flavor, preservative (sorbic acid), bleach (sulfites)
		I	ND	5.2	1.43	0.41	Sugar, papaya, bleach (sulfites)
		J	ND	12.9	1.90	0.45	Papaya, sugar, bleach (sodium sulfite)
		K	ND	ND	ND	ND	Papaya, sugar, preservative (sulfur dioxide), lime juice, lemon flavor, bleach (sulfites)
		L	ND	4.6	1.56	0.42	Sugar, papaya, bleach (sodium sulfite)
Canned papaya		A	34.6	7.0	1.07	0.62	Pineapple, papaya, guava, sugar, passion fruit juice, lemon juice
		B	24.9	237.8	1.90	2.14	Pineapple, papaya, *nata-de-coco*, sugar, citric acid
		C	37.8	4.1	0.75	0.59	Papaya, pineapple, guava, sugar, citric acid, calcium chloride
Papaya jam		A	28.8	13.1	1.77	0.60	Sugar, lemon, papaya, lemon juice
		B	38.4	4.0	1.93	0.15	Papaya, sugar
		C	25.2	120.4	1.86	1.97	Papaya, sugar, pectin, lemon juice
		D	ND	1.4	0.92	0.14	Papaya, sugar, passion fruit
		E	ND	ND	ND	ND	Sugar, hibiscus, papaya
		F	ND	9.1	1.54	0.36	Papaya, sugar, apple, ginger, lemon juice, pectin, vitamin C
		G	ND	21.6	1.49	0.60	Papaya, sugar, lemon juice

[a] Ct values greater than 48, at a threshold value of 0.2, were indicated as ND.
[b] Purification efficiency represents the amount of purified DNA per gram of starting material.

were transferred to a new tube and 2.5 mL of AP2 buffer were added. The cocktail was mixed sufficiently and kept for 15 min on ice. After centrifugation at 3000 × g for 35 min at room temperature, 8 mL of supernatant were applied to a QIA shredder column set in the tube. The tube was centrifuged at 3000 × g for 5 min at room temperature; 6.8 mL of supernatant and 10.2 mL of AP3 buffer containing 67% (v/v) ethyl alcohol were mixed, and the cocktail was applied to a DNeasy spin column. The mixed cocktail was centrifuged at 3000 × g for 15 min at room temperature and the eluate was discarded. The DNeasy spin column was washed with 12 mL of AW buffer, and transferred to a new tube. 1 mL of pre-warmed water (65 °C) was applied to the DNeasy spin column to elute the DNA. The eluate containing DNA and an equal volume of isopropyl alcohol were mixed thoroughly, and the subsequent operations were carried out according to the procedure using IER-100G.

2.6. Extraction and purification of genomic DNA by GM Quicker 3 Kit

DNA was extracted and purified from 1 g of homogenized dried papaya, using a silica membrane-type kit (GM Quicker 3; Nippon Gene, Tokyo, Japan) according to the "Protocol 2" described by the manufacturer. The procedure was as follows: 1 mL of GE1 buffer, 10 μL of RNase, 2 μL of α-amylase and 20 mL of proteinase K were added, and vortexed thoroughly, followed by incubation at 65 °C for 30 min. During incubation, samples were mixed twice by vortex. 200 μL of GE2-P buffer were added to the incubated cocktail and vortexed thoroughly. The cocktail was centrifuged at 4000 × g for 10 min at 4 °C; 800 μL of supernatant were transferred to a new tube and 600 μL of GB3 buffer were added. After mixing thoroughly by inverting the tubes, the cocktail was centrifuged at 10,000 × g for 5 min at 4 °C, and the eluate was discarded. 800 μL of supernatant were applied to a spin column and centrifuged at 10,000 × g for 0.5 min at 4 °C. The eluate was discarded and the spin column was transferred to a new tube; 50 μL of water were applied to the spin column and kept for 3 min at room temperature. The spin column and tube were centrifuged at 10,000 × g for 1 min at 4 °C. The eluate was used as the DNA sample solution.

2.7. Extraction and purification of genomic DNA by Wizard Cleanup Resin System

DNA was extracted and purified from 0.5 g of homogenized dried papaya, using a silica base-type kit (Wizard Cleanup Resin; Promega), as described in a previous report (Ohmori et al., 2008).

2.8. Evaluation of the purity and concentration of the extracts

The DNA concentration of the purified genomic DNA was measured by UV absorption at 260 nm, and the DNA purity was evaluated on the basis of the absorbance ratios at 230, 260 and 280 nm (A_{260}/A_{280}, and A_{260}/A_{230}), using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). To prepare DNA samples for real-time PCR assays, the purified genomic DNAs were diluted to 10 ng/μL using distilled water, whereas those at 10 ng/μL or lower concentrations were used directly in experiments.

2.9. Real-time PCR

Real-time PCR assays were performed using the ABI PRISM™ 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA, USA). The reaction mixture (25 μL) consisted of 2.5 μL of DNA sample solution, 12.5 μL of TaqMan® Universal PCR Master Mix (Life Technologies), 0.8 μM forward and reverses primers, and 0.1 μM probe. The PCR conditions were as follows: 2 min at 50 °C, then 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C, and finally 1 min at 60 °C under the 9600 emulation mode. To detect the papaya endogenous internal control gene, *chymopapain* (Chy; GenBank ID: AY803756), we used the following primers and probe (amplicon size, 72 bp), based on published reports (Guo et al., 2009; Nakamura et al., 2011).

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Forward primer: 5′-CCATGCGATCCTCCCA-3′ (Q-Chy-1F2)
Reverse primer: 5′-CATCGTAGCCATTGTAACACTAGCTAA-3′ (Q-Chy-2R)
Probe: 5′-FAM-TTCCCTTCAT (BHQ1)CCATTCCCACTCTTGAGA-3′ (Q-Chy-P)

Primers and probe were diluted with an appropriate volume of distilled water, and stored at −20 °C until use. Analyses were performed using SDS 2.1 Sequence Detection Software (Life Technologies) for ABI PRISM™ 7900 Sequence Detection System. The number of copies of Chy present in the DNA samples were estimated from the standard curve generated using a reference plasmid containing Chy amplicon inserted into a pGEM-T Easy vector (Promega). The sequence of the insert was verified using pUC/M13 forward and reverse sequencing primers.

2.10. Statistical analysis

A two-sided Dunnett test, with a significance level of 5% ($p < 0.05$), was performed to determine significant differences. IBM SPSS Statistics 20 software package (SPSS, 2011) was used for the analyses. Three or six replicates were tested.

3. Results and discussion

3.1. Sampling for the IER-100G method

Two combinations of sample weight and buffer volume were tested for the *IER-100G* method. The first included 10 g of homogenized dried papaya with 30 mL of buffer G2, and enzymes (20 μL of 100 mg/mL RNAse, 500 μL of cellulase and 200 μL of 20 mg/mL proteinase K). The second included 20 g of homogenized sample with 20 mL of buffer G2, and the same enzymes. The DNA yield of the former combination was approximately 700 ng/μL, but that of the latter was close to zero (data not shown). The cause of the result was considered as follows. QIAGEN Genomic-tips contain an anion-exchange resin which is based on the interaction between negatively charged phosphates of the DNA backbone and positively charged diethylaminoethyl (DEAE) groups on the surface of the resin. The salt concentration and pH conditions of the buffers used determine whether DNA is bound or eluted from the column (Qiagen, 2001). The 20 g of homogenized sample which mixed with 20 mL of buffer G2 possibly varied the salt concentration or pH conditions of the buffers. It was considered that the ability of DNA binding to the surface of the anion-exchange resin was inhibited with the 20 g of sample. Therefore, for the extraction and purification of DNA from processed papaya products using the IER-100G method, we set the weight of the sample to 10 g and the volume of buffer G2 to 30 mL.

3.2. DNA elution for the IER-100G method

For the dried papaya and canned papaya samples, we applied 1 mL of buffer QF five times on the IER-100G column, and the DNA concentration of each eluate was determined. At the second and third elution, 95% of the DNA was recovered (Fig. 1). Therefore, we improved the DNA elution protocol, by reducing the volume of buffer QF. After washing the IER-100G column with buffer QC, 1 mL of pre-warmed buffer QF (50 °C) was applied and discarded. Then, the IER-100G column was transferred to a fresh centrifuge tube, and 2 mL of pre-warmed buffer QF were added to elute the DNA, followed by the addition of an equal volume of isopropyl alcohol, and centrifugation, as described in Section 2.2. In this way, a single centrifugation for DNA collection could be accomplished using three 1.5 mL tubes.

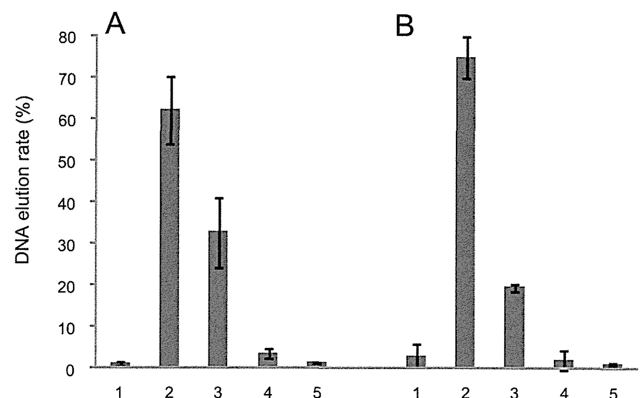

Fig. 1. DNA elution rate after the addition of QF buffer ($n = 3$). A, Dried papaya; B, Canned papaya. 1, first elution; 2, second elution; 3, third elution; 4, fourth elution; 5, fifth elution.

3.3. Effects of enzymes on the amount of extracted DNA

Processed foods contain many components, such as protein and sugar. Since these components could interfere with PCR, it is necessary to extract high purity DNA by removing the interfering components. α-Amylase hydrolyzes the α-bonds of α-linked polysaccharides, such as starch and glycogen, yielding oligosaccharides with varying lengths (Sales, Souza, Simeoni, & Silveira, 2012). Cellulase is an enzyme which hydrolyzes β-1,4-glucosidically linked cellulose chains, which form the cell walls of plants (Pang et al., 2009). Proteinase K is a non-specific, subtilisin-related serine protease, widely used to remove contaminants from the liquid formulations of nucleic acids by hydrolyzing the protein (Siezen & Leunissen, 1997). By adding proteinase K, it is possible to inactivate nucleases that quickly degrade nucleic acids. Since α-amylase and proteinase K are relatively expensive enzymes, we evaluated the effects of the added amounts of these enzymes on the yield and purity of DNA extracted from dried papaya (Fig. 2A). When no enzymes were added except for RNase a DNA concentration of 55 ± 3 ng/μL was obtained. When α-amylase or cellulose were added to the first incubation at 50 °C for 1 h, the obtained DNA concentrations were 29 ± 16 ng/μL and 45 ± 11 ng/μL, respectively. The concentration of extracted DNA was significantly higher when proteinase K was added to the second incubation at 50 °C for 1 h (702 ± 17 ng/μL). The highest DNA concentration was obtained when proteinase K, α-amylase and cellulase were added simultaneously (716 ± 27 ng/μL) (Fig. 2A). In terms of the purity of the extracted DNA, no significant differences were found in the A_{260}/A_{230} and A_{260}/A_{280} ratios of the DNA sample solutions (Fig. 2B). Therefore, these results suggest that proteinase K is necessary for a higher yield of DNA from dried papaya. On the other hand, we considered that α-amylase is not required for the extraction and purification of DNA from dried papaya. By the addition of cellulase, the concentration of the extracted DNA in the sample solution did not change; however, the clogging of the IER-100G column during DNA purification, especially in the case of dried papaya, was prevented. We thus consider that a cellulase digestion step is necessary for the extraction of papaya DNA.

3.4. Effect of proteinase K on DNA extraction

We found that proteinase K is indispensable for the extraction and purification of DNA from dried papaya. So, we examined the effect of the amount of proteinase K on the extraction of DNA from dried papaya (Fig. 3). There was no significant difference in the

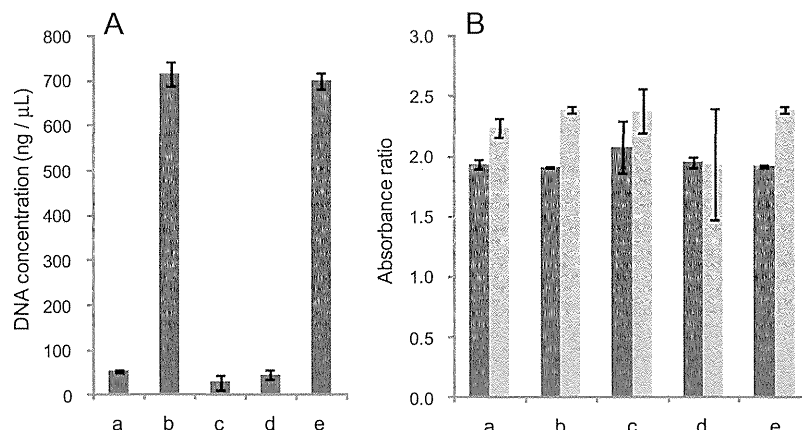

Fig. 2. Quantification of DNA extracted from dried papaya under different conditions, and evaluation of the quality of the extracted DNA ($n = 3$). a, Without added enzymes; b, With α-amylase, cellulase and proteinase K; c, With α-amylase; d, With cellulase; e, With proteinase K. A, DNA concentration; B, DNA purity, determined by the UV absorbance ratio ▩ A_{260}/A_{280} ratio; □ A_{260}/A_{230} ratio.

yield and purity of the extracted DNA when using 4 mg (final concentration 100 μg/mL) and 8 mg (final concentration 200 μg/mL) of proteinase K. However, the DNA yield of the sample extracted using 2 mg of proteinase K (final concentration 50 μg/mL) was 80% of that obtained with 4 mg of proteinase K (final concentration 100 μg/mL) (Fig. 3A). The A_{260}/A_{280} ratio of the DNA sample solution extracted with 2 mg of proteinase K was significantly smaller than that of the sample extracted with 4 mg of proteinase K (Fig. 3B). In addition, there was no significant difference in the estimated total number of copies of the papaya endogenous internal control gene, Chy, in the DNAs extracted with the three concentrations (50, 100, 200 μg/mL) of proteinase K (Fig. 3C). Therefore, we found that the addition of 4 mg of proteinase K, at final concentration of 100 μg/mL, was sufficient to extract DNA for PCR analysis.

3.5. Effect of α-amylase on DNA extraction

The effect of α-amylase on DNA recovery was described in Section 3.3. We examined the effect of α-amylase (20 μL) in the presence of proteinase K (4 mg) on the extraction of DNA from dried papaya, canned papaya and papaya jam. For dried papaya and canned papaya, there was no significant difference in the quality (the purity reflected in the absorbance ratios, A_{260}/A_{280} and A_{260}/A_{230}) and quantity (the total number of copies of Chy) of the DNA samples extracted with and without added α-amylase (Figs. 4 and 5). Also, there was no significant difference between the qualities of the DNA samples extracted from papaya jam with and without added α-amylase (Fig. 6A and B). However, the number of copies of Chy in the DNA samples extracted from papaya jam with added α-amylase was significantly higher than that obtained without α-amylase (Fig. 6C). Papaya jam contains pectins, which are rich in galacturonic acid. Homogalacturonans are linear chains of α-(1–4)-linked D-galacturonic acid, but pectin is a not digested by α-amylase. Thus, it was suspected that the PCR inhibitor was removed from the DNA sample solution at the α-amylase digestion step. For this reason, we consider that for the extraction of DNA from papaya jam, the incubation with α-amylase is essential, although the preparation from dried or canned papaya was carried out without the need of α-amylase digestion.

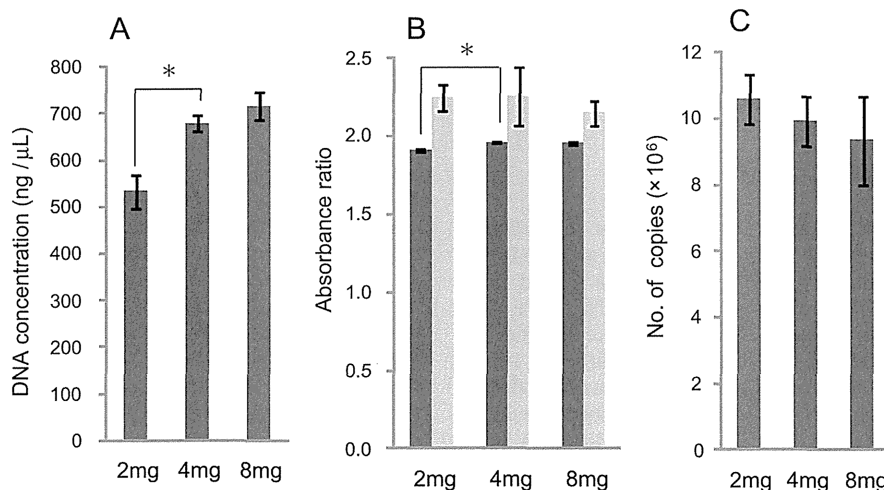

Fig. 3. Effect of proteinase K on the concentration and quality of DNA extracted from dried papaya ($n = 6$). The horizontal axis indicates the amount of proteinase K. A, DNA concentration; B, DNA purity, determined by the UV absorbance ratio; C, Number of copies of the Chy gene in 50 ng of DNA (threshold line, 0.2). ▩ A_{260}/A_{280} ratio; □ A_{260}/A_{230} ratio.

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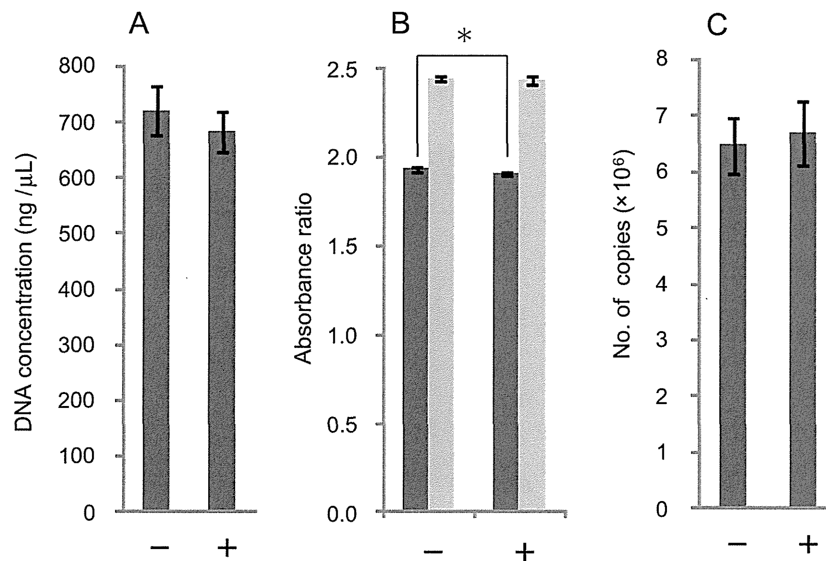

Fig. 4. Effect of α-amylase on the concentration and quality of DNA extracted from dried papaya ($n = 6$). −, without α-amylase; +, with α-amylase. A, DNA concentration; B, DNA purity, determined by the UV absorbance ratio; C, number of copies of the Chy gene in 50 ng of DNA (threshold line, 0.2). ■ A_{260}/A_{280} ratio; □ A_{260}/A_{230} ratio.

3.6. Comparative study of the six methods for DNA extraction

The final protocol for the extraction and purification of DNA from dried papaya, canned papaya and papaya jam is described in the Materials and methods section. The yield and purity of the DNAs extracted using the proposed IER-100G method were compared with those of the DNAs extracted using the IER-20G method, QIAamp DNA Stool Mini Kit, DNeasy Plant Maxi Kit, GM Quicker 3 Kit and Wizard Cleanup Resin System (Table 1). The DNA yield obtained from 1 g of dried papaya, using the proposed IER-100G method, was 1437 ng. The amount of DNA extracted using the IER-100G method was less than the amounts extracted using the QIAamp DNA Stool Mini Kit and the Wizard Cleanup Resin System. However, the estimated number of copies of Chy in 25 ng of DNA extracted using the proposed IER-100G method was 30 times higher than the number of copies obtained with the QIAamp DNA Stool Mini Kit and the Wizard Cleanup Resin System. DNA purification on IER-100G and IER-20G is based on the interaction between negatively charged phosphates of the DNA backbone and positively charged DEAE groups on the surface of the anion-exchange resin. The salt concentration and pH conditions of the buffers determine whether DNAs are bound or eluted from the column. Impurities such as RNA, protein, carbohydrates, and small metabolites are washed from IER-100G and IER-20G with medium-salt buffers, while DNA remains bound, until eluted with a high-salt buffer. Furthermore, the advantage of IER-100G and IER-20G arises from their exceptionally high charge density. The resin consists of defined silica beads with a particle size of 100 μm, a large pore size,

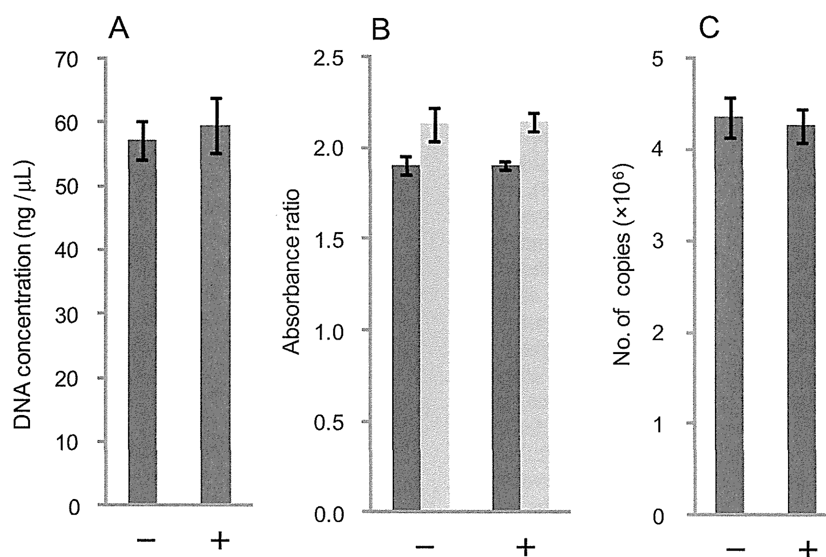

Fig. 5. Effect of α-amylase on the concentration and quality of DNA extracted from canned papaya ($n = 6$). −, without α-amylase; +, with α-amylase. A, DNA concentration; B, DNA purity, determined by the UV absorbance ratio; C, number of copies of the Chy gene in 50 ng of DNA (threshold line, 0.2). ■ A_{260}/A_{280} ratio; □ A_{260}/A_{230} ratio.

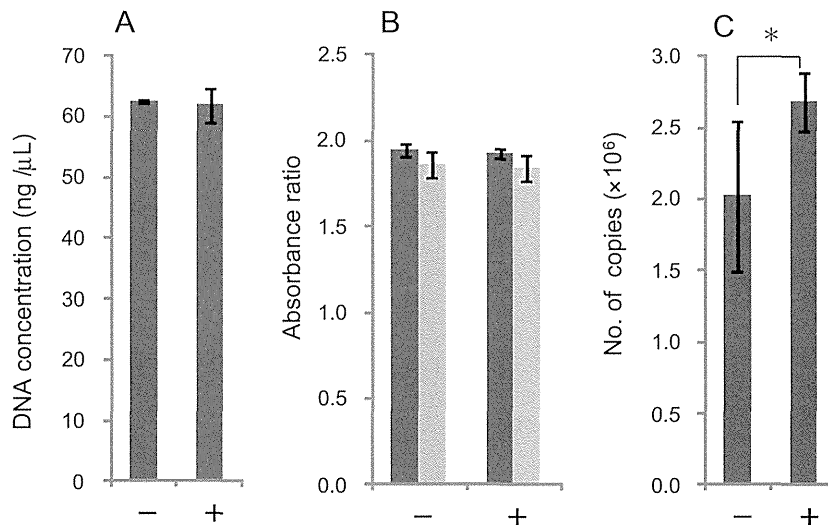

Fig. 6. Effect of α-amylase on the concentration and quality of DNA extracted from papaya jam ($n = 6$). −, without α-amylase; +, with α-amylase; *, $p < 0.05$, t-test. A, DNA concentration; B, DNA purity, determined by the UV absorbance ratio; C, number of copies of the Chy gene in 50 ng of DNA (threshold line, 0.2). ■ A_{260}/A_{280} ratio; □ A_{260}/A_{230} ratio.

and a hydrophilic surface coating. Meanwhile, the other 4 kits are silica membrane-type kits. DNA binds to the silica membrane and impurities are washed away. DNA bound to the silica membrane spin column is eluted using buffers with optimized salt concentrations and pH conditions. The true purity of DNA purified using anion-exchange resin might be higher than that obtained with silica membrane.

Furthermore, the DNA yield from 1 g of canned papaya, obtained with the proposed IER-100G method, was twice as high as that obtained with the IER-20G method. The DNA concentration of the sample prepared by the IER-20G method was less than 10 ng/μL, but that of the proposed IER-100G method was 66 ng/μL. From these results, we consider that the proposed IER-100G method is the best among the six evaluated methods for the extraction and purification of DNA from processed papaya products.

3.7. Applicability of the method to processed papaya products

The method for the extraction and purification of papaya DNA proposed in this report was applied to a total of twenty-one samples from five types of papaya processed products (Table 2). The DNA purification efficiency and purity of DNA were estimated from the absorbance measurements. For dried papaya A–E (unbleached, no sugar added), dried papaya G (candied, unbleached), canned papaya B and papaya jam C, the purification efficiency was higher than 100 ng of DNA per 1 g of sample, and the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} were higher than 1.8. Efficient DNA extraction and purification were achieved, as well as sufficient detection of Chy using real-time PCR, with Ct values lower than 26. On the other hand, dried papaya samples H–L (bleached with sulfites) yielded little DNA and Chy could not be detected with real-time PCR. It is well known that bleaching agents degrade DNA. It was reported that intra-nucleotide bonds of DNA were cleaved by concentrations ranging from 1 to 10 mM (104–1040 mg/L) sodium hydrogen sulfite solutions by a mechanism believed to involve free radical formation (Hayatsu & Miller, 1972). The dry fruits treated with sulfites in order to breach or preservatives were containing sulfites at concentrations ranging from 10 to 1000 mg/kg. The highest maximum level of sulfites for dry fruits was 2000 mg/kg in Australia and New Zealand (WHO, 2009). Therefore, we considered that the five DNA samples extracted from bleached papaya were degraded to fragments smaller than the amplicon size of Chy detection (72 bp). For those samples with a DNA purification efficiency lower than 20 ng/g (canned papaya A and C, and papaya jam A and B), Ct values of Chy were relatively high (Ct > 29). The Ct values of Chy for three of the DNA samples from papaya jam (A, B and C) were lower than 39, but for the other four samples, Chy could not be detected. This suggests that for highly processed jams, degradation of DNA is likely to occur to the limit of detection.

4. Conclusions

We evaluated different methods to obtain a high DNA yield and purity from three processed papaya products (dried papaya, canned papaya and jam). The extraction of DNA from dried papaya and canned papaya was successfully carried out with the addition of digestive enzymes, including RNase, cellulase and proteinase K. In the case of papaya jam, the extraction of DNA was carried out with the above-mentioned enzymes plus α-amylase. The DNA yield and purity obtained with the proposed IER-100G method was considerably higher than the ones obtained with the other five methods (Genomic-tip 20G Kit, QIAamp DNA Stool Mini Kit, DNeasy Plant Maxi Kit, GM Quicker 3 Kit and Wizard Cleanup Resin System). The IER-100G method was found suitable for the extraction and purification of DNA from processed papaya products.

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FOOD COMPOSITION AND ADDITIVES

Interlaboratory Study of Qualitative PCR Methods for Genetically Modified Maize Events MON810, Bt11, and GA21, and CaMV P35S

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Qualitative PCR methods for the genetically modified (GM) maize events MON810, Bt11, and GA21, and the 35S promoter (P35S) region of the cauliflower mosaic virus (CaMV), were evaluated in an interlaboratory study. Real-time PCR-based quantitative methods for these GM events using the same primer pairs had already been validated in previous studies. Fifteen laboratories in Japan participated in this interlaboratory study. Each participant extracted DNA from blind samples, performed qualitative PCR assays, and then detected the PCR products with agarose gel electrophoresis. The specificity, sensitivity, and false-negative and false-positive rates of these methods were determined with different concentrations of GM mixing samples. LODs of these methods for MON810, Bt11, and GA21, and the P35S segment, calculated as the amount of MON810 were 0.2, 0.2, 0.1, and 0.2% or less, respectively, indicating that the LODs of MON810, Bt11, and P35S were lower than 10 copies and the LOD of GA21 was lower than 25 copies of maize haploid genome. The current study demonstrated that the qualitative methods would be fit for the detection and identification of these GM maize events and P35S segment.

Genetically modified (GM) crops have been developed and commercialized since 1994. The cultivated area of GM crops has continuously increased and exceeded 160 million hectares in 2011 (1). Even though many countries have authorized the use of GM crops, some consumers have expressed concerns about the utilization of GM organisms (GMOs) in food or feed. In response, several countries have legislated labeling systems to indicate the presence of authorized GM products in crops or foods (2–4). To enforce the labeling system, the development of validated analytical methods is requisite for detecting the unintentional commingling of GMOs.

Maize (*Zea mays* L.) is one of the major crops in the world. GM maize has been emerging since the early days of GM development, and various kinds of GM maize events are currently distributed worldwide. The major traits of GM maize are herbicide tolerance (T25, NK603, GA21, etc.); insect resistance (MON863, MON810, etc.); and combinations of these traits (Bt11, TC1507, etc.).

In Japan, quantitative methods of five GM maize events (Bt11, Bt176, GA21, MON810, and T25) and a glyphosate-tolerant soybean (GTS) Roundup Ready soy (RRS) using real-time PCR were developed and validated in interlaboratory collaborative studies (5, 6) and adopted as the Japanese standard methods (7, 8). The real-time PCR system is widely used as a powerful tool to quantify GM amounts in samples and is also used for the qualitative detection of the GMO (9–11). The real-time PCR system is recognized as a useful tool but requires difficult and time-consuming procedures and expensive instruments and reagents. In comparison, conventional PCR systems are simple, relatively less time-consuming, and moderately priced. These systems are, thus, often used as qualitative analyses at the first stage of GMO monitoring to determine whether GMOs are present, and, if so, which GM events are included in test samples (12). In the Japanese standard method, the presence of GM maize in foods or food products is in fact primarily detected with a conventional PCR-mediated qualitative method. If the qualitative analysis shows positive results, quantitative analyses will be performed to assess the amount of GMO content.

Although there are no internationally harmonized guidelines to validate qualitative detection methods, several rules and criteria for collaborative procedures concerning validation have been proposed (13–15). A number of collaborative studies aiming to validate PCR-based qualitative methods have been reported including GMO detection methods (16–20).

In this study, we describe the results of an interlaboratory evaluation for the qualitative detection methods of three GM

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Table 1. Content of mixing test samples

MON810 and P35S analyses	Bt11 and GA21 analyses
1) non-GM (100%)	1) non-GM (100%)
2) MON810 (0.2%) + non-GM (99.8%)	2) Bt11 (0.2%) + GA21 (0.1%) + non-GM (99.7%)
3) MON810 (0.4%) + non-GM (99.6%)	3) Bt11 (0.4%) + GA21 (0.2%) + non-GM (99.4%)

events: MON810, Bt11, and GA21 as representatives of typical GM maize events, and the P35S segment in MON810.

Experimental

Plant Materials

We used F1 generation seeds of three GM maize events. MON810 was kindly provided by Monsanto (St. Louis, MO), and Bt11 and GA21 by Syngenta Seeds (Basel, Switzerland). MRX3 maize was purchased from Pioneer Hi-Bred International (Johnston, IA) and used as a non-GM maize sample.

Preparation of Test Samples and DNA Extraction

To prepare the mixed samples, the GM and non-GM seeds were independently ground. The ground seeds were lyophilized by a freeze drier and then mixed on a weight-to-weight basis. We prepared two sets of three mixing levels of test samples. One set consisted of 0, 0.2, and 0.4% of MON810 for the qualitative analyses of MON810 and P35S, and the other consisted of Bt11 and GA21 in non-GM maize, at the concentration of 0 and 0%, 0.2 and 0.1%, and 0.4 and 0.2% of Bt11 and GA21, respectively (Table 1). Each mixture sample weighed about 300 g and was divided into 1.0 g aliquots in a bottle. We prepared mixing samples in which the concentrations of GA21 were lower than those of the other two events, MON810 and Bt11. Our previous studies, which were conducted to validate quantitative methods for GM maize events (5, 6), suggested that the LOQ for GA21 were lower than those of MON810 and Bt11. We then assumed that GA21 would be detectable in a lower concentration than MON810 and Bt11. tbl1

Genomic DNA was extracted from those ground materials using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) with slight modifications (20). The concentration of the extracted DNA solutions was determined by measuring the UV absorbance at 260 nm. The quality of the DNA solutions was evaluated based on the 260/280 and 260/230 nm UV absorption ratios. Maize genomic DNA solutions were adjusted at a concentration of 10 or 20 ng/μL for the qualitative or quantitative analyses, respectively.

Oligonucleotide Primers

The primers and probes used in this study are listed in Table 2. The maize *starch synthase IIb* (*SSIIb*) gene (21) was used as a maize-specific endogenous reference DNA for experimental control (6). The specificity of these primers was already confirmed in our previous studies (22). The oligonucleotide primers were synthesized by FASMAC (Kanagawa, Japan). The TaqMan probes that were labeled with 6-carboxyfluorescein (FAM) at the 5′ ends and 6-carboxytetramethylrhodamin (TAMRA) at the 3′ ends were synthesized by Life Technologies (Carlsbad, CA). tbl2

Quantitative PCR

TaqMan real-time PCR assays were carried out using the ABI PRISM 7900HT (Life Technologies) in 25 μL final volume reactions consisting of 200 ng sample DNA instead of 50 ng template DNA that is used in our validated quantitative methods (5, 6), 12.5 μL Universal Master Mix (Life Technologies), 0.5 μM primer pairs, and a 0.2 μM probe. The step-cycle program was as follows: 2 min at 50°C, 10 min at 95°C, 45 cycles, 30 s at 95°C, and 1 min at 59°C. Each sample was measured in triplicate. A dilution series of calibrant

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Table 2. List of primers and TaqMan probes

Target	Name	Sequence	Length, bp	Ref.
zSSIIb	SSIIb 3-5′	CCA ATC CTT TGA CAT CTG CTC C		
	SSIIb 3-3′	GAT CAG CTT TGG GTC CGG A	114	6
	SSIIb-Taq	5′-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3		
MON810	MON810 3-5′	GAT GCC TTC TCC CTA GTG TTG A		
	MON810 2-3′	GGA TGC ACT CGT TGA TGT TTG	113	22
	MON810-Taq	5′-FAM-AGA TAC CAA GCG GCC ATG GAC AAC AA-TAMRA-3′		
Bt11	Bt11 3-5′	AAA AGA CCA CAA CAA GCC GC		
	Bt11 3-3′	CAA TGC GTT CTC CAC CAA GTA CT	127	22
	Bt11-2-Taq	5′-FAM-CGA CCA TGG ACA ACA ACC CAA ACA TCA-TAMRA-3′		
GA21	GA21 3-5′	GAA GCC TCG GCA ACG TCA		
	GA21 3-3′	ATC CGG TTG GAA AGC GAC TT	133	22
	GA21-2-Taq	5′-FAM-AAG GAT CCG GTG CAT GGC CG-TAMRA-3		
P35S	P35S 3-5′	ATT GAT GTG ATA TCT CCA CTG ACG T		
	P35S 3-3′	CCT CTC CAA ATG AAA TGA ACT TCC T	101	22
	P35S-Taq	5′-FAM-CCC ACT ATC CTT CGC AAG ACC CTT CCT-TAMRA-3		

Table 3. Homogeneity of simulated mixtures

	% (w/w)	Measured mean, %	*F*-value[a]	*P*-value
MON810	0.20	0.24	0.35	0.94
Bt11	0.20	0.18	1.36	0.32
GA21	0.10	0.08	1.12	0.43
MON810	0.40	0.29	0.66	0.73
BT11	0.40	0.42	0.25	0.97
GA21	0.20	0.17	0.42	0.90

[a] Critical value of F is 3.02 (α = 0.05).

plasmid, pMul5 (22), with 20, 125, 1500, 20 000, and 250 000 copies, was used to prepare the calibration curves.

Qualitative PCR

The amplifications were carried out in 25 µL volume reaction solutions, with 25 ng sample DNA, 200 µM **[AU: SPELL OUT] dNTP**, 1.5 mM $MgCl_2$, 0.625 U AmpliTaq Gold polymerase (Life Technologies), and 0.25 µM of each primer. For the detection of Bt11, 1.2 mM $MgCl_2$ was used instead of 1.5 mM (23). The reactions were buffered with the PCR buffer II (Life Technologies) and amplified using a thermal cycler, GeneAmp PCR system 9700 (Life Technologies) with the following step-cycle program: 10 min at 95°C, 40 cycles, 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by a final extension at 72°C for 7 min. Five microliters of PCR products were electrophoresed on 3.0% agarose gel supplemented with 0.5 µg/mL of ethidium bromide in Tris-acetate-EDTA (TAE) buffer.

Interlaboratory Evaluation: Blind Test

The interlaboratory study was designed and the results were analyzed as previously described (20). Fifteen laboratories in Japan participated. The experimental protocols and test samples were prepared and provided by the National Food Research Institute (NFRI). Other experimental reagents such as the DNeasy Plant Mini Kits, AmpliTaqGold DNA polymerase; PCR reaction buffer; dNTP; $MgCl_2$; primer pairs for MON810, Bt11, GA21, and P35S; and GM maize detection positive control plasmids, pMul5, were prepared and supplied by the Food and Agricultural Materials Inspection Center (Saitama, Japan). Each laboratory received 36 blind coded samples including all concentrations of GM maize events.

All participants were first requested to extract DNA from the samples using a DNeasy Plant Mini Kit. The concentrations and qualities were evaluated as described above. The qualitative PCR analyses for MON810, Bt11, GA21, and P35S were then conducted. In addition, one positive and two negative controls were prepared. As the positive control, GM maize detection positive control plasmid (NIPPON GENE, Tokyo, Japan) was used as a template instead of extracted maize DNA. The same PCR reactions were performed for the negative controls as for the positive control, except that the reactions were either performed without template or without primers. The results of

Table 4. Results of interlaboratory study for detection of MON810

	Test samples																				
	Positives														Negatives						
	0.2%							0.4%							0.0%						
	Replicate No.							Replicate No.							Replicate No.						
Labs	1	2	3	4	5	6	No. of positive	1	2	3	4	5	6	No. of positive	1	2	3	4	5	6	No. of negative
1	+[a]	+	+	+	+	+	6	+	+	+	+	+	+	6	–[b]	–	–	–	–	–	6
2	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
3	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
4	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
5	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
6	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
7	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
8	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
9	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
10	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
11	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
12	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
13	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
14	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
15	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6

[a] Positive response.

[b] Negative response.

Table 5. Results of interlaboratory study for detection of Bt11

	Test samples																				
	Positives														Negatives						
	0.2%							0.4%							0.0%						
	Replicate No.							Replicate No.							Replicate No.						
Labs	1	2	3	4	5	6	No. of positive	1	2	3	4	5	6	No. of positive	1	2	3	4	5	6	No. of negative
1	+[a]	+	+	+	+	+	6	+	+	+	+	+	+	6	–[b]	–	–	–	–	–	6
2	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
3	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
4	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
5	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
6	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
7	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
8	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
9	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
10	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
11	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
12	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
13	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
14	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
15	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6

[a] Positive response.

[b] Negative response.

Table 6. Results of interlaboratory study for detection of GA21

	Test samples																				
	Positives														Negatives						
	0.1%							0.2%							0.0%						
	Replicate No.							Replicate No.							Replicate No.						
Labs	1	2	3	4	5	6	No. of positive	1	2	3	4	5	6	No. of positive	1	2	3	4	5	6	No. of negative
1	+[a]	+	+	+	+	+	6	+	+	+	+	+	+	6	–[b]	–	–	–	–	–	6
2	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
3	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
4	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
5	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
6	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
7	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
8	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
9	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
10	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
11	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
12	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
13	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
14	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
15	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6

[a] Positive response.

[b] Negative response.

Author proof

Table 7. Results of interlaboratory study for detection of P355

	Test samples																				
	Positives														Negatives						
	0.2%							0.4%							0.0%						
	Replicate No.							Replicate No.							Replicate No.						
Labs	1	2	3	4	5	6	No. of positive	1	2	3	4	5	6	No. of positive	1	2	3	4	5	6	No. of negative
1	+[a]	+	+	+	+	+	6	+	+	+	+	+	+	6	–[b]	–	–	–	–	–	6
2	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
3	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
4	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
5	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
6	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
7	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
8	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
9	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
10	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
11	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
12	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
13	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
14	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6
15	+	+	+	+	+	+	6	+	+	+	+	+	+	6	–	–	–	–	–	–	6

[a] Positive response.

[b] Negative response.

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the PCR were judged acceptable when the positive control was detected and the negative controls were not. All of the resulting data of the DNA measurements, electrophoresis images, and qualitative judgments were submitted to the NFRI, and the submitted data of detection were analyzed by Cochran's Q-test to remove laboratories with a different proportion of correct responses (15, 24).

Results and Discussion

Homogeneity of Test Samples

Confirmation of the homogeneities of test samples for qualitative analyses would be difficult (25). We evaluated the homogeneities of test samples by the validated real-time PCR-mediated quantitative methods for MON810, Bt11, and GA21 (5, 6) using the averages of the one-way analysis of variance (ANOVA) for each mixing level as described previously (20). All mixing samples were divided into 300 independent aliquots, and two sets of 10 sample tubes were randomly selected. The DNAs were then extracted with a DNeasy Plant Maxi Kit (QIAGEN), and quantitative PCRs were performed using MON810, Bt11, and GA21 specific quantification systems (5, 6, 22). The calculated copy number ratios of the GMO amount (%) were converted into weight ratios using conversion factors determined by Kodama et al. (6). One-way ANOVA was then conducted to compare the between- and within-sample variances referring to the previous reports (5, 6, 20). The smallest P-value, 0.32, obtained from 0.2% of the Bt11 sample was larger than 0.05 (Table 3). Therefore, the contents of MON810, Bt11, and GA21 of all the test samples were sufficiently homogeneous and met the requirements for the following interlaboratory study. tbl3

Blind Test: Detection of MON810, Bt11, GA21, and P35S

As one of the criteria for a collaborative study regarding qualitative analysis methods, McClure proposed that Lm^2

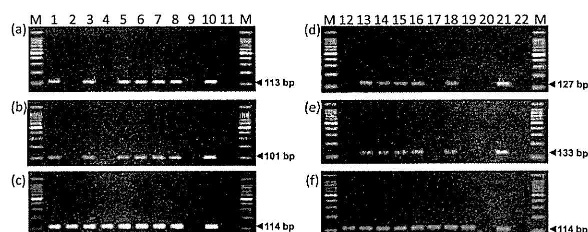

Figure 1. Typical results of agarose gel electrophoresis of PCR products corresponding to (a) MON810, (b) P35S, (c) and (f) SSIIb, (d) Bt11, and (e) GA21 from blind samples. Lanes 1, 5, and 8, amplification of 0.2% MON810 sample; lanes 2 and 4, amplification of 0% MON810 sample; lanes 3, 6, and 7, amplification of 0.4% MON810 sample in (a)–(c); lanes 12 and 17, amplification of 0% Bt11and GA21 samples; lanes 13, 16, and 18, amplification of 0.4% Bt11 and 0.2% GA21 samples; lanes 14 and 15, amplification of 0.2% Bt11 and 0.1% GA21 samples in (d) and (e); lanes 9 and 20, negative control (no template DNA); lanes 10 and 21, positive control (plasmid pMul5); lanes 11 and 22, negative control (no primer); and M, 100 bp ladder size marker.

Table 8. Statistical evaluation of data obtained in this interlaboratoy study

	Concn level, %	Sensitivity, %	Specificity, %	False negative rate, %	False positive rate, %	Accordance, %	Concordance, %	COR[a]
MON810	0	—	100	—	0	100	100	1
	0.2	100	—	0	—	100	100	1
	0.4	100	—	0	—	100	100	1
Bt11	0	—	100	—	0	100	100	1
	0.2	98.9	—	1.1	—	97.8	97.8	1
	0.4	100	—	0	—	100	100	1
GA21	0	—	100	—	0	100	100	1
	0.1	98.9	—	1.1	—	97.8	97.8	1
	0.2	100	—	0	—	100	100	1
P35S	0	—	100	—	0	100	100	1
	0.2	100	—	0	—	100	100	1
	0.4	100	—	0	—	100	100	1

[a] COR = Concordance odds ratio.

should be over 362, where L is the number of laboratories and m is the number of test portions per laboratory (14). Fifteen laboratories participated in the present study, and the number of samples at the same concentration in each laboratory was six. Therefore, $Lm^2 = 540$ and met the criterion.

Each participating laboratory first received a total of 36 blind samples, which consisted of two sets of 18 blind samples comprising six replications of three mixing levels of MON810 or Bt11 and GA21. DNA was extracted from a total of 540 blind samples by the 15 participants. The quality and quantity of all DNA samples were acceptable (data not shown).

Detection of MON810, Bt11, GA21, and P35S was conducted with qualitative PCR methods, and the data submitted from the participants were summarized (Tables 4–7). No participant obtained significantly different proportions from those of the other participants by Cochran's Q-test. Tables 4–7 show the results of MON810, Bt11, GA21, and P35S, respectively. Although participant No. 7 did not detect 0.2% of Bt11 and 0.1% of GA21 just once in six repetitions, all remaining results were correct. Typical electrophoresis results are shown in Figure 1. The sensitivity, specificity, false-negative, and false-positive rates for MON810, Bt11, GA21, and P35S were calculated (Table 8). The values of the specificity were 100% for all four methods, indicating the high specificities of these primer pairs that have been used in practical monitoring in Japan. The interlaboratory study revealed that the four detection methods also have high sensitivities. The MON810 and P35S detection methods showed 100% sensitivities, even at lower concentrations. In the Bt11 and GA21 methods, the sensitivities were increased when the samples containing higher GMO amounts (%) were analyzed. tbl4-7, fig1, tbl8

In collaborative trials for qualitative methods, the accordance value, which is an agreement within laboratories meaning repeatability, and the concordance value, which is an agreement between laboratories meaning reproducibility, have often been evaluated (26). We calculated the accordance and concordance values in each method, and all obtained values were sufficiently high, over 97.8%. The concordance odds ratio (COR) value, which is expressed as the combination of accordance and concordance values, was also evaluated. The closer the value is to 1.0, the higher the likelihood of obtaining the same results from two identical samples that are analyzed at the same or different laboratories (26). All obtained COR values in the four methods were almost 1.0, indicating that the interlaboratory variations of our qualitative PCR method were acceptable.

The LODs of the MON810, Bt11, and GA21, and P35S segments in MON810 in these methods were determined to be 0.2, 0.2, 0.1, and 0.2% or less, respectively. The maize genome was considered to be 2.3-gigabase (27), corresponding to approximately 2.52 pg per haploid genome. In this study, 25 ng of maize genome DNA was used as a template in each qualitative analysis. When the F1 hybrid of a GM maize includes the single copy of transgene per genome, 0.1% corresponds to 4.96 copies. The LODs of the MON810, Bt11, and GA21, and P35S segments were then also expressed as 9.92, 9.92, 24.8, and 9.92 copies or less, respectively. In our previous international validation studies, the LOQs of MON810, Bt11, and GA21 were 0.5, 0.5, and 0.1%, respectively (5, 6), and these LOQs correspond to 49.6 copies, indicating that the determined LODs were around one-fifth to one-half of the LOQs of previously established methods in the copy number ratios. From these results, we concluded that the qualitative methods were demonstrated to be reliable and practical for the detection of GM maize events to monitor the food labeling systems.

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Author Proof

Development and Interlaboratory Validation of Quantitative Polymerase Chain Reaction Method for Screening Analysis of Genetically Modified Soybeans

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A novel real-time polymerase chain reaction (PCR)-based quantitative screening method was developed for three genetically modified soybeans: RRS, A2704-12, and MON89788. The 35S promoter (P35S) of cauliflower mosaic virus is introduced into RRS and A2704-12 but not MON89788. We then designed a screening method comprised of the combination of the quantification of P35S and the event-specific quantification of MON89788. The conversion factor (Cf) required to convert the amount of a genetically modified organism (GMO) from a copy number ratio to a weight ratio was determined experimentally. The trueness and precision were evaluated as the bias and reproducibility of relative standard deviation (RSDR), respectively. The determined RSDR values for the method were less than 25% for both targets. We consider that the developed method would be suitable for the simple detection and approximate quantification of GMO.

Key words screening; quantification; genetically modified; real-time polymerase chain reaction; soybean

In the past few decades, a huge variety of genetically modified (GM) crops have been developed and cultivated worldwide.[1] Several countries legislate the labeling systems of authorized GM crops, or their derived foods and feeds. To enforce the labeling system, it is indispensable to define a practical threshold level of GM content and to develop validated quantitative methods for the estimation of unintentional commingling of GM organisms (GMO). GM crops are generally classified in terms of event which is identified by what transgene was inserted, and where on the chromosome it was inserted. There are many GM events approved in Japan.[2]

The quantitative methods of a glyphosate-tolerant soybean (GTS) Roundup ready soy (RRS) and five GM maize events, *i.e.*, Bt11, Bt176, GA21, MON810, and T25, using real-time polymerase chain reaction (PCR) were developed and validated by an interlaboratory collaborative study,[3] and adopted as Japanese standard methods.[4]

For DNA-based detection of GM crops, screening tests are highly cost- and time-effective methods. The Ministry of Health, Labour and Welfare (MHLW) of Japan announced a combinational quantification of P35S and GA21 maize as a quantitative screening method for GM maize and it has been officially adopted.[4] Among approved GM soybean events, RRS has been cultivated since in 2001, and the commercial cultivation of a glufosinate-tolerant soybean, A2704-12 and the second generation of GTS, MON89788, have recently started. To analyze these novel GM soybean events, we have developed individual event-specific quantitative methods.[5,6] A practical quantitative screening method for GM soybeans is needed because many food products derived from soybeans are listed for labeling and then frequently analyzed in Japan.[7]

The authors declare no conflict of interest.

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In this study, we first developed a new quantitative screening method for RRS, A2704-12 and MON89788. The developed method was evaluated with an interlaboratory collaborative study.

MATERIALS AND METHODS

Plant Materials MON89788 and RRS seeds were kindly provided by Monsanto (St. Louis, MO, U.S.A.), and A2704-12 was kindly provided by the developer. Dry soybean seeds harvested in the U.S.A. in 2004 were purchased from Ryokoku Shoji (Hiroshima, Japan) and used as a non-GM soy sample.

Preparation of Oligonucleotide Primers, Probes and Calibrant Plasmids The primers and TaqMan probes used in this study are listed in Table 1. We used three standard plasmids, pMulSL2, pLLS and pSCS for the individual quantification of RRS, A2704-12 and MON89788, respectively. pSCS was also used for the P35S quantification. These primers, probes and plasmids were prepared according to the previous reports.[5,6,8]

Preparation of Test Samples and DNA Extraction To evaluate the quantitative method, we prepared five test samples that contained equal concentrations of the three GM soybean events: RRS, A2704-12 and MON89788, and each test sample consisted of a different mixing level of test materials, *i.e.*, 0%, 0.50%, 1.0%, 5.0%, and 10.0%. The mixed samples were prepared as described previously.[3] Genomic DNA was extracted from those ground materials using a DNeasy Plant Maxi kit (Qiagen, Hilden, Germany). The homogeneities of the samples were confirmed using the averages of the one-way analysis of variance according to the previous reports for all mixing levels of each GM soy event.[3,5,6] The concentrations and qualities of the extracted DNA solutions were evaluated

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Table 1. Primers and TaqMan Probes for Real-Time PCR Systems

Target	Name	Sequence	Length	Reference
MON89788	MON89788-F	5′-TCC CGC TCT AGC GCT TCA AT-3′	139 bp	5)
	MON89788-R	5′-TCG AGC AGG ACC TGC AGA A-3′		
	MON89788-P	5′FAM-CTG AAG GCG GGA AAC GAC AAT CTG-TAMRA3′		
P35S	P35S 1-5′	5′-ATT GAT GTG ATA TCT CCA CTG ACG T-3′	101 bp	8)
	P35S 1-3′	5′-CCT CTC CAA ATG AAA TGA ACT TCC T-3′		
	P35S-Taq	5′FAM-CCC ACT ATC CTT CGC AAG ACC CTT CCT-TAMRA3′		
Le1 (soybean endogenous)	Le1n02-5′	5′-GCC CTC TAC TCC ACC CCC A-3′	118 bp	8)
	Le1n02-3′	5′-GCC CAT CTG CAA GCC TTT TT-3′		
	Le1-Taq	5′FAM-AGC TTC GCC GCT TCC TTC AAC TTC AC-TAMRA3′		
RRS	RRS 01-5′	5′-CCT TTA GGA TTT CAG CAT CAG TGG-3′	121 bp	8)
	RRS 01-3′	5′-GAC TTG TCG CCG GGA ATG-3′		
	RRS-Taq	5′FAM-CGC AAC CGC CCG CAA ATC C-TAMRA3′		
A2704-12	KVM175	5′-GCA AAA AAG CGG TTA GCT CCT-3′	64 bp	6)
	SMO001	5′-ATT CAG GCT GCG CAA CTG TT-3′		
	TM031	5′FAM-CGG TCC TCC GAT CGC CCT TCC-TAMRA3′		

as described previously.[5)]

Quantitative PCR and Interlaboratory Study TaqMan real-time PCR assays were carried out using the ABI PRISM 7900HT (ABI7900) or the ABI 7500 (ABI7500) (Life Technologies). The interlaboratory study consisted of a measurement of the conversion factor (Cf) values and a blind test. These studies were performed as described previously.[3,5,6)]

RESULTS AND DISCUSSION

Determination of the Cf Values for MON89788 and P35S The Cf value for MON89788 was determined by measuring the copy numbers of endogenous gene *Le1* and MON89788. Meanwhile, to determine the Cf value for P35S, we had two choices of GM events that contain the P35S region, that is, RRS and A2704-12. RRS contains the single insertion of the transgene cassette. On the other hand, A2704-12 contains two copies of the whole recombinant segments that include P35S.[6)] We first calculated the Cf values for P35S derived from RRS and A2704-12 in a single laboratory examination with the ABI7900 (Table 2). In the real-time PCR analysis, the obtained amplification plots from RRS and A2704-12 targeting the P35S segment were clearly separated (data not shown). As expected, the evaluated Cf values for P35S from RRS and A2704-12 were close to 1.0 and 2.0, respectively, which are the theoretically estimated copy number ratios of the recombinant per taxon specific region.

It is true that screening quantitative methods can often yield an overestimation of the GMO amount. In the practical monitoring, however, it is most important to exclude the risk of underestimation by which the commingling GMO amounts surpassing the defined labeling threshold could be estimated unduly low. Therefore, we chose RRS to determine the Cf value for P35S. The Cf values for MON89788 and P35S were determined using ABI7900 and ABI7500 independently, from the results of 11 laboratories for ABI7900 and 4 laboratories for ABI7500. The measurement was repeated twice, and the Cf value was determined as the mean of values measured by these laboratories (Table 3).

Interlaboratory Evaluation of the PCR Quantification We performed an interlaboratory evaluation of the developed screening quantitative method as a blind test performed by 11 laboratories using ABI7900. The blank sample, with 0% RRS, A2704-12, and MON89788 was used to determine outlier laboratories, but no laboratory was eliminated. All the submitted data were subjected to Cochran's test ($p<0.025$) and Grubb's test ($p<0.025$) to remove outlier laboratories according to the harmonized guidelines of AOAC as previously described.[9–11)] Three Cochran outliers were detected in 0.5% of the MON89788 and 2.0% and 20.0% of the P35S quantifications. After removing these outliers, we conducted further statistical analyses. The trueness and precision were determined as previously described.[3,5,6)] The mean, bias, repeatability of relative standard deviation (RSDr), and reproducibility of RSD (RSDR) of blind samples were measured (Table 4). The determined RSDR were similar to or within a narrower range than those in previously reported GMO events.[3,5,6)] The determined biases in MON89788 quantification were also similar to those in the previously established methods. As envisaged in advance, the bias values obtained from P35S quantification were significantly high. These high bias values must be attributed to the difference of P35S copy numbers between RRS and A2704-12. The P35S contents derived from A2704-12 must have been overestimated by nearly double.

We developed a screening method for GM soybeans which quantitatively detected P35S and MON89788. Screening methods are designed on the assumption that the qualitative or quantitative tests analyze samples that may contain more

Table 2. Single Laboratory Estimation of Cf Values for P35S

	RRS		A2704-12	
	Mean	RSD	Mean	RSD
P35S	0.83	7.22	1.80	5.16

RSD: Relative standard deviation.

Table 3. Summary of Cf Values for ABI PRISM 7900 and 7500

	7900		7500	
	Mean	RSD	Mean	RSD
MON89788	1.32	8.06	1.33	6.27
P35S	0.74	8.50	0.74	7.54

RSD: Relative standard deviation.

Table 4. Summary of Accuracy and Precision Statistics for Real-Time PCR Using ABI PRISM 7900

		Trueness		Precision					Trueness		Precision		
		Mean	Bias			Detection limit			Mean	Bias			Detection limit
% (w/w)	Retained labs	GMO amount, %	True value, %	RSDr,[a)] %	RSDR,[b)] %	Below 20 copies[c)]	% (w/w)	Retained labs	GMO amount, %	True value, %	RSDr, %	RSDR, %	Below 20 copies[c)]
		MON89788							P35S (RRS+A2704-12)				
0.50	10	0.61	21.1	13.6	21.9	0/20	1.0	11	1.95	95.4	9.4	11.8	0/22
1.0	11	1.09	9.4	16.3	20.1	0/22	2.0	10	3.74	87.2	8.2	10.7	0/20
5.0	11	5.36	7.1	11.2	16.3	0/22	10.0	11	20.18	101.8	12.4	12.4	0/22
10.0	11	11.53	15.3	8.8	9.9	0/22	20.0	10	37.14	85.7	6.5	7.1	0/20

a) RSDr: Repeatability relative standard deviation. *b)* RSDR: Reproducibility relative standard deviation. *c)* Below 20 copies is expressed as the ratio of the number of retained data below 20 copies/the total number of retained data.

than one GM event using PCR-target sequences shared within targeted events. Nevertheless, there are few reports that assess screening methods using test samples containing plural GM events for common target segments. In this study, we quantified and evaluated test samples that contained both RRS and A2704-12 targeting the P35S region, and the obtained trueness and precision showed predictable results.

In the practical monitoring, the total amount of the three types of GM soybean content can be estimated by the developed quantitative screening method whether the commingling level is less than the labeling threshold or not. If the GM content exceeds the threshold level, individual quantitative analysis for RRS, A2704-12 and MON89788 would be performed for final assessment. We concluded that the developed method would be reliable and practical for the primary stage of the monitoring.

On the other hand, the commercial utilization of other approved GM soybean events such as insect resistant and high oleic acid soybeans would be started at anytime. In the future, it will be necessary to add new detection and quantification methods for novel approved GM soybean events depending on the situation of their commercial cultivation and distribution and, furthermore, other screening methods using commonly introduced promoters or terminators into them may be required, while the time- and cost-effectiveness of P35S quantification will remain.

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水耕栽培システムによる生薬の生産とその評価

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超高齢社会の日本では、漢方薬を処方される例が増え、漢方薬市場は急成長している。複数生薬より構成される漢方薬は一生薬でも欠けると製造できないため、漢方処方の70%以上に配合されている生薬「甘草」は最も重要であり、また「甘草」は、食品添加物や化粧品原料としても汎用されている。しかし、甘草の供給は、ほぼ100%海外に依存し、主生産国の中国における採取や輸出の規制、中国国内需要の伸び、「遺伝子資源へのアクセスと利益配分」のルールづくり等により、今後、甘草の確保が困難になり、「第2のレアアース」になる恐れが顕在化している。日本漢方生薬製剤協会の2010年の調査によると、生薬の国内自給率はわずか12%であり、甘草をはじめ、生薬を国内で確保・供給できるシステムを実用化することは喫緊の国家的課題である。

甘草の人工水耕栽培の実用化へむけて

我々は産学官連携の研究成果として、水耕栽培により1～1年半の短期間で安定的に高品質な甘草を生産する手法を世界で初めて開発し、甘草の人工水耕栽培の実用化に向けての基盤を整備した。本成果は産学官連携で研究成果をあげた好例として高い評価を受け、2011年9月22日第9回産学官連携功労者表彰において厚生労働大臣賞を受賞した。

この研究成果を基盤に、確実に実用化を推進するため、平成24年度より3カ年計画で、厚生労働科学研究費補助金（創薬基盤推進研究事業）「人工水耕栽培システムにより生産した甘草等漢方薬原料生薬の実用化に向けた実証的研究（H24-創薬総合-一般-007）」を開始した。人工水耕栽培は、圃場栽培や野生植物の利用と比較し優れた点がある（右図）が、実用化に際しては、経済性や汎用性など、クリアすべき課題があり、また、人工水耕栽培により生産した生薬の製品化事例はないため、そのような新技術で生産された生薬に対しての潜在的不安があるのは否めない。

そこで本研究では、人工水耕栽培システムによる生薬生産の着実な実用化を推進するため、まずは、甘草をはじめとして、1）「甘草」等の種苗生産システムの構築、2）ハイテク「甘草」等生産システムの構築、3）生薬「甘草」等の評価（品質・有効性・安全性）を、また、生薬の国内生産基盤構築と推進モデル実証のため、4）地域企業との連携によるブランド生薬の開発を、富山県において実施中である。

現在までに、1)については、甘草の有効成分であるグリチルリチン酸の生合成酵素遺伝子の多型を利用した優良株の育成に成功し、また、水耕栽培で得られた地上部を利用した大量増殖法を開発した（医薬基盤研究所、丸善製薬）。2)については、人工栽培環境において、光源の種類や紫外線の種類及びその照射方法の最適化や、収穫後の根の貯蔵・乾燥方法の最適化により、効率的に薬用成分濃度を高められることを明らかとした（医薬基盤研究所、鹿島建設、千葉大学）。3)については、甘草の市場流通品と水耕栽培品について比較を行った。品質については、日本薬局方試験を実施し、水耕栽培品も市場流通品と同様に日本薬局方規格を満たすことを確認した（医薬基盤研究所、東京生薬協会）。また、それぞれの熱水抽出エキス及びメタノールエキスについて、高速液体クロマトグラフ質量分析計（LC/MSMS）による多変量解析を行い、薬用成分だけでなく、その他の多くの成分についても、水耕栽培品は市場流通品と化学的同等性が高いことを確認した。有効性については、それぞれの熱水抽出エキスを接触性皮膚炎モデルマウスに投与して抗アレルギー作用を調べ、水耕甘草エキスは市場流通甘草エキスと同様に抗アレルギー作用を示すことを確認した（医薬基盤研究所、国立医薬品食品衛生研究所）。安全性については、熱水抽出エキスの復帰突然変異試験（Ames test）及びそれぞれの甘草に含まれる有害元素（ヒ素、カドミウム、水銀、鉛）の定量を実施し、いずれの甘草も遺伝子突然変異誘発性は認められないこと、有害元素のうち、ヒ素、カドミウム、水銀は水耕栽培品が市場流通品に比べて著しく低く（定量下限または検出限界以下）、鉛はいずれの甘草も基準値以下で同程度であることを確認した（医薬基盤研究所、国立医薬品食品衛生研究所）。4)については、シャクヤク、ダイオウ、エゾウコギについて、ブランド生薬の開発を実施中である（医薬基盤研究所、富山大学等）。

本研究は平成26年度が最終年度であるが、少なくとも甘草については完全人工水耕栽培により生産した生薬が、高品質、安心・安全で、効果が確かなものであることは示せたと考えている。国内で製造される漢方薬の原料となる生薬は、まだまだ数多く存在する。我々の研究成果が、生薬の国内自給率の向上に貢献できれば幸いである。

図　水耕栽培システムによる薬用植物（生薬）の生産の背景と利点

- 薬用植物の供給の約80%以上は野生植物資源の採取に依存
- 環境変化や人為的環境撹乱等により薬用植物資源（特に良品）が激減
- 国内自給率は重量ベースで12%（2010年）
- 国交や相手国の情勢による影響大（例：輸出規制、政情不安）
- 資源国での規制強化

恒常的な需要を満たすには不安定な状態

水耕栽培システムによる甘草等漢方薬原料生薬の生産

- 自然環境（気温、日照量、降水量、土質等）の影響を受けず安定的に生産可能
- 植物種が明確で品質が安定した薬用植物の供給が可能
- 農薬、土壌汚染や人為的環境撹乱を回避できる
- 連作障害がなく、計画栽培・多角栽培が可能
- 人手がかからず短期間で収穫可能
- 野外・水耕栽培に適した優良苗の育成が短期間で可能

薬用植物の安心・安全な安定供給