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Research activities for the monitoring of genetically modified organisms in Japan*

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Abstract: The Japanese government introduced a labeling system for genetically modified (GM) foods. To ensure the authenticity of the labeling system, we have developed and validated detection methods for newly approved GM events. One was the development of quantitative analytical methods utilizing plasmid DNAs as calibrators, which enabled us to obtain an unlimited supply of calibrators of consistent quality and also to obtain a stable standard curve to quantify GM organisms (GMOs) in samples. The significance of quality control has been recognized among relevant stakeholders, and in response we launched a project to distribute certified reference materials (CRMs) to the users of our methods for the purpose of internal quality control. In addition to these activities, we have developed time- and cost-effective detection methods, such as a new screening method to simultaneously detect the sequence of Cauliflower mosaic virus 35S promoter (p35S) and the construct-specific sequence of GA21 event utilizing multiplex real-time polymerase chain reaction (PCR). We also developed a qualitative nonaplex PCR detection method, which allows the simultaneous detection of eight events of GM maize lines. Because the influx of any unapproved and unknown GMOs into the Japanese market is not permitted, we continue to explore this issue.

Keywords: certified reference materials; genetically modified organisms; labeling; monitoring; polymerase chain reaction.

INTRODUCTION

While the worldwide demand for grain has been increasing along with an expanding population and a rising income level, the production of primary grains has not been sufficient to meet the demand, and the world stocks-to-use ratio, that is, the grain supply measured against grain demand, has been declining. In parallel, a shortage of water resources, continued global warming, and many other factors of instability have been threatening grain production. In addition, increasing demand for biofuels may have a huge impact on the supply and demand for food in the world. In such a food situation, the production of genetically modified (GM) crops has been increasing since the start of full-scale commercialization of GM crops in 1996, as both industrial countries and developing countries have attempted to secure the desired amount of production and benefit farmers. In fact, the global area of GM crops exceeded 125 million hectares in 25 countries in 2008, and is expected to spread even further [1].

Meanwhile, in Japan, the country's food self-sufficiency ratio by supplied-calorie basis has decreased year by year and is currently about 40 %, which is at the lowest level among developed

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countries. It is apparent that the Japanese population is highly dependent on imported foods. Regarding maize, soy, and canola, which are the leading GM crops, our ratio of imports is extremely high. For example, Japan is the world's largest importer of maize, and almost all of our maize for domestic consumption comes from abroad. In addition, we are dependent on the United States for more than 95 % of imported maize, where GM maize has been cultivated in more than 80 % of the maize area. For Japan, where demand for non-GM crops is high, it is possible that it will become more difficult to ensure a stable supply of such products in the future.

SAFETY ASSESSMENT AND LABELING POLICY FOR GM PRODUCTS IN JAPAN

The World Health Organization (WHO), Organization for Economic Cooperation and Development (OECD), and other organizations have developed scientific guidelines for the safety assessment of foods and food additives produced by recombinant DNA techniques. In 1991, the Ministry of Health and Welfare, Japan (MHW) formulated the "Guideline for Safety Assessment of Foods and Food Additives Produced by Recombinant DNA Techniques" based on these international guidelines. In the beginning, assessments of GM foods were conducted by the Food Sanitation Investigation Council on a voluntary basis. As the development of GM foods and their commercial use have rapidly spread internationally, the Ministry of Health, Labour, and Welfare, Japan (MHLW), the successor to MHW, decided to legally impose the safety assessment of GM foods. Therefore, the safety assessment of GM foods has been mandatory under the Food Sanitation Law since 1 April 2001, and GM foods that have not undergone the safety assessment have been forbidden to be exchanged commercially in Japan. In 2003, the Food Safety Commission (FSC) was established in the Cabinet Office. The safety assessment of GM foods has since been conducted by FSC at the request of MHLW. Within FSC, the Expert Committee of Genetically Modified Foods conducts safety assessments based on three standards and three policies (available at the following URL: <http://www.fsc.go.jp/english/index.html>) regarding the safety assessment of GM organisms (GMOs) and their products. As of April 2009, 98 kinds of GM crops have been authorized and can be introduced into the Japanese market as foods [2].

Along with the expansion of GM crop production, consumers have started to express their concerns over the newly introduced technology, and have demanded appropriate information and labeling for foods derived from GM crops. As a result, a new labeling system has been introduced in Japan [3]. GM foods that have passed through the safety assessment and have been authorized by MHLW shall be labeled as "GM food" when they are in the market. As of October 2007, seven agricultural products, i.e., soy, maize, potatoes, canola, cotton, alfalfa, and sugar beets, and their processed foods have been designated as mandatory labeling items. Food items subject to mandatory GM labeling are designated as those in which GM DNA or proteins derived from the DNA can be detected even after processing, or those whose compositions or nutritional values differ in comparison to their conventional counterparts. Processed foods in which the DNA and protein have been removed or highly degraded, such as cooking oil, are exempted from the labeling. In the case that products are ensured not to contain GM products through identity preserve (IP) handling, they can be labeled as, for example, "soy beans (non-GM)" or "not containing GM products". Here, IP handling has been defined as a management method in which GM products and non-GM products are not commingled at any stage of production, distribution, or processing from farms to food manufacturers. The non-GM status must be verified by documents indicating that the IP management has been conducted. And as long as the non-GM food is handled properly according to IP handling, the adventitious presence of GM crops can be accepted up to 5 % [4].

DEVELOPMENT OF GM DETECTION METHODS IN JAPAN

Japanese standard methods

Quantitative methods using plasmids as calibrators

The development of accurate, reliable, and rapid methods for the detection of GM crops is of paramount importance for the affirmation of labeling authenticity. Since the threshold of the adventitious presence of GM crops has been defined as 5 % in Japan, not only qualitative but also quantitative detection methods have been required. We have developed and validated many detection methods for newly approved GM events, since the enactment of laws requiring a labeling system for foods produced by the recombinant DNA technique in Japan. One of the highlights of our work was the development of new quantitative detection methods based on a real-time polymerase chain reaction (PCR) technique utilizing plasmid DNAs as calibrators [5]. The methods were based on the quantification of targets' copy numbers by PCR and meant for the detection of five lines of GM maize, i.e., MON810, Event 176, Bt11, T25, and GA21, and a GM soy, Roundup Ready. The copy numbers of GM-specific target sequence and taxon-specific sequence were quantified individually, and a GM amount (%) in a sample was subsequently calculated using the following formula (1):

$$\text{GM amount (\%)} = \text{copy number of recombinant DNA (r-DNA) sequence in sample/copy number of taxon-specific sequence in sample/Cf} \times 100 \quad (1)$$

Here, the ratios of GM-specific target sequence and taxon-specific sequence in each genuine seed were calculated as follows and defined as coefficient value (Cf):

$$\text{Cf} = \text{copy number of r-DNA sequence in DNA extracted from genuine GM seed/copy number of taxon-specific sequence in DNA extracted from genuine GM seed} \quad (2)$$

For the quantification of the copy number for each target, a precise calibration curve is essential. To draw a calibration curve, finely ground GM seeds accurately weighed and mixed into ground non-GM seeds are usually prepared. However, it is unlikely that the quality of such reference materials (RMs) prepared from agricultural products could be maintained at the same level all of the time, because various factors, such as variety, growing area, and year, the different genotypes of embryos and albumen, may affect the quality and extractability of DNAs from the seeds. To overcome this problem, we constructed two plasmids as RMs for the detection of GM maize and GM soy, in which target sequences were ligated together in a plasmid vector. The molecules contained the DNA sequences of a specific region found in each GM event, the universal sequences introduced in GM crops, such as Cauliflower mosaic virus 35S promoter (p35S), nopalin synthase terminator (tNOS), and the endogenous DNA sequences of maize or soy (Fig. 1). The development of these plasmids made it possible to provide RMs of consistent quality to all laboratories.

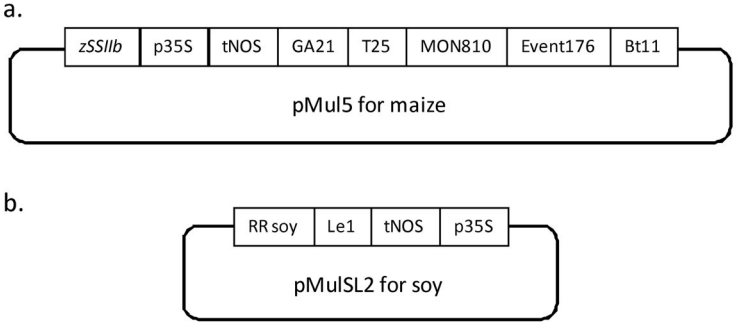


Fig. 1 Schematic diagrams of plasmid DNAs as calibrators. (a) Plasmid calibrator for the detection of GM maize, and (b) plasmid calibrator for the detection of GM soy.

These methods were validated through an interlaboratory evaluation study with more than 13 participating laboratories from Japan, Korea, and the United States, and they were officially adopted as national standard methods in Japan and Korea [6–8]. Meanwhile, global standardization of detection methods for GMOs has proceeded primarily in the International Organization for Standardization (ISO), and these quantitative PCR methods have been adopted and published in the Annex of ISO21570 and recognized internationally [9].

Certified reference material (CRM) production

Quality assurance of analytical measurements has been recognized and emphasized among relevant stakeholders as a significant aspect of food analysis. To assure the quality of an analytical measurement, it is necessary to use a validated method, to participate in appropriate proficiency testing, to perform an internal program of quality control, and to meet the requirements according to ISO 17025. Having CRMs is essential for meeting these criteria. To provide some measures for the quality control of the GM analysis, we launched a project to distribute CRMs to the users of the Japanese standard methods. The CRMs may also be utilized for proficiency tests conducted by public institutes. The CRMs that we are producing are prepared by quantitative mixing of GM and non-GM ground seeds. These CRMs are characterized with a quantified value (GM %) obtained by central laboratories using a validated method. Only the quantified GM % would appear as a certified value, and the original weight ratio of GM/non-GM would not be indicated in the case of distributable CRMs. To be a producer of CRMs, the National Food Research Institute (NFRI) was accredited to comply with ISO Guide 34: 2000 (JIS Q 0034: 2001) – General requirements for the competence of reference material producers, and ISO/IEC 17025: 2005 (JIS Q 17025: 2005) in 2007. We are currently offering Roundup Ready Soybean (RRS)-containing CRMs. A set of CRMs named "CRM: GM soy" consists of three kinds of CRMs, of which one does not include RRS and two include RRS at different concentrations.

The purpose of this project is to produce and distribute CRMs that enable the user to verify the trueness of the measurement values obtained by the relevant standard method and to perform internal quality control. Therefore, the CRMs are only available to third parties who use Japanese standard methods to ensure compliance with the GM labeling regulation defined by the "JAS Law" [3] and " Food Sanitation Law" [10]. The use of CRMs is limited for the internal quality control, and intended users are also limited to the following three categories: (1) public research/inspection institutes, (2) private sectors performing the quality control of their products and requiring an internal quality control, and (3) private sectors offering inspection service for the quality control of their customers' products.

Multiplex PCRs for efficient analysis

Duplex real-time PCR for quantitative screening

Maize is one of the major agricultural crops to which GM technology has been applied. In Japan, 18 events of GM maize and 27 varieties of their hybrid progenies have been authorized as of 2009 [2]. Analysis of each individual event is time-consuming and not practical for monitoring purposes. Analysis of the r-DNA constructions of 8 major GM maize events elucidated that all the events except GA21 maize contained the same constitutive promoter, p35S. MHLW announced a combinational method in which the quantification of p35S and a construct-specific quantification of GA21 maize would be individually conducted, and subsequently the quantitative values of both sequences would be combined. The method has been officially used as a screening method for GM maize [8].

To improve the efficiency of the screening procedure, we developed a new screening method utilizing duplex real-time PCR, in which the sequences of p35S and the event-specific sequence of GA21 were simultaneously quantified [11]. As an efficient alternative to the existing screening method, we chose p35S and an event-specific segment for GA21 as the targets of this method. We could have chosen p35S and tNOS, which is the most popular terminator used for the development of GM crops, and such a system might have been able to detect a wider range of GM maize. However, because there

are many events with both the p35S and the tNOS segments, we chose the duplex real-time PCR method targeting p35S and GA21 to avoid overestimation caused by p35S and tNOS segments. Meanwhile, the development and authorization of new GM crops have been continuously progressing, and concurrently the number of GM events not covered by the screening method and the number of combined event products that can be overestimated by the screening method is expected to continue to increase. New approaches accommodating this situation are in high demand.

Multiplex PCRs for qualitative screening

Because the number of GM crops has been continuously increasing, multiplex PCR will be a useful method for the qualitative screening of GM events contained in raw materials and/or processed foods. We have been continuously developing multiplex PCR systems for the simultaneous detection of GM maize [12,13]. Onishi et al. [14] also developed a multiplex PCR system for the simultaneous detection of up to eight events of GM maize in a single reaction (Fig. 2). The eight detection primers for the construct-specific detection of eight GM events, i.e., Bt 11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507, and a primer pair for an endogenous reference gene, *ssIIb*, were developed for this nonaplex (9plex) PCR system. The amplification products of this system were distinguished by agarose gel (Fig. 2a) or capillary electrophoresis (Fig. 2b) based on their different lengths. The system enabled us to reliably amplify all nine fragments in a simulated GM mixture containing as little as

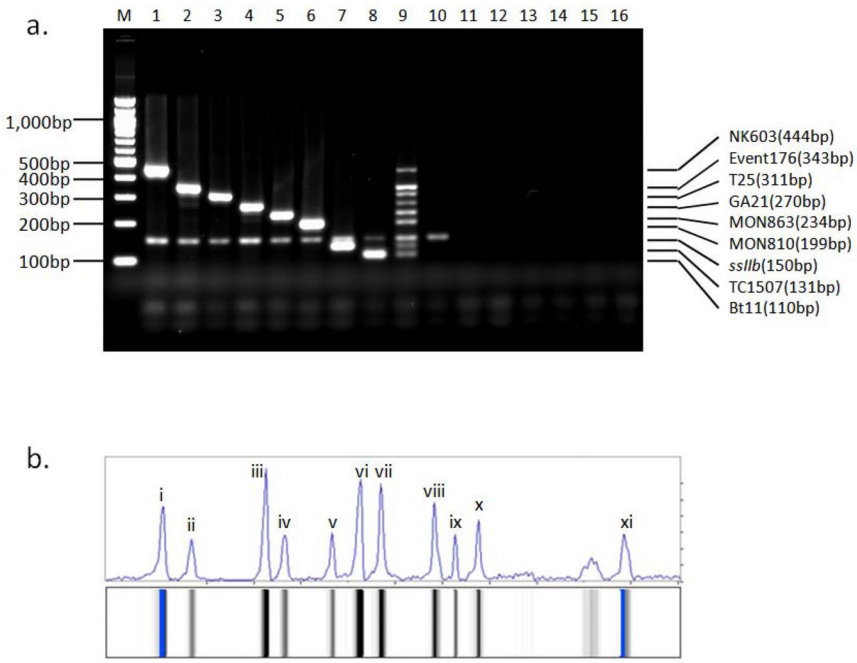


Fig. 2 Nonaplex PCR for 8 events of GM maize. (a) Lanes 1–8, amplification of DNAs extracted from the seeds of each event of GM maize: NK603, Event 176, T25, GA21, MON863, MON810, TC1507, and Bt11, respectively; lane 9, the simulated GM mixture containing 1 % of each event of 8 GM maize; lane 10, non-GM maize; lanes 11–15, non-GM soy, RRsoy, rice, wheat, and barley, respectively; lane 16, negative control (no DNA); and M, 100 bp ladder size standard. PCR products were electrophoresed on 3 % agarose gel. (b) Amplification products from a simulated GM mixture containing 1 % each of above described 8 events of GM maize were subjected to capillary electrophoresis analysis. One microliter aliquots of 10-fold dilutions of PCR products were electrophoresed with internal control fragments (10 and 500 bp) at a constant voltage (350 V) for 4.5 min. i: internal control (500 bp), ii: NK603, iii: Event 176, iv: T25, v: GA21, vi: MON863, vii: MON810, viii: ssIIb, ix: TC1507, x: Bt10, xi: internal control (10 bp). The peak between Bt11 and internal control (10 bp) attributes to primer dimer.

0.25 % (w/w) each of eight events of GM maize (data not shown). The sensitivity of multiplex PCR, however, might be affected when there is a big concentration gap between events. The amplification from dominant events might inhibit the amplification of underrepresented GM events. Although the developed multiplex PCR systems have shown a great performance and potential as screening methods for GM maize, an interlaboratory collaborative study is needed to determine the practical limits of detection (LOD) of this method, as well as the applicability of this method to processed food samples. We have been continuously developing new multiplex screening methods for newly approved events.

Emerging problems with GMOs: Unapproved/unknown GMOs

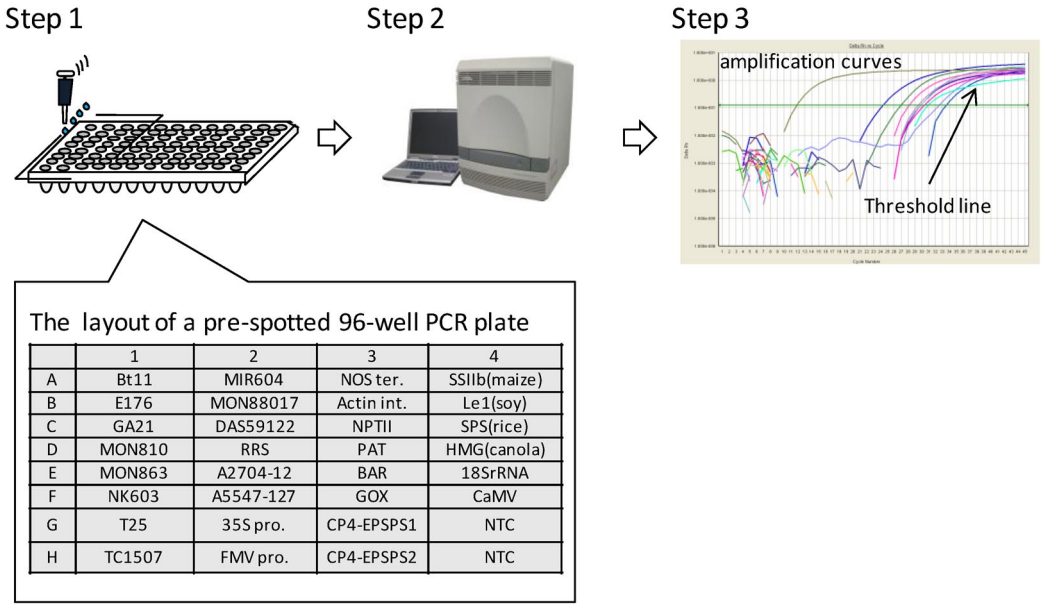
DNA segment-specific screening system

Countermeasures against unapproved/unknown GMOs have been required since the advent of GMOs. Only approved GM crops have been allowed to be distributed in the Japanese market, and the commercialization of unapproved GMOs has been strictly restricted. However, incidents of unapproved/unknown GMOs have occurred sporadically, as in the case of StarLink, Bt10, LLRice601, Chinese Bt rice, and so on. Since the influx of any kinds of unapproved/unknown GMOs into the Japanese market is not permitted, the development of monitoring methods for both approved and unapproved GMOs has been strongly demanded. However, unless the sequence data of an unapproved/unknown GMO is available, it is quite difficult to develop a detection method to monitor it.

DNA cassettes introduced into GM crops consist of the combination of r-DNA segments, such as functional genes, promoter and terminator regions, and intron sequences, to confer new traits. Some of the segments are frequently introduced into different GM crops. Therefore, the detection of r-DNA segments may be utilized for the screening of GM crops, including unauthorized GMOs. From this viewpoint, we took a comprehensive segment detection approach, which consisted of combinational simplex PCRs [15]. In this study, we designed 14 primer pairs for the detection of individual segments derived from exogenous organisms and evaluated the specificity of their detection of each of the introduced DNA segments. The results suggested that this approach could provide useful information about the existence of GM crops, including unapproved events, and that it could be effectively used for the screening of GM crops.

Comprehensive detection for the assumption of unapproved/unknown GM events

An increase in the number of targets brought about by one newly introduced GM event after another has made the above-mentioned approach less practical. We developed a novel type of PCR array as a platform for the comprehensive and semiquantitative detection of GM crops [16]. Thirty primer-probe sets for the specific detection of GM events, r-DNA segments, endogenous reference genes, and donor organisms were synthesized, and a 96-well PCR plate was prepared with a different primer-probe in each well as the real-time PCR array. The conceptual scheme of this approach is depicted in Fig. 3. The specificity and sensitivity of the array were evaluated. A comparative analysis with the data and publicly available information on GM crops approved in Japan allowed us to make an assumption of the possible contamination of unapproved GM crops. Furthermore, we designed a Microsoft Excel spreadsheet application, Unapproved GMO Checker version 2.01, which helps process all the data of real-time PCR arrays for the easy assumption of unapproved GM crop contamination. The spreadsheet is available free of charge at <http://cse.naro.affrc.go.jp/jmano/index.html>. In addition, in the development of an analytical method for regulatory use for the monitoring of GMOs, a validation study among participating laboratories is required to evaluate the performance. Validation studies tend, however, to be time- and cost-consuming. Given this situation, a universal detection system that permits the simultaneous implementation of many individual validated methods would be an efficient and useful tool for GM analysis. Therefore, the developed system with PCR array and the spreadsheet application would make it easy to supply suitable GM testing methods to testing laboratories in a flexible and impromptu manner so that they will be ready to deal with the increasing number of approved GM crops.



	1	2	3	4
A	Bt11	MIR604	NOS ter.	SSIIb(maize)
B	E176	MON88017	Actin int.	Le1(soy)
C	GA21	DAS59122	NPTII	SPS(rice)
D	MON810	RRS	PAT	HMG(canola)
E	MON863	A2704-12	BAR	18SrRNA
F	NK603	A5547-127	GOX	CaMV
G	T25	35S pro.	CP4-EPSPS1	NTC
H	TC1507	FMV pro.	CP4-EPSPS2	NTC

Fig. 3 Conceptual scheme of new platform for real-time PCR based comprehensive detection of GM crops. This multitarget system allowed the simultaneous detection of 30 targets in a single experiment. A 96-well PCR plate was pre-spotted with all primer-probe sets for the individual detection of all targets as a real-time PCR array. The system consisted of the following three steps: (1) extracted sample DNA is mixed with TaqMan Universal PCR Master Mix and the mixture is loaded into each well; (2) the thermal cycling is carried out; (3) data analysis is performed. For the assumption of unapproved GM crops, a Microsoft Excel spreadsheet application, Unapproved GMO Checker version 2.01 is available.

Emerging problems with GMO: Combined-trait GM maize

Kernel-based detection

A plant with stacked genes contains two or more transgenes, usually as a result of the crossing of two transgenic plants with different transgenes, and double- and triple-stacked GMOs are now common. In fact, 78 % of GM maize, 75 % of GM cotton, and 41 % of all GM crops in the USA in 2008 were stacked products [1]. Since stacked genes confer multiple traits in a single variety, the global area of stacked GMOs is expected to increase, and also higher combinations of stacked GMOs, such as up to octuple-stacked genes, are anticipated to come into the market in the future.

Stacked GMOs have posed a problem for detection. As mentioned above, a threshold of adventitious commingling of GMOs has been set, such as at 0.9, 3, and 5 % in the European Union [17], Korea [18], and Japan [4], respectively. At least in Japan, the threshold has been defined as the weight per weight (w/w) percentage of GM material in non-GM material. Currently available quantitative PCR methods convert the relative copy number of a specific r-DNA sequence to that of a taxon-specific DNA sequence into GM amounts (w/w). Therefore, the GM amount (w/w) in a maize sample containing a stacked variety of GM maize may be overestimated, and overestimation of GM content (w/w) with stacked traits could lead to unjustified legal consequences for affected stakeholders. As a solution to this matter, Akiyama et al. [19] developed a rapid PCR system with a multiplex real-time PCR method on a single-kernel basis.* The equipment for the grinding of individual kernels and a silica membrane-

*A kernel-based detection method based on the ref. [19] was officially adopted as a standard method by MHLW as of 3 August 2009; Notification No. 308 (3 August 2009), Ministry of Health, Labour and Welfare, Japan <http://www.mhlw.go.jp/topics/yunyu/hassyutu/2009/dl/h08a.pdf>.

based 96-well DNA extraction kit were both significantly revised and optimized for this particular purpose. Later, the combined use of multiplex PCR and capillary gel electrophoresis (CGE) was also proposed as a rapid and prospective method for multiplexing for multiple samples [20].

CONCLUSION AND PERSPECTIVES

The Japanese government introduced a labeling system for foods produced by the recombinant DNA technique in 2001. To ensure the authenticity of the labeling system, reliable and cost-effective methods for GMO detection are essential. We have thus developed and validated many detection methods for newly approved GM events since the enactment of the system.

There have been numerous positive effects from the cultivation and commercialization of GM crops over the last 10 years, especially for farmers, including reduced workload, facilitated pest control, and reduced applications of herbicides and pesticides. The next generation of GM crops is anticipated to provide advantages for the consumer, such as enhanced nutritional value, and tolerance to harsh environments, such as drought and salinity. In conjunction with the increasing GMO production worldwide, numerous incidents of the involuntary release of GMOs have been sporadically occurring. Moreover, the number of new-comers involved in GMO development, both in the public and private sectors in the world, has been increasing, but information about their products is not necessarily available to the public. Countermeasures against unapproved/unknown GMOs will thus be needed more strongly than ever before.

Since there is currently no domestic cultivation of GM crops in Japan, the monitoring of GM crops is performed primarily for imported commodities at a waterfront when they are unloaded from the ship. However, given that the commercial cultivation of GM crops starts in Japan, we should manage not only foreign but also domestic commodities in terms of the labeling law. Under such circumstances, it will be necessary to consider the "coexistence" of GM products with conventional and organic products, and to implement a rule for "coexistence". Since the adventitious commingling of GMOs into a non-GM product can occur at various stages of the food supply chain, analyzing and identifying where adventitious commingling occurs along the food supply chain would be required to comply with the labeling law. In addition, since the domestic cultivation of GM crops would require more fast-track and efficient logistics than imported commodities, more cost- and time-effective detection methods will be needed.

As pointed out above, since stacked genes confer multiple traits in a single variety, stacked GMOs meet the economic and environmental needs of farmers, and are increasingly deployed in many countries. Stacked GMOs have, however, posed a significant problem with respect to the quantification of GMOs. As mentioned above, the threshold of adventitious commingling of GMOs has been set at 5 % (w/w) in Japan. Existing quantitative PCR methods are incapable of accurately quantifying the GM % on a weight per weight basis, and may lead to overestimation of the GM contents of a sample containing stacked varieties. Although a countermeasure, in which a single-kernel basis analysis is exploited, has been proposed, more effective methods are required for the efficient quantification of stacked varieties.

To keep up with the rapid progress in the development of GM crops, including stacked varieties, more work will be required, and, concurrently, international cooperation in the development of GM detection must be encouraged.

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