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REVIEW

Development and Standardization of Analytical Methods for Increasing Varieties of Genetically Modified Crops

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Abstract

The varieties of commercially available genetically modified (GM) crops are rapidly increasing, and this situation demands analytical methods capable of detecting recently developed GM crops. Here we review our research activities to develop and validate new analytical methods for recently distributed GM crops. For the screening analysis of GM content in analytical samples, we developed real-time PCR-based quantitative methods for two GM maize events, MIR604 and MIR162. To accurately analyze GM content irrespective of the commingling of stacked GM events, we developed the group testing method. For the comprehensive analysis of various GM events, the real-time PCR array method was established. In November 2016, the Consumer Affairs Agency of Japan released the standard testing manual including these new testing methods to ensure the validity of the food labeling system in Japan. Given the expected increase in the number of GM events to be analyzed in the future, we need to keep working toward the realization of simple and comprehensive detection and quantification methods that can be used for the increasing number of these events.

Discipline: Food
Additional key words: GM crop, GMO, detection, standard testing method

Introduction

Many genetically modified organisms (GMOs) including microorganisms, animals, and plants are already in practical use, and the varieties of commercially available genetically modified (GM) crops are rapidly increasing (James 2016). In Japan, the total number of GM events approved by the Japanese government for food under the Food Sanitation Act is continuously increasing, and reached 335 as of November 1, 2017 (Ministry of Health, Labour and Welfare 2017) (Fig. 1).

GM crops intended for commercial use have not been cultivated in Japan. Large amounts of such approved GM crops as maize, soybean, and canola have been imported from various countries. To give consumers in Japan the freedom of choice between GM and conventional food products, food-labeling regulation on GMOs was implemented in 2001. Food-labeling regulation is currently mandated under the Food Labeling Act issued in April 2015 (Consumer Affairs Agency 2015). The regulation permits "non-GMO" labeling only when the final products are made from non-GMO materials produced and distributed in accordance with an identity-preserved (IP) handling system. In general, almost all maize and soybean foods subject to the labeling regulation are labeled as "non-GMO" in food markets in

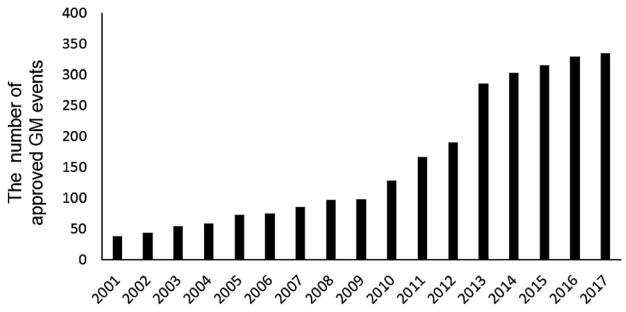

Fig. 1. The cumulative number of GM events approved under the safety assessment system for foods in Japan

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Japan. Conversely, non-IP corn and soybean containing a lot of GMOs are used for producing oil, corn syrup, and animal feed, which do not need the labeling of GMOs. The labeling system in Japan tolerates unintentional GMO commingling up to 5% of the content for maize and soybean. Therefore, the quantitative analysis of GM crops is required to ensure the validity of "non-GMO" labeling.

Our group developed real-time PCR-based quantitative methods for several GM maize and soybean events (Kuribara et al. 2002). These analytical methods were validated and have been used as standard testing methods in Japan since 2001 (Shindo et al. 2002). However, the more recent GMO scenario in which the number of approved GM events is continuously increasing has required the renewal of these testing methods. In this report, we review the research activities conducted to develop and validate the new analytical methods for recently distributed GM crops, and also discuss the current situation of standard testing methods regarding food-labeling regulation in Japan.

Event-specific real-time PCR-based quantitative analytical methods

The most common technique for GMO quantification in maize and soybean grain is the quantitative real-time PCR analysis of bulk sample homogenates. One of the characteristics of GMO quantification methods in the Japanese standard testing manual is the use of calibrant DNAs, which are plasmid DNAs including a sequence consisting of tandemly connected GM targets and endogenous reference genes (Kuribara et al. 2002). The calibrant DNAs permit the reproducible preparation of calibration curves. In addition, Japanese standard methods measure the ratio of copy numbers of the GM target sequence to that of endogenous reference sequence, with the ratio finally being converted to GMO content on a weight/weight basis by using a conversion factor (Cf) as shown in Figure 2 (Kuribara et al. 2002). The conversion factor is the ratio of copy numbers of the GM target to the endogenous reference sequence obtained by analyzing genuine GM materials corresponding to 100% as the weight percentage.

In 2001 when food-labeling regulation started in Japan, the commercially distributed GM maize events were Bt11, Event176, MON810, GA21, and T25 (Shindo et al. 2002). We therefore used the 35S promoter (P35S) that is introduced in Bt11, Event176, MON810, and T25, and a GA21-specific sequence as analytical targets for the present GM maize analysis. Currently at least DAS-59122-7, NK603, MON863, MON88017, MON89034, TC1507, MIR604, and MIR162 have been distributed in addition to the first five GM maize events (Akiyama et al. 2011). Because MIR604 and MIR162 were not detected by P35S, we developed event-specific quantitative PCR methods for these GM events (Mano et al. 2012a, Takabatake et al. 2014).

To check the fitness for the purpose of the analytical methods, we evaluated the performance of the methods using an interlaboratory collaborative trial. The international standard regarding GMO quantitation, ISO 24276 (International Organization of Standardization 2006), defines the limit of quantitation (LOQ) as being the lowest level of analyte with RSD_R of ≤ 25%. Therefore, we determined the LOQs of the MIR604 and MIR162 quantitation methods as being 0.5%. The trueness, precision, and LOQ of these methods were comparable to those of previously established Japanese standard methods (Shindo et al. 2002). We thus concluded that the developed methods were successfully validated.

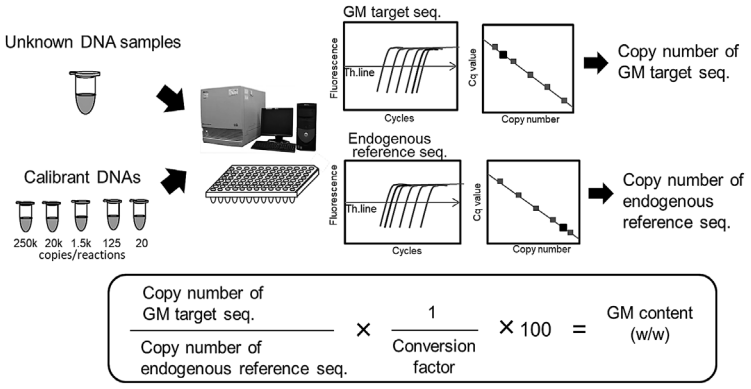

Fig. 2. Overview of the real-time PCR-based quantitative methods used in Japan

The conversion factor is a predetermined constant value used to calculate weight-based GMO content from the ratio of copy numbers of the GM target to endogenous reference sequences.

Group testing and its validation

In terms of maize, many types of stacked-event seeds have been produced by crossing two or more single GM events, and have already been widely used (Akiyama et al. 2011). In Japan, a safety assessment is performed for each combination of single events, and 182 stacked events have been approved as of November 1, 2017 (Ministry of Health, Labour, and Welfare 2017).

The regulation of GMO labeling in Japan expresses the amounts of GM material in terms of weight/weight percentages. Because the GM stacked events contain the recombinant DNA sequences corresponding to two or more single GM events, the GMO content measurement by real-time PCR leads to an overestimation compared to the actual weight-based GMO content. Given the growing use of GM stacked events, an accurate measurement of weight-based GMO content would become difficult to achieve. We developed a group testing method in which a predetermined number of groups is taken from a larger bulk sample, while each group contains a defined number of kernels. The GMO content is then evaluated statistically based on the qualitative results from multiple small pools of grains (Fig. 3) (Mano et al. 2011). The testing method consists of a sample pretreatment step in which a group of 20 maize kernels is ground in a lysis buffer by a household food processor. The next step is a PCR assay in which the lysed sample is directly analyzed as a DNA template by qualitative PCR.

For qualitative PCR analysis, we developed two duplex real-time PCR assays: a GM maize screening assay and an experimental control assay. The GM maize screening assay detects P35S and the NOS terminator region (TNOS) that have been widely introduced into commercially available GM maize events. The experimental control assay is designed to check that the reaction mixture contains sufficient amounts of extracted DNA and no PCR inhibitors. The target sequences of the experimental control assay are the starch synthase IIb gene from *Zea mays* (SSIIb, as the endogenous reference sequence) and an artificial sequence in small amounts of plasmid DNA (as an internal positive control; IPC). We achieved the efficient evaluation of GMO content on a weight/weight basis, regardless of the presence of stacked-event products.

After we developed the group testing method, we tried to validate the analytical method by using a collaborative trial (Mano et al. 2011). According to AOAC guidelines, we prepared groups consisting of two GM kernels and 18 non-GM kernels (group A), groups consisting of one GM kernel and 19 non-GM kernels (group B), and groups consisting of 20 non-GM kernels (group C). As a set of blind samples for a laboratory, six A groups, six B groups, and six C groups were sorted at random and numbered from 1 to 18. A set of blind samples was provided to each of 12 laboratories. Each laboratory analyzed the blind samples in one experiment according to the testing protocol. Almost all the results of that trial showed the expected positive/negative determinations corresponding to the presence/absence of GM kernel(s) in each group. The results indicated that the qualitative detection method for group testing accurately detected the presence of GM without cross-contamination between groups. There were no false-negative results for group samples including a GM kernel, which fulfilled the criterion for the limit of detection for qualitative GMO detection methods as described in the ISO standard regarding GMO analysis (International Organization of Standardization 2006).

In the collaborative trial, we obtained a false-negative result in PCR analysis to detect IPC, presumably due to PCR inhibition. We therefore developed a new PCR reagent including an enzyme and buffer more tolerable to PCR inhibition materials (Mano et al. 2014).

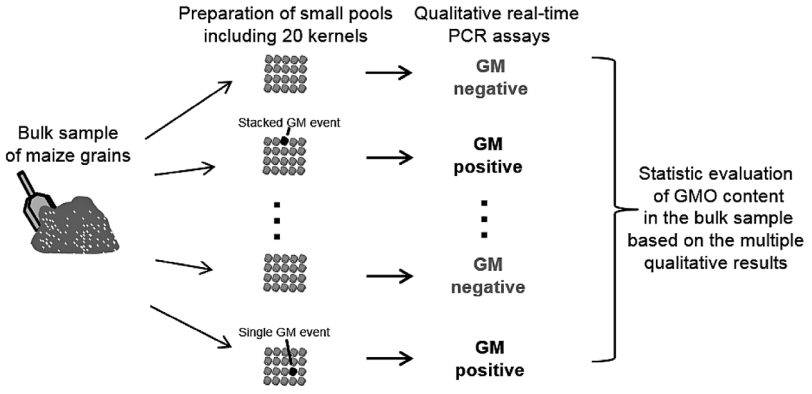

Fig. 3. Schematic representation of the group testing method
This figure is reprinted from Mano et al. (2016).

By using this new PCR reagent, the false negative detection caused by PCR inhibition in group testing is expected to be reduced. We modified the PCR reagent and part of the primer sequences, and then re-evaluated the method's performance using an in-house blind test (Mano et al. 2016). All blind samples were correctly analyzed and the method's performance was confirmed as being appropriate for standard testing methods.

The real-time PCR array and its validation

Due to the increasing number of commercially available GM crops, it was necessary to develop testing methods capable of collecting a great deal of information on GMOs in foods at one time. Several research groups have reported simultaneous multiple target detection methods for GM crops, such as multiplex PCR methods (James et al. 2003, Onishi et al. 2005) and DNA chips (Leimanis et al. 2006, Rudi et al. 2003). Multiplex PCR is recognized as one of the most efficient and least laborious techniques for multi-target detection. However, the multiplex reaction may be difficult to apply in practical testing as false-positive amplifications occur more often than in the simplex reaction (Markoulatos et al. 2002, Rudi et al. 2003, Schmidt et al. 2008). An interaction between individual reactions occurs in the multiplex system, and this causes unstable testing results when there is a large gap between the amounts of target DNAs (Elnifro et al. 2000, Ratcliff et al. 2007).

In the development of a GM analytical method for regulatory use, validation by an interlaboratory study is required to evaluate the method's performance. Such validation studies entail much time and costs for both the study conductor and participants. When one individual reaction is added to a validated multiplex reaction system, a substantial effort to re-evaluate the whole system would be required. These points make it difficult to supply suitable GM testing methods that use multiplex reactions to testing laboratories in a flexible and impromptu manner. 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.

We therefore developed a real-time PCR array (i.e., a 96-well PCR plate prepared with a different primer-probe set in each well) as a universal platform for GM crop detection (Fig. 4) (Mano et al. 2009). We designed primer-probe sets for the comprehensive detection of GM crops. In the method, genomic DNA prepared from an agricultural product or food is subjected to a lineup of various qualitative real-time PCR assays targeting individual GM events, recombinant DNA (r-DNA) segments, taxon-specific DNAs, and donor organisms of the respective r-DNAs on one 96-well PCR plate. In our previous investigation, 40 primer-probe sets were prepared as component PCR assays constituting the real-time PCR array (Mano et al. 2012b).

In GMO analyses, TaqMan probe-based real-time PCR assays are recognized as the gold standard method. By referring to various relevant research articles and the subsequent evaluation of the reactions involved, we could introduce new component PCR assays to the existing lineup of assays. For cost reduction, the volume of reaction mixtures was set at 10 μL, which is the smallest volume recommended by the manufacturer of the real-time PCR instruments. It takes three hours to complete

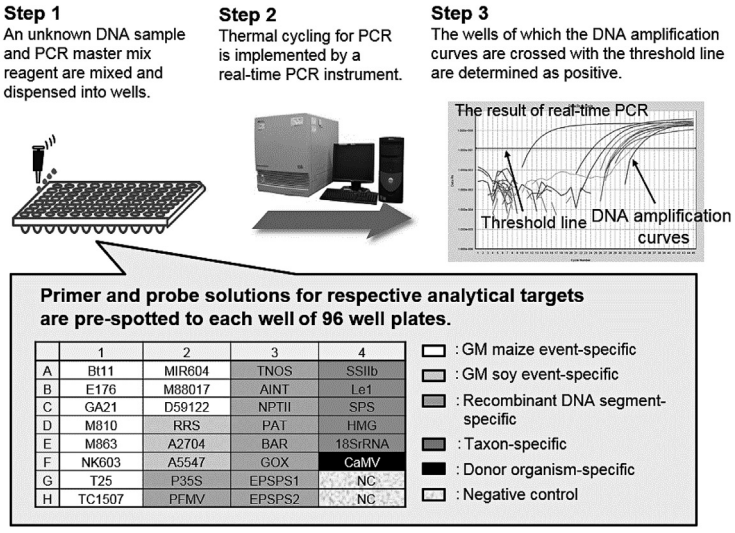

Fig. 4. Overview of the real-time PCR array method

all experimental work for one assay, including preparation of the PCR mixtures, thermal cycling, and data analysis.

We then evaluated both the DNA extraction methods and component PCR assays at the single-laboratory level (Mano et al. 2012b). The performance of the methods satisfied the criteria set based on the previous reports and guidelines. We concluded that the DNA extraction methods and component PCR assays were successfully validated. The real-time PCR array method is an updatable system in terms of sample matrixes and PCR targets. Following an update of this system, the newly developed DNA extraction methods and/or component PCR assays can be validated the same way as for existing methods/assays.

The real-time PCR array approach offers high specificity of detection, a wide dynamic range of detection, time efficiency, easy manipulation, high updatability, and high customizability. Another advantage of this approach is that the proposed method requires no extra investment for equipment in many GMO testing laboratories. Further updating of this system by editing of the detection targets depending on the purpose of a given investigation would provide appropriate testing methods for both regulatory and commercial use.

The new standard testing manual for the food labeling system in Japan

The Consumer Affairs Agency (CAA) of the Government of Japan supervises the food-labeling regulation for GMOs. The CAA reviewed the recent research on GMO analyses and released a new standard testing manual in November 2017 to ensure the validity of GMO labeling (Consumer Affairs Agency 2016). In this testing manual, the newly developed analytical methods were adopted as testing methods for maize grains.

Figure 5 shows an overview of the analytical scheme for maize grains. DNA will be extracted and the real-time PCR-based quantification method targets P35S, GA21, MIR604, and MIR162. If the total GM content by these four assays is over 4.5%, the sample is subjected to the next step. The analytical condition is basically the same as that described in the previous reports (Mano et al. 2012a, Shindo et al. 2002, Takabatake et al. 2014). The conversion factors used to calculate weight-based GMO content were slightly modified from those reports, and the modified conversion factors in the testing manual (Consumer Affairs Agency 2016) must be checked. As an alternative to the screening step, the ΔΔCq method reported by Noguchi et al. (2016) may be used. In the ΔΔCq method, GMO content is semi-quantitatively evaluated based on the difference of PCR cycle numbers to detect GMOs in unknown samples and in a reference material. Group testing is subsequently set for a definitive analysis of GMO content. The analytical condition of group testing is the same as that described by Mano et al. (2016).

For group testing, a group is comprised of 20 maize kernels, and 10 groups are analyzed for the first stage. If seven or more groups are found to be GM-positive in the first analysis, an additional 10 groups will be analyzed in the second stage. If the total number of GM-positive groups in the two stages (20 groups) combined is ≤ 12, then GMO content of the bulk sample is determined to be below 5% (Mano et al. 2016). As an alternative to group testing, a single-kernel-based method (Akiyama et al. 2005) can be used. According to statistical analyses (Laffont et al. 2005, Remund et al. 2001), we found that both the testing conditions and acceptance criteria of group testing and the single-kernel-based method have equivalent accuracy of analysis (Mano et al. 2011). In the standard testing manual, subsequent to group testing, the real-time PCR array method is provided to confirm the GM event(s) included in each group sample prepared in

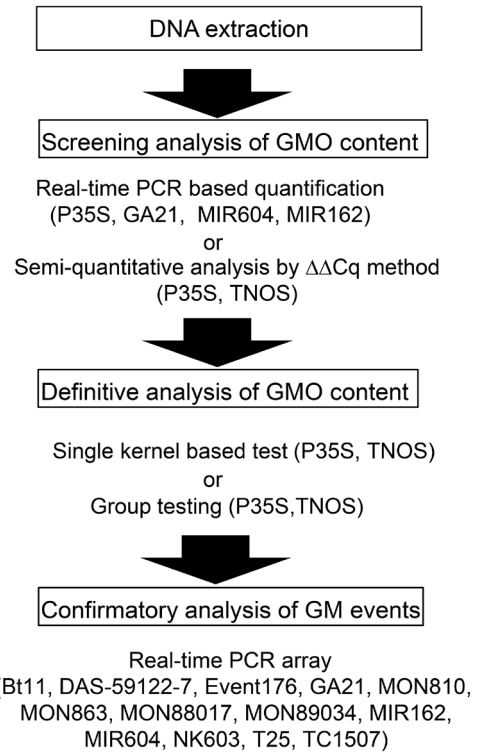

Fig. 5. The new standard testing manual for maize grains released from the Consumer Affairs Agency
The analytical targets for the respective detection methods are shown in parentheses.

the group testing. The analytical procedure for DNA purification from the residual cell lysate generated during group testing and real-time PCR array is as described (Mano et al. 2016).

Conclusions

Along with the increase of approved GM events and their stacked varieties, we have successfully updated the analytical methods to verify the validity of non-GMO labeling. As some of the GM events approved under safety evaluations as foods are not used for commercial distribution, the analytical targets in the Japanese standard testing method are basically limited to actually distributed GM events. If the newly released GM events completely replace 'old' GM events, the number of actually distributed GM crops may be constant, allowing us to continuously use the analytical methods based on the group testing and real-time PCR array strategy. However, even the oldest GM events such as MON810 maize and Roundup Ready Soybean continue to be used, and the number of GM events to be detected will also continue to increase. Moreover, many GM events released recently by biotech companies have no sequences commonly introduced among various GM events, such as P35S and TNOS. This means that it would not be possible to make an analysis simpler by detecting such common sequences. To maintain the standard testing methods that prevent fraud in GMO labeling systems, we may need a breakthrough that provides a simple and comprehensive analysis of hundreds of GM events.

Acknowledgements

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