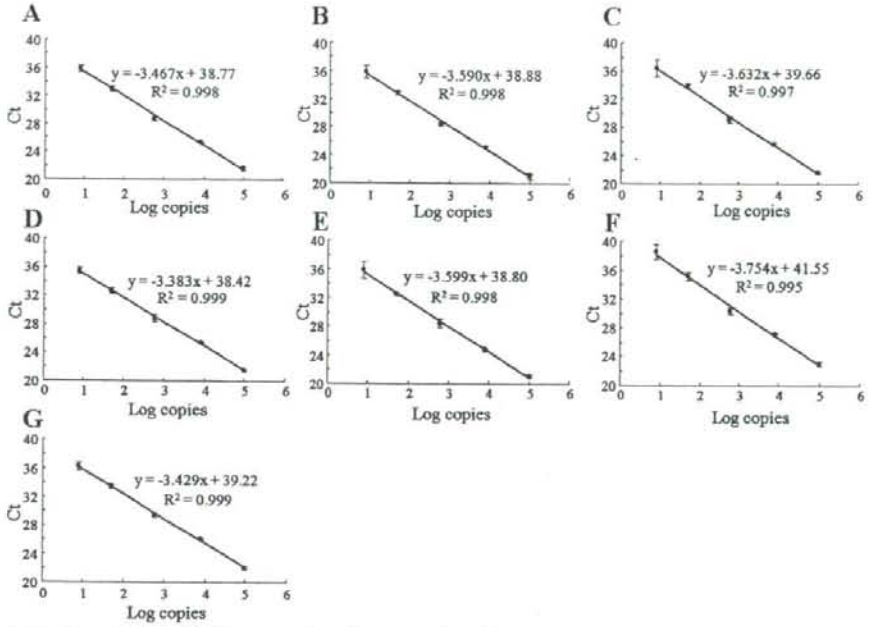

Figure 3. Evaluation of linearity of amplification in real-time PCR array. The calibration plasmids with five different copy numbers were assayed as described in the Materials and Methods section. The mean Ct values with standard deviations, derived from the amplification data of the reactions listed below, are plotted against the log values of the copy number of the control plasmids. The equation and correlation coefficient value for each linear regression curve are also indicated: (A) the Bt11 detection; (B) the E176 detection; (C) the GA21 detection; (D) the M810 detection; (E) the P35S detection; (F) the TNOS detection; and (G) the SSIIb detection.

Development of the Spreadsheet Application, Unapproved GMO Checker. We developed Unapproved GMO Checker version 2.01 as a spreadsheet application for the assumption of unapproved GM crop contamination (**Figure 1**). In the development of the application, unapproved GM crops were conceptually defined as (Unapproved GM crops) = (All GM crops) − (Approved GM crops). Approved GM crops could be selectively detected using GM line-specific detections, while r-DNA segment-specific detections detected various kinds of GM crops ranging from approved to unapproved. Therefore, the assumption of unapproved GM crop contamination is achieved by comparing the results of r-DNA segment-specific detection with those of GM-line specific detection in the real-time PCR array. In the present investigation, unapproved GM crop lines were defined as GM crops that have not been approved for open-field cultivation or provision as food, feed, or ornamental plants under Cartagena Protocol domestic law in Japan. LLRICE62 is an unapproved GM crop line. On the basis of the results obtained by the present real-time PCR array (**Figure 2C**), the contamination of unapproved GM crop(s) was assumed, as shown in **Figure 1**. This result was obtained by the detection of P35S and BAR as r-DNA segments. The result demonstrated no discrepancy between the obtained data and publicly available information regarding GM crops. The application is available and downloadable online (http://cse.naro.affrc.go.jp/jmano/index.html).

Application of the Real-Time PCR Array to the Assumption of Unapproved GM Crops. For the assumption of unapproved GM crop contamination, GM maize, GM soy, and GM rice were selected as targets. Our assumption could be accomplished only when the appropriate results in all wells of the real-time PCR array were perfectly obtained. The results of the present sensitivity evaluation indicated that a contamination level of 0.5% would be sufficient to obtain reliable data without false-negative results. Thus, an analytical sample of fewer than 200 seeds may be preferable. Because unapproved GM crops that have become major concerns such as CBH351 maize, Bt10 maize, LLRICE601, and Bt-rice containing r-DNA segments were selected as target DNA in our investigation, our analytical system may have the potential to discern the novel types of unapproved GM crops as well as the already known unapproved GM crops. However, the present method does not necessarily promise the absolute detection of unapproved GM crops, because crops constructed of completely unknown r-DNA segments or r-DNA segments with modified nucleotide sequences cannot be detected. Also, GM line-specific detection does not completely cover all the approved GM crops at present. Furthermore, if approved and unapproved GM crops were mixed in a sample and both crops shared all r-DNA segments, the unapproved GM crop would be masked by the approved GM crop. If unapproved GM crop contamination is suspected, further analysis, such as sequencing of the r-DNA flanking regions, may be required. Despite its many restrictions, the proposed system would serve as an excellent tool to detect unapproved GM contamination. In addition, since the system is able to add new detection sets, it has great potential for expanding its analytical capacity, making use of feedback information from users about frequently detected approved and unapproved GM lines. A differential quantitative PCR technique was recently reported by Cankar et al. (*33*) as a new approach to unapproved/unknown GMO detection. Their method is based on quantitative assay, and the accuracy of quantitation with real-time PCR is indispensable. Our strategy is based on qualitative results, and higher reproducibility of the assumption of unapproved GM crop contamination is expected. For low concentrations of GM contents, the present qualitative assay strategy is advantageous.

In addition, our system may detect a much broader range of unapproved GM crops by the various r-DNA segment-specific detections. The utilization of semiquantitative analysis in the present method provides great potential for discovery of unapproved GM crops.

In the present investigation, we proposed a universal platform for GM detection. The developed real-time PCR array allows the comprehensive detection of GM crops and the assumption of contamination by unapproved GM crops. This approach is attractive in terms of the specificity of detection, the dynamic range of detection, time efficiency, easy manipulation, updatability, and customizability. Another important factor for the dissemination of this new technology is that the proposed method requires no extra investment for equipment in many GMO testing laboratories. Further updating of this system by editing detection targets depending on the purpose of a given investigation would provide appropriate testing methods for both regulatory and commercial use.

ABBREVIATIONS USED

AOCS, American Oil Chemists' Society; A2704, A2704-12; A5547, A5547-127; EPSPS1, a region of 5-enolpyruvylshikimate-3-phosphate synthase gene introduced into NK603, M88017, and RRS; EPSPS2, a region of 5-enolpyruvylshikimate-3-phosphate synthase gene introduced into RT73; GOX, a region of the glyphosate oxidoreductase gene derived from *Ochrobactrum anthropi* strain LBAA; HMG, a region of the high-mobility-group protein I/Y gene of rapeseed; Le1, a region of the lectin 1 gene of *Glycine max*; NPTII, a region of the neomycin phosphotransferase II gene; PAT, a region of the phosphinothricin-*N*-acetyltransferase gene derived from *Streptomyces hygroscopicus*; BAR, a region of the phosphinothricin-*N*-acetyltransferase gene derived from *Streptomyces viridochromogenes*; SSIIb, a region of the starch synthase IIb gene of *Zea mays*; SPS, a region of the sucrose phosphate synthase gene of *Oryza sativa*; 18SrRNA, a region of the 18S rRNA gene common in crop plants; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamin; CaMV, *Cauliflower mosaic virus*; CRM, certified reference material; Ct, cycle threshold; D59122, DAS-59122; E176, Event176; GM, genetically modified; GMO, genetically modified organism; IRMM, Institute for Reference Materials and Measurements; LMO, living modified organism; M810, MON810; M863, MON863; M88017, MON88017; PCR, polymerase chain reaction; r-DNA, recombinant DNA; RRS, Roundup Ready Soybean; AINT, intron region of the rice Actin 1 gene; P35S, 35S promoter region derived from CaMV; PFMV, 35S promoter region of *Figwort mosaic virus*; TNOS, terminator region of the nopaline synthase gene derived from *Rhizobium radiobacter*; UV, ultraviolet.

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