Development and evaluation of rapid screening detection methods for genetically modified crops using loop-mediated isothermal amplification

Original Research Article

Title: Development and Evaluation of Rapid Screening Detection Methods for Genetically Modified Crops Using Loop-Mediated Isothermal Amplification

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No. of black and white figures: 1

No. of tables: 5

No. of supplementary materials: 3

ABSTRACT

We developed new loop-mediated isothermal amplification (LAMP)-based detection methods for the screening of genetically modified (GM) maize and soybean events. The LAMP methods developed targeted seven sequences: cauliflower mosaic virus 35S promoter; *5-enolpyruvylshikimate-3-phosphate synthase gene from Agrobacterium tumefaciens strain CP4* (*cp4epsps*); *phosphinothricin acetyltransferase* (*pat*) gene; mannose-6-phosphate isomerase gene; *Pisum sativum* ribulose 1, 5-bisphosphate carboxylase terminator; a common sequence between *Cry1Ab* and *Cry1Ac* genes; and a GA21 construct-specific sequence. We designed new specific primer sets for each target, and the limit of detection (LOD) was evaluated using authorized GM maize and soybean events. LODs for each target were ≤ 0.5%. To make the DNA extraction process simple and rapid, we also developed a direct LAMP detection scheme using crude cell lysates. The entire process, including pretreatments and detection, could be completed within 1 hour.

Key words: Loop-mediated isothermal amplification (LAMP); Genetically modified (GM); Direct LAMP; Rapid qualitative analysis

1. Introduction

The cultivation area of genetically modified (GM) crops continues to expand. The global area of GM crops reached 179.7 million hectares in 2015, from 1.7 million hectares in 1996 (James, 2015). However, some consumers still express concerns about the utilization of genetically modified organisms (GMs) in food or feed and, in response, many countries and regions, including Japan, have legislated labeling systems to indicate the presence of authorized GM crops.

The number of GM events is also increasing. As of November 2015, 26 GM crops and 363 GM events had been approved for use as food or feed or for environmental release in 40 countries (James, 2015). In Japan, 201 varieties of GM maize and 22 varieties of GM soybean have been authorized (MHLW, 2016). To deal with the increased number of GM events, an efficient screening detection method for comprehensive GMO inspection is required. Polymerase chain reaction (PCR) is a reliable, robust, and sensitive technique that has been used in many countries as a gold standard for GMO detection (Holst-Jensen, Ronning, Lovseth, & Berdal, 2003; http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx; ISO21570:2005; ISO21571:2005; Kuribara et al., 2002; Notification 201, 2012). However, PCR takes a relatively long time, and requires expensive instruments and reagents. In contrast, loop-mediated isothermal amplification (LAMP) is a rapid, inexpensive and highly specific isothermal DNA amplification technique that uses DNA polymerase with high strand displacement activity (Notomi et al., 2000). Several techniques have been developed to detect LAMP products. Pyrophosphate ions, which are a by-product of DNA amplification, yield a white magnesium pyrophosphate precipitate in the reaction mixture, the turbidity of which can be used for detection (Tomita, Mori, Kanda, & Notomi, 2008). Alternatively, to visualize the presence of LAMP products, dyes such as calcein, hydroxynaphtol blue, and SYBR Green I have been employed (Goto, Honda, Ogura, Nomoto, & Hanaki, 2009). Electrochemical detection by current response, using redox molecules, has also been developed for

LAMP product detection (Ahmed, Hasan, Hossain, Saito, & Tamiya, 2010; Safavieh et al., 2016).

LAMP techniques have also been adapted for GMO detection. As in other uses of LAMP for DNA amplification, detection is based on either turbidity (Fukuta et al., 2004; Huang, Chen, Xu, Ji, Zhu, & Chen, 2014), or SYBR Green I mediated fluorescence (Chen et al., 2012; Huang, et al., 2014; Randhawa, Singh, Morisset, Sood, & Zel, 2013; Wang et al., 2015; Zhang et al., 2013). In addition, Kiddle et al. (2012) reported a detection method using a bioluminescent real-time reporter coupled with LAMP amplification. These methods better PCR-based approaches in terms of their simplicity, but they do not permit discernment between specific and non-specific amplification products.

To overcome these drawbacks, we have developed a novel screening method using a real-time fluorometer, Genie II (OptiGene, UK), that permits the rapid and robust detection of LAMP products. With the Genie II system, annealing curve analysis can be performed to confirm the presence of specific LAMP products because the annealing temperature is unique to the amplified sequence. Moreover, the developed method covers almost all approved GM maize and soybean events in Japan, and uses common segments that have been introduced into many GM events, such as sequences derived from the 35S promoter of cauliflower mosaic virus (P35S), *mannose-6-phosphate isomerase* (pmi) gene, and *Pisum sativum* ribulose 1, 5-bisphosphate carboxylase terminator, referred to as the E9 terminator (tE9) by Coruzzi et al. (1984). Despite this universality, the method is highly specific and sensitive. Also, our newly-designed primer set, targeting P35S, is more sensitive than the previously reported primer sets. Finally, to reduce the sample preparation time, we developed a direct LAMP amplification scheme using crude extracts derived directly from ground seed samples, instead of purified DNA. From a practical point of view, our methods are expected to provide significant value for GMO testing.

2. Materials and Methods

2.1. Plant materials

The GM soybean and maize seeds, MON810, MON863, MON88017, MON87460, MON89034, NK603, MON89788, MON87701, MON87705, MON87769 and 40-3-2 (RRS), were kindly provided by Monsanto Co. (St. Louis, MO, USA). The maize seeds, 3272, Bt11, Event176, GA21, MIR162, and MIR604, were kindly provided by Syngenta Seeds AG (Basel, Switzerland). The maize seeds, TC1507 and DAS59122, were kindly provided by Pioneer Hi-Bred International (Johnston, IA). The soybean seed A2704-12 was kindly provided by its developer, and the maize seed T25 was directly imported from the USA. QC9651 maize, from Quality Technology International (Elgin, IL), was used as a non-GM maize. Seeds for wheat, barley, *Lotus japonicus*, alfalfa, buck wheat, sorghum, rye, and oat were kindly provided by the NARO Genebank Project (Ibaraki, Japan).

2.2. DNA extraction

Maize and soybean genomic DNA were extracted using a DNeasy Plant Maxi kit (Maxi kit) (Qiagen, Hilden, Germany) according to the protocol provided in the JAS analytical test handbook (http://www.famic.go.jp/technical_information/jashandbook/gmo/manual_3.pdf). The concentration and quality of the extracted DNA were evaluated by ultraviolet (UV) absorbance using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The concentration of genomic DNA was adjusted to 50 ng/μL, and 100 ng was used as the template for LAMP analyses.

For the evaluation of specificity, genomic DNA from other plants was also extracted, as described.

2.3. The design of the LAMP primers

The primers used in this study are listed in Table 1. For the detection of each target sequence, a set of six primers consisting of two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LoopF and LoopB) was used. We designed the primer set for each segment using LAMP

Designer 1.13 (PREMIER Biosoft, Palo Alto, CA). The oligonucleotide primers were synthesized by FASMAC (Kanagawa, Japan). We used *starch synthase IIb* (SSIIb) and *lectin1* (Le1) genes, as maize-specific and soybean-specific endogenous sequences, respectively.

2.4. Sample preparation

To evaluate the limit of detection (LOD), mixed DNA samples, which are easy to prepare, were used. Genomic DNAs were extracted from each GM maize and soybean event and the non-GM maize and soybeans, and adjusted to 50 ng/μL. GM and non-GM DNAs were mixed by volume ratios to create samples containing GM maize at 0%, 0.1%, 0.3%, and 0.5% and GM soybean at 0%, 0.05%, 0.1%, and 0.5%.

For the direct LAMP analyses, we used weight-based mixed samples. The weight-based mixed samples are listed in Table S1 in the Supplementary material. We used the following GM maize samples: ① 0.5% of MON810 and 0.5% of GA21, ② 0.4% of Bt11 and 0.2% of GA21, and ③ 0.5% of MIR604. We also used a GM soybean sample containing ④ 0.5% of RRS, 0.5% of A2704-12, and 0.5% of MON89788. All four were prepared as described previously (Mano et al., 2012a; Takabatake et al., 2011, 2013a, b). We also prepared the following: ⑤ 0.5% of MON88017, and ⑥ 0.5% of RRS and 0.5% of MON87701. To prepare these samples, we ground GM and non-GM seeds separately, and mixed them on a weight-basis, then confirmed the homogeneities, as described previously (Takabatake et al., 2011).

2.5. LAMP assay

LAMP reactions were performed with Genie II (OptiGene Ltd., Horsham, UK). The reactions were conducted in a 25-μL volume using 2.0 μL of template DNA, 15 μL of isothermal master mix (OptiGene), 10 mM Tris-KCl (pH 8.0), 1 mM DTT, and primers at the final concentrations of 0.02 μM for F3 and B3, 0.16 μM for FIP and BIP, and 0.08 μM for LoopF and LoopB. For the detection of P35S, a concentration

of 0.32 μM rather than 0.16μM was used for FIP and BIP. The amplification conditions were as follows: amplification at 65°C for 30 min, followed by annealing from 98°C to 80°C with a ramp rate of 0.05°C /sec. The LAMP assay was repeated 21 times for each target and each sample, and a no-template control assay was also performed for all the primer sets.

2.6. Direct LAMP detection

We used GenCheck® DNA Extraction Reagent (FASMAC), which was originally developed for direct PCR analyses (http://www.fasmac.co.jp/GM/kit/DER.html), for the sample-direct LAMP analyses. For sample preparation, 400 μl of lysis buffer was added to 40 mg and 20 mg of ground maize and soybeans samples, which were heated for 10 min at 100°C and then chilled on ice. The samples were centrifuged at 15,000 × g for 5 min, and the resulting supernatants were used directly as templates for LAMP analyses.

3. Results

3.1. Specificity evaluation of the primer sets for each target

LAMP amplification was detected as fluorescence intensity with the isothermal Genie II system. Genie II is a compact, portable instrument that is suitable for real-time fluorescence detection and annealing analyses for LAMP products. First, we checked the specificity of the primer sets and the results are summarized in Table 2. Seven targets for LAMP assays were newly designed: (1) cauliflower mosaic virus 35S promoter (P35S), (2) *cp4epsps* gene (EPSPS), (3) *pat* gene (PAT), (4) mannose-6-phosphate isomerase gene (pmi), (5) *Pisum sativum* ribulose 1, 5-bisphosphate carboxylase E9 terminator (tE9), (6) a common sequence between *Cry1Ab* and *Cry1Ac* genes (Cry1Ab/Cry1Ac), and (7) a GA21 construct specific sequence (GA21). The amplifications were observed from only GM events that contained each target: Fig. 1A, C, E, G, and Fig. S1A, C, E, G, I, K, M. In each, only a single peak was detected, and no

unexpected amplification was observed from the no-template control, non-GM maize or soybean, or from other GM events that did not include target segments. We also designed LAMP target sequences for the SSIIb and Le1 genes, as maize- and soybean-specific sequences, because species-specific endogenous sequences are generally needed, as internal positive controls, for the development of detection methods for both authorized and unauthorized GM crops. To evaluate the specificity, we prepared genomic DNAs from other plants including rice, wheat, barley, *Lotus japonicus*, alfalfa, buck wheat, sorghum, rye, and oat. The LAMP amplifications of SSIIb and Le1 were only observed in maize and soybean genomic DNAs, respectively, and there were no non-specific amplifications with other plants (Fig. 1I and J). These data confirmed that the LAMP assays developed were highly specific for the target sequences (Table 2).

3.2. LOD evaluation of the primer sets for each target

To evaluate the limit of detection (LOD) of the newly developed methods, we used mixed DNA samples prepared from genomic DNA from individual GM events and non-GM maize or soybeans. For the qualitative analyses, we set the criterion for LOD determination as being positive ≥ 20 times in 21 analyses, meaning the false-negative rate had to be ≤ 5%, as described previously (Mano et al., 2009, 2012b). This conforms with the criterion for collaborative study in ISO24276. The LODs determined for each target and each GM event are listed in Table 3. All the LODs were ≤ 0.5% for both GM maize and soybean events. Notably, the LODs for GM soybeans were ≤ 0.1%, except for MON87705 and MON87769 targeting tE9.

The detection time was defined as the point at which the second derivative of an amplification curve peaked: examples are shown in Fig. S2. For each, the signal started to appear at approximately 10-15 min and detection times were within 25 min (Table 3). After amplification, we analyzed the LAMP products using annealing curve analyses. Since the annealing temperature is unique to the amplified sequence,

annealing curve analysis can confirm the specificity of LAMP products. Single peaks were detected in each target (Fig. 1B, D, F, H, and Fig. S1B, D, F, H, J, L, N). The values for each target were very close in both maize and soybean, which clearly indicated that the amplifications were specific.

Several sequences targeting common GM segments, such as P35S and NOS terminator (TNOS), have been applied previously in LAMP analyses (Fukuta et al., 2004; Kiddle et al., 2012; Randhawa et al., 2013; Wang et al., 2015; Zhang et al., 2013). Using our LAMP system, we compared the sensitivity of our newly-designed primer set for LAMP amplification, using Genie II targeting P35S, to those of three primer sets previously reported for LAMP amplification targeting P35S (Table 4). Primer sets for LAMP amplification targeting P35S in Zhang et al. (2013), Randhawa et al. (2013), and Wang et al. (2015) were named P35S-1, P35S-2, and P35S-3, respectively. As shown in Table 3, when using our primer set for P35S, the LODs for MON810 and RRS were 0.3% and 0.05%, respectively. The positive rates for all three previous sets, P35S-1, P35S-2 and P35S-3, were not acceptable, even at 0.5% of MON810 or 0.1% of RRS (Table 4). These results suggest that our primer set was more sensitive than the previously reported primer sets, at least when used with the Genie II detection system.

3.3. Direct LAMP detection

To shorten sample preparation time, we developed a direct LAMP detection scheme using crude cell lysates prepared directly from ground maize or soybean seed samples without any DNA extraction or purification steps. We used the GenCheck® DNA Extraction Reagent (GenCheck reagent) for direct LAMP detection analyses, which was, originally developed for PCR amplifications from blood, animal cells, plant cells, microorganisms, and food samples. In our direct LAMP detection scheme, sample preparation procedures took less than 20 min and included very few steps, mainly heat treatment and centrifugation. We tested the applicability of the GenCheck reagent to LAMP analysis. Six distinct GM mixed samples were used in our evaluation (Table S1). 0.5% of MON810, 0.5% of MON88017, 0.4% of

192 Bt11, 0.5% of MIR604, 0.4% of Bt11, and 0.5% of GA21 were used for detection of P35S, EPSPS, PAT,
193 pmi, Cry1Ab/Cry1Ac and GA21. 0.5% of RRS, 0.5% of RRS, 0.5% of A2704-12, 0.5% of MON89788,
194 and 0.5% of MON87701 were used for detection of P35S, EPSPS, PAT, tE9, and Cry1Ab/Cry1Ac. The
195 results are summarized in Table 5. Twenty-milligram samples were sufficient for the GM soybean
196 analyses. On the other hand, for the GM maize events, when 20-mg samples were used, positive detection
197 rates were not acceptable (< 95%) at 0.5% of MON810 and MIR604 (Table 5). The sensitivities were
198 improved using 40-mg samples, meaning 40 mg was sufficient for GM maize analyses. These targets
199 could be detected within 25 min at concentrations near the LOD. The entire detection process, including
200 sample preparation and LAMP detection, was completed within one hour.
201
202 **4. Discussion**
203 The commercialization of GM crops has brought huge economic benefit, but concerns about the new
204 technology have arisen among consumers, leading to the introduction of GMO labeling systems in many
205 countries. Our research group has developed several real-time PCR-based screening detection methods
206 (Oguchi et al., 2009; Takabatake et al., 2013a), but in recent years, the number of varieties of GM maize
207 and soybean events have increased. Thus, it has become difficult to find common sequences that cover
208 many events and allow development of efficient PCR-mediated screening detection methods.
209 To resolve these problems, additional time- and cost-effective technologies are needed. LAMP is one
210 such relatively new DNA amplification technique and, under certain circumstances, is simpler, quicker,
211 and costs less than PCR. In fact, LAMP has the potential to replace PCR as an initial screening for
212 comprehensive GMO detection.
213 In this study, we developed screening methods for GM maize and GM soybeans using a LAMP assay
214 targeting seven sequences, namely P35S, EPSPS, PAT, pmi, tE9, Cry1Ab/Cry1Ac, and GA21. To detect
215 LAMP products, we used a Genie II system that can confirm specific amplifications through annealing

curve analyses. Genie II and the reagents for Genie II are also reasonably priced compared to other DNA detection systems, such as real-time PCR. Results from this study demonstrate that our newly-developed methods are rapid and cost-effective, and the LODs equal to or less than 0.5%. Thus, the LODs were equivalent to or better than those reported previously for PCR-based qualitative screening methods (Kodama et al., 2011; Takabatake et al., 2013). We also developed a direct LAMP amplification scheme, using crude extracts derived from ground seed samples. In terms of just the basic sample-preparation procedures, DNA extraction and purification usually take about 60 min or more. In our direct LAMP detection scheme, however, sample preparation required less than 20 min, and the entire detection process, including sample preparation and LAMP detection, was completed within one hour. Therefore, we conclude that our new methods are applicable for the detection of GM crops to monitor the validity of food labels in many countries.

Acknowledgements

We thank the Genetic Resources Center, National Agriculture and Food Research Organization (NARO). The biological genetic resources, JP16471, JP21313, JP31147, JP31346, JP38350, JP51518, JP77857, JP79656, JP209867 and JP212442, were distributed from the NARO Genebank Project.

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Research project for Genomics-based Technology for Agricultural Improvement GRA201-1).

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Fig. 1. Representative results of the specificity tests for LAMP analyses from GM maize and GM soybean events (A-H), and non GM crops (I) and (J), obtained with Genie II. The amplification profiles are shown in (A), (C), (E), (G), (I), and (J), and annealing curves are shown in (B), (D), (F), and (H). The peaks in the annealing curves indicate the annealing temperature of the LAMP products. For GM maize, Bt11, Event176, MON810, T25, GA21, NK603, MON863, TC1507, DAS59122, MON88017, MIR604, MON89034, MIR162, 3272, and MON87460 were used as templates, and for GM soybean, RRS, MON89788, A2704-12, MON87701, MON87705, and MON87769 were used. For SSIIb and Le1, non-GM maize, soybean, rice, wheat, barley, *Lotus japonicus*, alfalfa, buck wheat, sorghum, rye, and oat were used as templates.

Fig. S1. Representative results of the specificity tests for the LAMP analyses from GM maize and GM soybean events obtained with Genie II. The amplification profiles are shown in (A), (C), (E), (G), (I), (K) and (M), and annealing curves are shown in (B), (D), (F), (H), (J), (L) and (N). The peaks in the annealing curves indicate the annealing temperatures of the LAMP products. For GM maize, Bt11, Event176, MON810, T25, GA21, NK603, MON863, TC1507, DAS59122, MON88017, MIR604, MON89034, MIR162, 3272, and MON87460 were used as templates, and for GM soybean, RRS, MON89788, A2704-12, MON87701, MON87705, MON87769 were used as templates.

Fig. S2. Representative results of the second derivatives of the amplification curves for LAMP analyses from GM maize and GM soybean events. The amplification profiles of 14 repeated analyses of 0.5% of Bt11 targeting P35S, RRS targeting P35S, MIR162 targeting pmi, and MON89788 targeting tE9 are shown in panels (A)-(D), respectively.

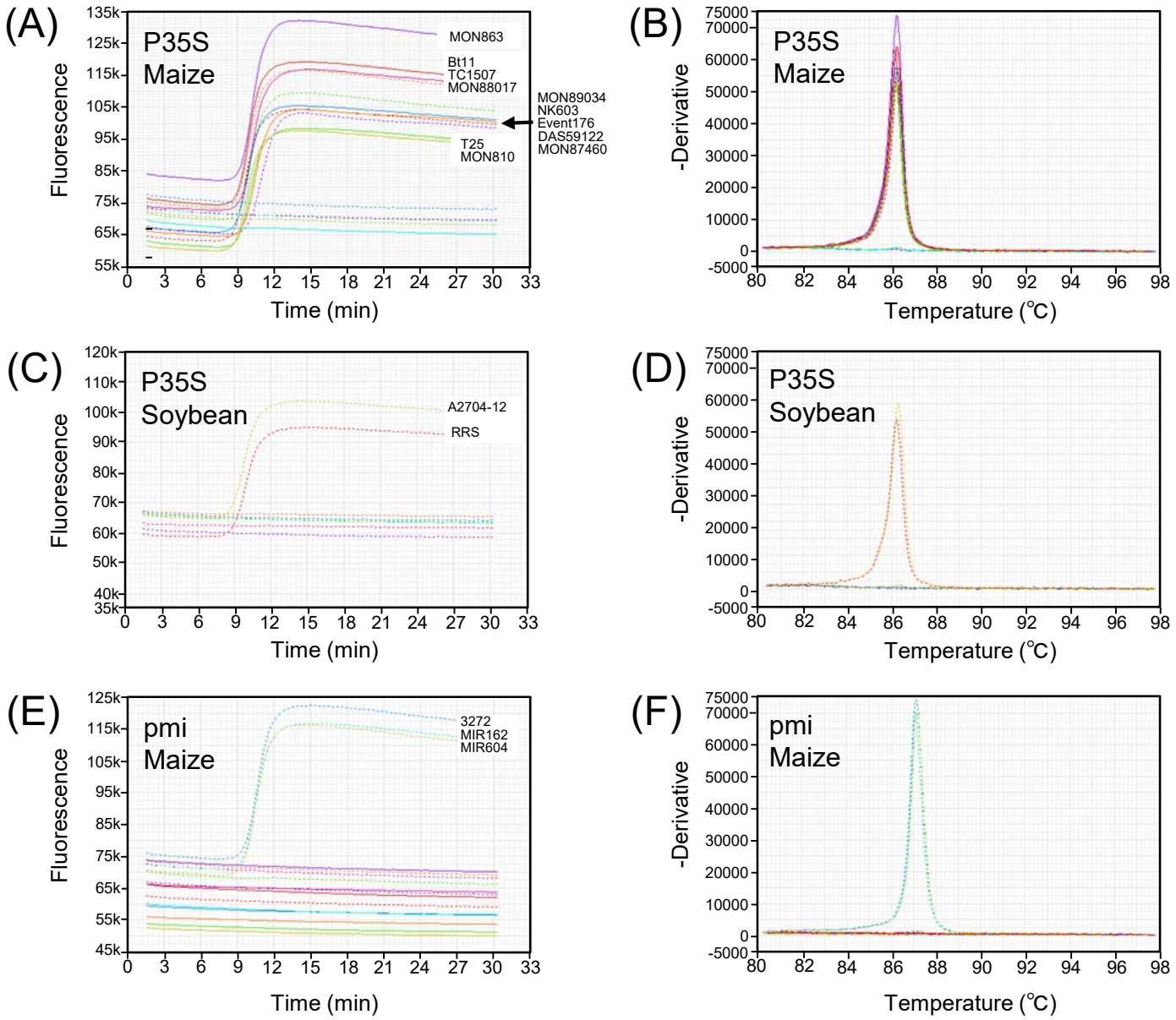

(A)
P35S
Maize
MON863
Bt11
TC1507
MON88017
MON89034
NK603
Event176
DAS59122
MON87460
T25
MON810
Fluorescence
Time (min)
(B)
P35S
Maize
-Derivative
Temperature (℃)
(C)
P35S
Soybean
A2704-12
RRS
Fluorescence
Time (min)
(D)
P35S
Soybean
-Derivative
Temperature (℃)
(E)
pmi
Maize
3272
MIR162
MIR604
Fluorescence
Time (min)
(F)
pmi
Maize
-Derivative
Temperature (℃)

Fig. 1

Fig. 1 (continued)

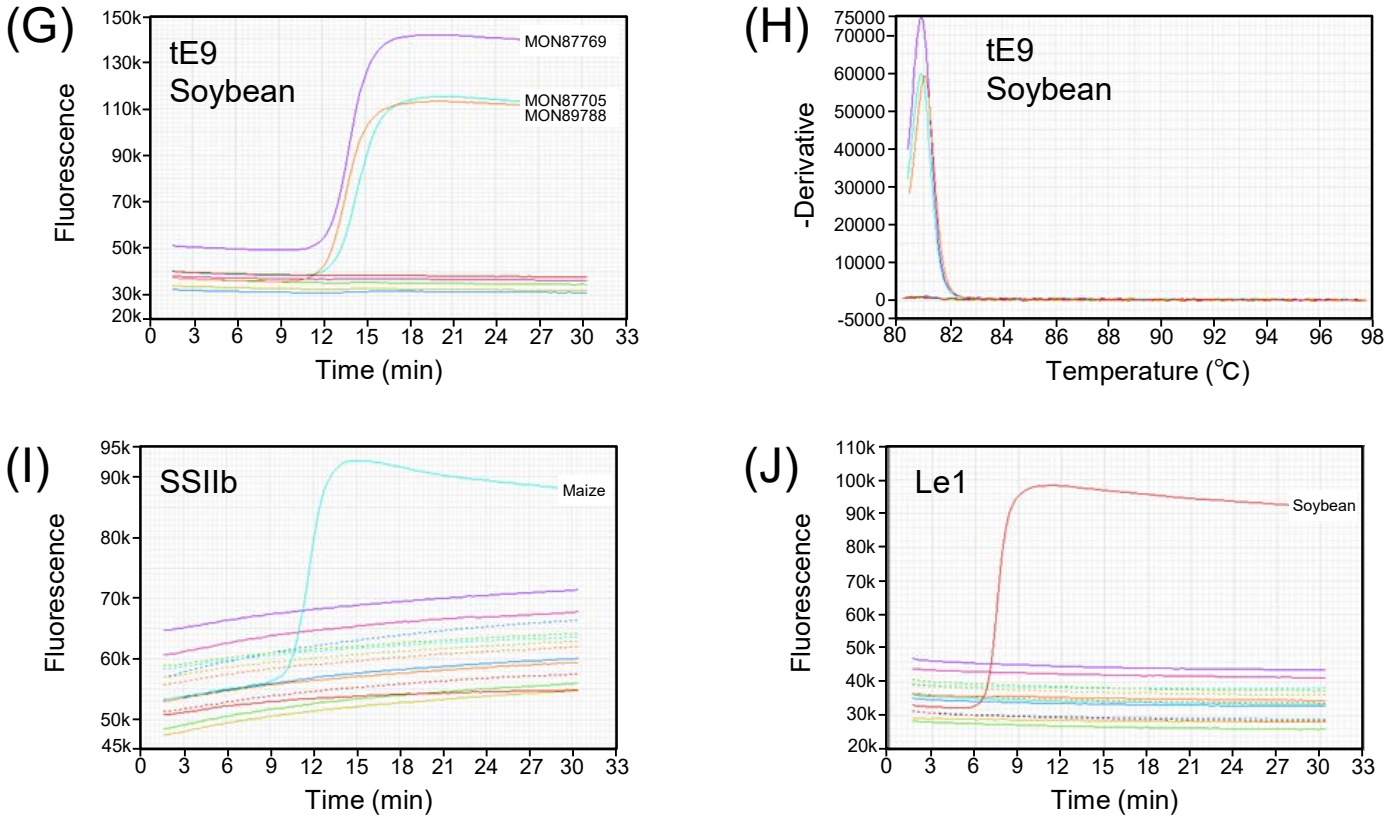


Table 1
The oligonucleotide primers used for the LAMP analyses

Target		Sequence	Ref.
P35S	F3	5'-ATTGCGATAAAGGAAAGGCTATCG-3'	This study
	B3	5'-ACTTCCTTATATAGAGGAAGGGTC-3'	
	FIP	5'-GAAGACGTGGTTGGAACGTCTTCTTAGTGGTCCCAAAGATGGA-3'	
	BIP	5'-GCAAGTGGATTGATGTGATATCTCCTTGCGAAGGATAGTGGGA-3'	
	LoopF	5'-TTTCCACGATGCTCCTCG-3'	
	LoopB	5'-CGTAAGGGATGACGCACA-3'	
EPSPS	F3	5'-GAATATCCGATTCTCGCTGT-3'	This study
	B3	5'-AGCCTTCGTATCGGAGAG-3'	
	FIP	5'-TCATCGCAATCCACGCCATGAACGGTCTGGAAGAACTC-3'	
	BIP	5'-TCACCGCATCGCCATGAGGCCATCAGGTCCATGAAC-3'	
	LoopF	5'-TGAGCTTGAGGCCATTGGCGAC-3'	
	LoopB	5'-GATGCCACGATGATCGC-3'	
PAT	F3	5'-CGTTAACCATTACATTGAGACG-3'	This study
	B3	5'-TGCGCCTCCATAGACTTA-3'	
	FIP	5'-GCCACAACACCCTCAACCTCACAAGAGTGGATTGATGATCT-3'	
	BIP	5'-CCTGGAAGGCTAGGAACGCTTGATGCCTATGTGACACG-3'	
	LoopF	5'-GCAACCAACCAAGGGTATCTA-3'	
	LoopB	5'-ACGATTGGACAGTTGAGAGTAC-3'	
pmi	F3	5'-CAGTTCACGAGTGCAGAAT-3'	This study
	B3	5'-CGGCTTGTGGTTAGGATC-3'	
	FIP	5'-GAAAGGCAGTTCGCCAAAGCCGTGATGTGATTGAGAGTGATA-3'	
	BIP	5'-CAGCACAGCCACTCTCCATTTGGCAAAACCGATTTCAGA-3'	
	LoopF	5'-TCTCCGAGCAGAGTCGAT-3'	
	LoopB	5'-CAGGTTCATCCAAACAAACACA-3'	
tE9	F3	5'-ACACCAGAATCCTACTGAGT-3'	This study
	B3	5'-GAATCTGACAAGGATTCTGGAA-3'	
	FIP	5'-CCATCCATTTCCATTTCACAGTTCGTGAGTATTATGGCATTGGGA-3'	
	BIP	5'-AAATGTGTCAAATCGTGGCCTCTAGCCTAGTGAATAAGCATAATGG-3'	
	LoopF	5'-CAAGCACAACAAATGGTACAAG-3'	
	LoopB	5'-TGACCGAAGTTAATATGAGGAG-3'	
Cry1Ab/Cry1Ac	F3	5'-TGATGGACATCTTGAACAGC-3'	This study
	B3	5'-CATAGGCGAACTCTGTTCC-3'	
	FIP	5'-CGCTGAATCCAACTGGAGAGGTCTACACCGATGCTCACA-3'	
	BIP	5'-AACGCCGCTCCACAACAAGGAAGACAAGGTTCTGTAGAC-3'	
	LoopF	5'-GTGTCCAGACCAGTAATACTCTC-3'	
	LoopB	5'-TATCGTTGCTCAACTAGGTCAG-3'	

Table 1 (continued)

GA21	F3	5’-GGACTACTGCATCATCACG-3’	This study
	B3	5’-TGATAATCATCGCAAGACCG-3’	
	FIP	5’-CGGCAAGGGAGAAAGCCATGAGAAGCTGAACGTGACG-3’	
	BIP	5’-GCTGAGCACTTTCGTCAAGAATTAAGTGCCAAATGTTTGAACGATC-3’	
	LoopF	5’-CATCCTGTGGTCGTCGTAC-3’	
	LoopB	5’-CTCTAGAAGAAGCTTCGACGAA-3’	
SSIIb	F3	5’-CCGAAGCAAAGTCAGAGCG-3’	This study
	B3	5’-GCATCAGCCTTAGCATCCA-3’	
	FIP	5’-ATCAGCTTTGGGTCCGGACACGCAATGCAAAACGGAACGAG-3’	
	BIP	5’-AGAAATCGATGCCAGTGCGGTGGCGATGCCTATGCTTTCCA-3’	
	LoopF	5’-GCGCGGCGGTGCT-3’	
	LoopB	5’-AAGCCAGAGCCCGCAGG-3’	
Le1	F3	5’-CCAGAATGTGGTTGTATCTCT-3’	This study
	B3	5’-TTGTCCCAAATGTGGATGG-3’	
	FIP	5’-TTCGGCACGAACTTGTTCCATTGGTACTGGTGCTACTGA-3’	
	BIP	5’-TGATCCTCCAAGGAGACGCTCCGTTTTCGTCAACCTTATTG-3’	
	LoopF	5’-TTTCCGCTGAGTTTGCCT-3’	
	LoopB	5’-TGACCTCCTCGGGAAAGT-3’	
P35S-1	F3	5’-AGGAAGGGTCTTGCG-3’	Zhang et al.
	B3	5’-ATAAAGGAAAGGCCATCG-3’	
	FIP	5’-GTCTTCAAAGCAAGTGGGGATAGTGGGATTGTGCG-3’	
	BIP	5’-TTCCACGATGCTCCTCGCCTCTGCCGACAGTGG-3’	
	LoopF	5’-ATTGATGTGATATCTCCACTGACGTAAGGGATGA-3’	
	LoopB	5’-TGGGTGGGGGTCCATCTTTGGGA-3’	
P35S-2	F3	5’-CTCCTCGGATTCCATTGC-3’	Randhawa et al.
	B3	5’-TCTACAGGACGGACCATG-3’	
	FIP	5’-ACGATGCTCCTCGTGGGTCATCGTTGAAGATGCCTCT-3’	
	BIP	5’-CGTTCCAACCACGTCTTCAAGTCTTGCGAAGGATAGTGG-3’	
	LoopF	5’-ATCTTTGGGACCACTGTCG-3’	
	LoopB	5’-TGATATCTCCACTGACGTAAGG-3’	
P35S-3	F3	5’-CAAAGATGGACCCCCACC-3’	Wang et al.
	B3	5’-CGGACCATGGAGATCTGCTA-3’	
	FIP	5’-TGCGTCATCCCTTACGTCAGTGAAGAAGACGTTCCAACCACG-3’	
	BIP	5’-TCCTTCGCAAGACCCTTCCTCTGTCAGCTTGTCAGCGTGT3’	
	LoopF	5’-GAGATATCATATCAATCCACTTGCTTTGAAGA-3’	
	LoopB	5’-ATATAAGGAAGTTCATTTCATTTGGAGAGG-3’	

Table 2
The results of the specificity evaluation

Target / GM event	P35S	EPSPS	PAT	pmi	tE9	Cry1Ab/ Cry1Ac	GA21
GM maize							
Bt11	+	-	+	-	-	+	-
Event176	+	-	-	-	-	-	-
MON810	+	-	-	-	-	-	-
T25	+	-	+	-	-	-	-
GA21	-	-	-	-	-	-	+
NK603	+	+	-	-	-	-	-
MON863	+	-	-	-	-	-	-
TC1507	+	-	+	-	-	-	-
DAS59122	+	-	+	-	-	-	-
MON88017	+	+	-	-	-	-	-
MIR604	-	-	-	+	-	-	-
MON89034	+	-	-	-	-	-	-
MIR162	-	-	-	+	-	-	-
3272	-	-	-	+	-	-	-
MON87460	+	-	-	-	-	-	-
GM soybean							
RRS	+	+	-	-	-	-	-
MON89788	-	-	-	-	+	-	-
A2704-12	+	-	+	-	-	-	-
MON87701	-	-	-	-	-	+	-
MON87705	-	-	-	-	+	-	-
MON87769	-	-	-	-	+	-	-

Table 3
Summary of evaluations for individual LAMP assays

Target	GM event	LOD	Detection Time (min)	Annealing Temperature (℃)
P35S	Bt11	0.1%	18.56±3.30	86.22±0.035
	Event176	0.5%	19.17±3.19	86.21±0.099
	MON810	0.3%	18.09±1.57	86.20±0.048
	T25	0.3%	16.43±2.25	86.15±0.059
	NK603	0.3%	18.24±3.28	86.14±0.057
	MON863	0.3%	20.21±2.58	86.11±0.082
	TC1507	0.5%	16.51±2.20	86.27±0.080
	DAS59122	0.3%	17.14±2.30	86.14±0.054
	MON88017	0.3%	21.50±2.33	86.09±0.085
	MON89034	0.5%	17.22±4.09	86.30±0.077
	MON87460	0.5%	20.13±2.52	86.25±0.064
	RRS	0.05%	18.23±4.32	85.96±0.064
	A2704-12	0.05%	17.13±3.41	85.97±0.063
EPSPS	NK603	0.3%	21.28±2.43	93.63±0.158
	MON88017	0.3%	21.40±2.54	93.63±0.095
	RRS	0.1%	21.12±2.03	93.55±0.075
PAT	Bt11	0.3%	12.31±2.56	87.17±0.047
	T25	0.3%	13.21±2.56	87.16±0.050
	TC1507	0.3%	14.15±3.17	87.16±0.046
	DAS59122	0.3%	11.09±2.04	87.15±0.070
	A2704-12	0.05%	12.59±2.28	87.07±0.097
pmi	MIR604	0.3%	16.32±3.13	87.03±0.046
	MIR162	0.3%	16.43±3.12	87.02±0.084
	3272	0.5%	13.36±2.02	87.12±0.040

Table 3 (continued)

tE9	MON89788	0.1%	20.29±2.17	80.73±0.053
	MON87705	0.5%	18.42±1.50	80.73±0.039
	MON87769	0.5%	18.42±1.15	80.79±0.043
Cry1Ab/ Cry1Ac	Bt11	0.3%	15.34±3.49	87.00±0.048
	MON87701	0.1%	11.44±1.24	86.89±0.029
GA21	GA21	0.1%	14.50±3.37	90.73±0.030

Table 4
Comparison of the sensitivities of the four P35S targeting primers

		P35S	P35S-1	P35S-2	P35S-3
0.5% MON810	Positive/Total	21/21	19/21	3/21	19/21
	Positive rate	100%	90.5%	14.3%	90.5%
0.3% MON810	Positive/Total	21/21	18/21	3/21	11/21
	Positive rate	100%	85.7%	14.3%	52.4%
0.1% RRS	Positive/Total	21/21	13/21	4/21	18/21
	Positive rate	100%	61.9%	19.0%	85.7%
0.05% RRS	Positive/Total	20/21	13/21	3/21	10/21
	Positive rate	95.2%	61.9%	14.3%	47.6%

Table 5
Summary of the evaluations for sample direct detections

Target	GM event	GM (%)	Positive/Total	Positive rate	Detection Time (min)	Annealing Temperature (℃)
P35S	MON810 (40 mg)	0.5%	21/21	100%	15.03±2.09	86.18±0.047
	MON810 (20 mg)	0.5%	18/21	85.7%		
	RRS (20 mg)	0.5%	21/21	100%	16.59±1.19	86.22±0.046
EPSPS	MON88017 (40 mg)	0.5%	21/21	100%	17.57±0.36	93.72±0.048
	RRS (20 mg)	0.5%	21/21	100%	24.08±0.23	93.96±0.884
PAT	Bt11 (40 mg)	0.4%	21/21	100%	10.01±0.34	87.23±0.042
	A2704-12 (20 mg)	0.5%	21/21	100%	11.34±0.14	87.23±0.033
pmi	MIR604 (40 mg)	0.5%	21/21	100%	16.48±3.45	87.00±0.047
	MIR604 (20 mg)	0.5%	15/21	71.4%		
tE9	MON89788 (20 mg)	0.5%	21/21	100%	24.21±0.44	80.72±0.049
Cry1Ab/ Cry1Ac	Bt11 (40 mg)	0.4%	21/21	100%	10.18±0.28	87.00±0.036
	MON87701 (20 mg)	0.5%	21/21	100%	12.33±0.48	87.03±0.039
GA21	GA21 (40 mg)	0.5%	21/21	100%	13.57±1.55	90.78±0.032

Supplementary material

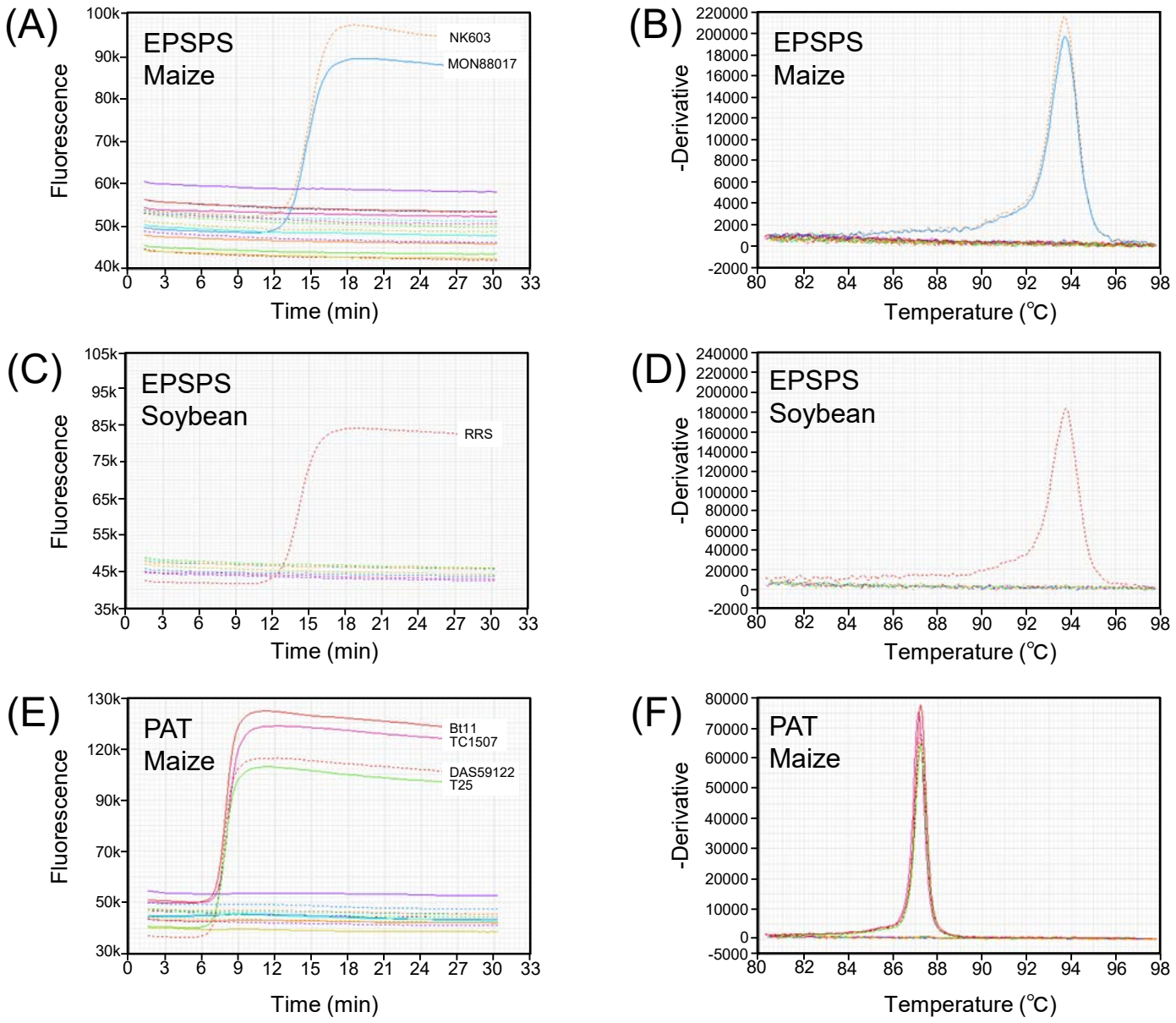


Fig. S1

Fig. S1 (continued)

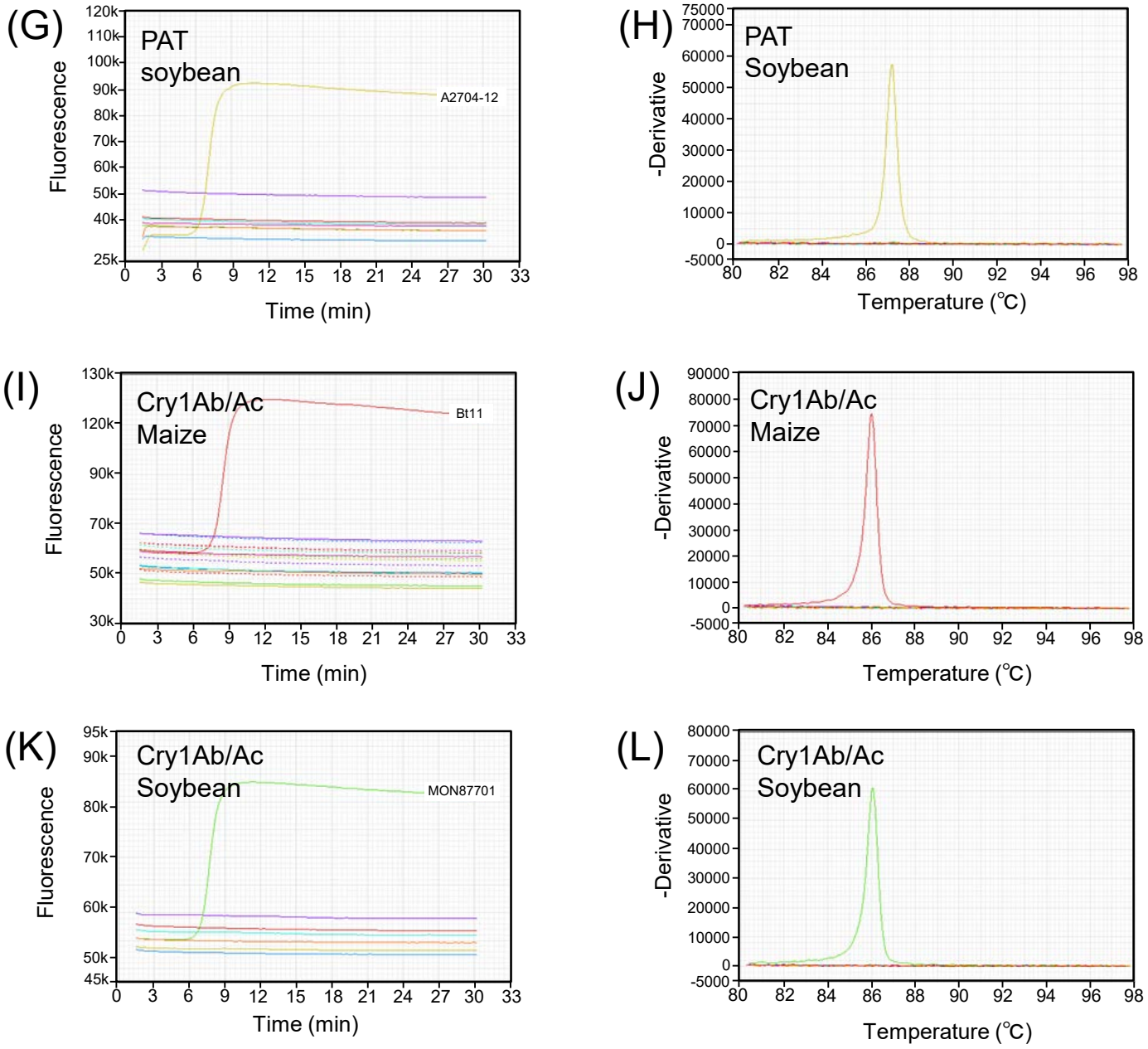
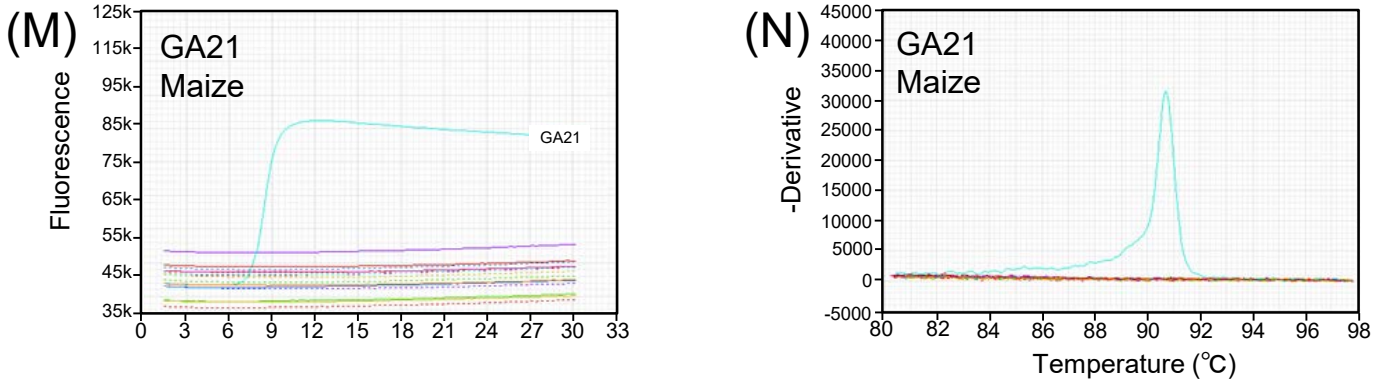


Fig. S1 (continued)



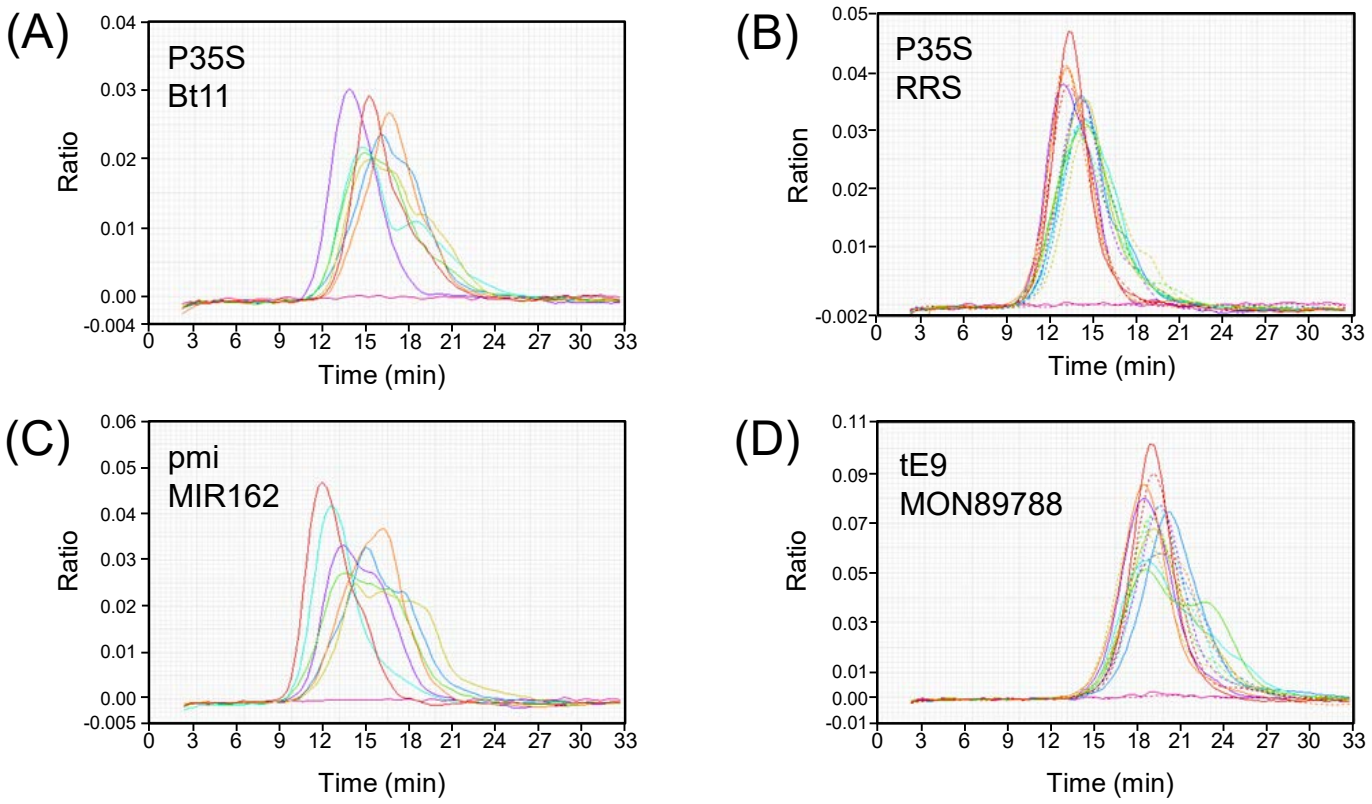

(A)
P35S
Bt11
Ratio
Time (min)
(B)
P35S
RRS
Ration
Time (min)
(C)
pmi
MIR162
Ratio
Time (min)
(D)
tE9
MON89788
Ratio
Time (min)

Fig. S2

Table S1

Contents of the weight-based mixing samples

Target	P35S	EPSPS	PAT	pmi	tE9	Cry1Ab/Cry1Ac	GA21
Maize	① MON810(0.5%)+ GA21(0.5%)	⑤ MON88017(0.5%)	② Bt11(0.4%)+ GA21(0.2%)	③ MIR604(0.5%)		② Bt11(0.4%)+ GA21(0.2%)	① MON810(0.5%)+ GA21(0.5%)
Soybean	⑥ RRS(0.5%)+ MON87701(0.5%)	④ RRS(0.5%)+ A2704-12(0.5%)+ MON89788(0.5)%	④ RRS(0.5%)+ A2704-12(0.5%)+ MON89788(0.5)%		④ RRS(0.5%)+ A2704-12(0.5%)+ MON89788(0.5)%	⑥ RRS(0.5%)+ MON87701(0.5%)	

Underlined GM events contain each target segment.

A circled number preceding at event names corresponds to the sample number in the text.