A Histochemical Method Using a Substrate of β-Glucuronidase for Detection of Genetically Modified Papaya

(Received August 22, 2003)

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A histochemical assay for detecting genetically modified (GM) papaya (derived from Line 55-1) is described. GM papaya, currently undergoing a safety assessment in Japan, was developed using a construct that included a β-glucuronidase (GUS) reporter gene linked to a virus coat protein (CP) gene. Histochemical assay was used to visualize the blue GUS reaction product from transgenic seed embryos. Twelve embryos per fruit were extracted from the papaya seeds using a surgical knife. The embryos were incubated with the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) in a 96-well microtiter plate for 10-15 hours at 37°C. Seventy-five percent of GM papaya embryos should turn blue theoretically. The histochemical assay results were completely consistent with those from a qualitative polymerase chain reaction (PCR) method developed by this laboratory. Furthermore, the method was validated in a five-laboratory study. The method for detection of GM papaya is rapid and simple, and does not require use of specialized equipment.

Key words: genetically modified papaya; recombinant DNA; GUS assay; PCR; detection method; embryo

Introduction

In recent years, there has been great progress in food biotechnology, including transgenic crop breeding and genetic modification for food production. The Ministry of Health, Labor and Welfare (MHLW) in Japan announced a mandatory safety assessment of genetically modified (GM) crops and processed foods containing GM ingredients. Since April 1, 2001, any GM foods which have not been authorized must not be imported or sold in Japan. Therefore, qualitative detection methods for regulated unauthorized GM foods were required. We reported the detection methods for GM soybean, GM maize, GM potato (NewLeaf Plus, New Leaf Y) and GM papaya (Line 55-1 or its derivatives) using qualitative polymerase chain reaction (PCR) methods. Since qualitative PCR methods involve several steps and are costly for routine analyses, a rapid method to detect GM foods was sought.

GM papaya (Line 55-1) has not yet been authorized in Japan. The GM papaya expresses the Papaya ringspot virus (PRSV) coat protein (CP), which confers tolerance to PRSV, neomycin phosphotransferase II (NPTII), and β-glucuronidase (GUS). The GUS gene, which was derived from E. coli, encodes the enzyme β-glucuronidase. Jefferson et al. developed a histochemical assay for GUS expression in transformed plants using the enzyme activity of GUS towards 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) as a substrate. In the present study, we describe a rapid and
simple method for the qualitative detection of GM papaya (Line 55–1 derivatives) using the GUS assay. The method was validated in a five-laboratory study.

**Materials and Methods**

**Samples**
Seeds from non-genetically modified (non-GM) papaya (Kapoho) and GM papaya (Rainbow, an F₁ hybrid of the PRSV CP gene homozygous Line 55–1 × Kapoho) were obtained from the Ministry of Health, Labor and Welfare (MHLW) in Japan. The fruit and the seeds were stored at room temperature, 4°C, or 20°C for one week to 6 months.

**Reagents**
4-Bromo-5-chloro-3-indolyl-β-D-glucuronide (X-Gluc) and 4-bromo-5-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Agarose and a DNA marker (Cat. No. 3407A) were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). DNA polymerase (AmpliTaq Gold) was purchased from Applied Biosystems (Foster City, CA, USA). All other reagents were of special grade. Water was purified using a Millipore Milli-Q Synthesis A 10 super water purifier and then autoclaved at 121°C for 20 minutes.

**Preparation of reagents**
A freshly prepared 200 mmol/L sodium phosphate buffer (pH 7.0) was used for each test. A 20 mg sample of X-Gluc powder was transferred into a microcentrifuge tube (1.5 mL) and dissolved in 1 mL of dimethylformamide (X-Gluc solution). The X-Gluc solution was stored at −20°C. The substrate solution was prepared by adding the X-Gluc solution to 200 mmol/L sodium phosphate buffer (pH 7.0) to give a final concentration of 1 mmol/L. The solution was thoroughly mixed using a vortex mixer (MY-51, Yamato Co., Ltd., Tokyo, Japan). The substrate solution was prepared following excision of the embryos.

**Procedure for identifying genetically modified papaya (Line 55–1 derivatives)**
A 50 μL aliquot of 200 mmol/L sodium phosphate buffer (pH 7.0) was added to each well in a 96-well microtiter plate. The required number of wells for the assay is the number of papayas × 12 since 12 embryos per papaya were tested.

The fresh papaya was cut in half lengthwise and a dark brown seed was picked at random. The viscous outer seed coat was removed with a surgical knife on a glass plate. A lengthwise cut was then made on the seedcoat in a straight line with a surgical knife. The tip of the knifepoint was placed into the cut to dislodge the seed coat, and the white endosperm enclosing the embryo was removed (Fig. 2). Careful examination of the endosperm revealed a transverse white line indicating the embryo. The endosperm was slit lengthwise adjacent to the white line. The freed embryo was carefully extracted with forceps, examined for nicks, and placed in the buffer in the microtiter plate (Fig. 3). For reaction with X-Gluc, the embryos should not be injured to prevent false-positives. Embryos from non-transgenic papaya can turn blue if the embryos are cut or broken. Twelve embryos were extracted from each test fruit. Seeds without an endosperm or seeds with

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**Fig. 1.** Reaction between the GUS enzyme and X-Gluc

**Fig. 2.** Procedure for removing the seed coat
(a) Photograph of cutting the papaya seed to make the nick. (b) Schematic structure of the papaya seed cut for removing the seed coat.
white seed coats were not used. After placing all of the embryos in the wells, the sodium phosphate buffer was replaced with 50 μL of the substrate solution (1 mmol/L X-Gluc in 200 mmol/L sodium phosphate buffer, pH 7.0). The 96-well microtiter plate was placed under vacuum for 15 minutes using an aspirator to improve substrate infiltration into the tissue. The 96-well microtiter plate was sealed and incubated at 37°C in an incubator (BNA-111; ESPEC Co., Ltd., Osaka, Japan) for 10–15 hours. After incubation, the reaction was stopped by adding 50 μL of 70% ethanol to each well. Finally, the embryos that were stained blue were counted and the percentage that expressed GUS was calculated using the following formula:

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\text{The percentage of embryos expressing GUS} = \frac{\text{the number of embryos that turned blue}}{12} \times 100.
\]

**Assessment of the results**

If the seed samples were from Rainbow papaya, a self-pollinated fruit, an average of 75% of the F2 embryos was expected to turn blue (9/12 embryos). Embryos from non-transgenic papaya do not turn blue. Since the sample size was small, the percentage of GUS-expressing embryos was expected to vary. When the percentage expressing GUS was more than 33.3% (i.e., more than 4 embryos turned blue), the sample was regarded as positive. When GUS expression was less than 25% (i.e., less than 3 embryos turned blue), the sample was regarded as negative.

**Qualitative PCR method**

The qualitative PCR method was carried out according to the previous report. The seeds were removed from the papaya, and the flesh was cut into 10 mm cubes, and freeze-dried. The freeze-dried flesh was ground using a mixing mill (SCM-40A, Shibata Co., Ltd., Tokyo, Japan). DNA was extracted using a silica-gel membrane-type kit (QIAGEN DNeasy Plant Mini) with some method modifications. An 80 mg sample of ground sample was transferred into a 2 mL microcentrifuge tube. A 400 μL aliquot of AP1 buffer previously warmed to 65°C and 4 μL of RNase A were added to the tube, which was then incubated at 65°C for 15 minutes. AP2 buffer (195 μL) was added, and the mixture was cooled on ice for 5 minutes and centrifuged at 10,000×g for 5 minutes at room temperature. The supernatant was applied to a QIAshredder spin column, and the column centrifuged for 2 minutes at 10,000×g at room temperature. The eluate was transferred to a 2 mL microcentrifuge tube. The eluate was added to the centrifuge tube with 1.5 times the volume of the AP3 buffer/ethanol mixture and the mixture was stirred for 10 seconds using a vortex mixer. A 500 μL aliquot of the mixture was applied to the mini spin column and the column was centrifuged at 10,000×g for 5 minutes at room temperature. The eluate was discarded. 500 μL aliquots of the remaining mixture were applied to the same column and the column was centrifuged, discarding the eluate after each centrifugation. The procedure was repeated until all of the mixture had been applied. A 500 μL aliquot of the AW buffer was added to the column and the column was centrifuged at 10,000×g at room temperature for 5 minutes. The eluate was discarded. The AW buffer was added to the column and the procedure was repeated. After the eluate was discarded, the column was centrifuged at 10,000×g or more for 15 minutes to dry it. The column was connected to the kit’s centrifuge tube and 50 μL of
previously warmed water was added to the column. The column was allowed to equilibrate for 5 minutes and then centrifuged at 10,000×g for 1 minute to elute the DNA. Water was added and the same procedures were repeated. The solution of combined eluates was used as the DNA sample stock solution.

The PCR reaction mixture was prepared in a PCR reaction tube. The reaction volume of 25 µL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 3 mmol/L MgCl₂, 0.2 µmol/L of 5’ and 3’ primers and 0.625 units of Taq DNA polymerase. Reactions were buffered with PCR buffer II (Applied Biosystems, Foster City, CA, USA). Amplification was performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) according to the following PCR step-cycle program: pre-incubation at 95°C for 10 minutes, denaturation at 95°C for 0.5 minutes, annealing at 60°C for 0.5 min, and extension at 72°C for 0.5 minutes. The cycle was repeated 40 times followed by a final extension at 72°C for 7 minutes.

After PCR amplification, agarose gel electrophoresis of the PCR reaction product was carried out according to the previous report. Samples of 7.5 µL of each PCR reaction mixture were electrophoresed at constant voltage (100 V) on a 4% agarose gel in the TAE buffer solution (40 mmol/L Tris-HCl, 40 mmol/L acetic acid, and 1 mmol/L EDTA, pH 8.0). After electrophoresis, the gel was stained in the TAE buffer solution with 0.5 µg/mL ethidium bromide for 30 min and de-stained in distilled water for 30 min. The gel was photographed with a Chemi-lumi Image Analyser (Chemi-lum Image Analyser with “Diana” system as the analytical software, Raytest, Germany).

Inter-laboratory validation

Inter-laboratory validation was performed in five laboratories including the National Institute of Health Sciences (NIHS). We requested all participants to follow the test protocol described above. The materials, reagents, the test protocol, and three blind samples (GM papaya seeds and non-GM papaya seeds) were supplied by the NIHS. Additional reagents and materials were supplied by participating laboratories. The data were compared and analyzed statistically.

Results and Discussion

Examination of GUS detection method conditions for detecting GM papaya

The GUS assay has been used worldwide to identify transgenic plants expressing the β-glucuronidase transgene. Crops awaiting approval in Japanese safety assessments, for example, GM papaya expressing GUS gene, could be monitored with a histochemical assay. Adoption of a GUS assay protocol would simplify identification of GM papaya by replacing the PCR method. Figure 1 shows the GUS reaction. When X-Gluc is added to plant tissues expressing GUS, X-Gluc is de-esterified by GUS and the indoxyl derivative monomer is released. The blue indigotin dye (insoluble in water) is generated by oxidative polymerization of these monomers. Seed embryos of GM papaya that contain the GUS gene would turn blue. A rapid detection method for GM papaya, developed using the GUS assay of embryos, was based on a protocol prepared by the United States Department of Agriculture (USDA)9). The procedure for extracting embryos from seed is shown in Figs. 2 and 3. According to the protocol prepared by the USDA, we have to excise 36 embryos from the seeds.

To judge the result rapidly, we proposed to take only 12 embryos from the seeds and to test 12 embryos because the embryo extraction from the seeds takes the longest time during the procedure. Although it takes a few hours to excise 36 embryos from the seeds, it should take only an hour to excise 12 embryos. If we test 12 embryos, the possibility of misjudgment (false-positive or -negative) in the GM papaya could be 1/416667. Thereafter, we tested only 12 embryos from each papaya.

We examined the conditions for the reaction between the expressed GUS of the embryo and the substrate X-Gluc. We found that incubation at 37°C for 10–15 hours with 1 mmol/L X-Gluc gave the clearest blue on the embryos from the GM papaya versus white on the embryos from the non-GM papaya. When we incubated the embryos from non-GM papaya for more than 15 hours, some embryos appeared slightly blue. Therefore, incubation for longer than 15 hours can result in false positives. Figure 4 shows typical results of the method following the protocol described. Only the embryos from the GM papaya turned blue, while none of the embryos from non-GM papaya did.

Effects of the storage conditions of GM papaya on the proposed method

Since storage conditions of the GM papaya could affect GUS activity in the seeds, the effects of storage conditions were examined (Table 1). GM and non-GM papayas, stored from one week to six months at 4°C or −20°C, were tested. Non-GM papayas were never GUS-positive. Furthermore, GM papayas stored for up to three weeks at 4°C and three months at −20°C were correctly identified. However, embryos from the seeds of GM papaya stored for six months at −20°C never stained blue using this method.

Comparative study of the proposed method and qualitative PCR method

Previously, we reported the detection of recombinant DNA from GM papaya using a qualitative PCR method5). The qualitative PCR method and the proposed method for detecting GM papaya were compared. Fifty-two papaya samples were investigated using both the proposed method and the qualitative PCR method. The results from the proposed method were completely

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9 Letter from the Commodities Branch to the Papaya Administrative Committee COM-01-205
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**Table 1.** Effects of Temperature and Period for Storage on the GUS Activity

<table>
<thead>
<tr>
<th>Temperature</th>
<th>0 day</th>
<th>1 w</th>
<th>2 w</th>
<th>3 w</th>
<th>4 w</th>
<th>6 w</th>
<th>6 m</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT non-GM</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GM</td>
<td>69.4</td>
<td>79.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4°C non-GM</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>GM</td>
<td>72.2</td>
<td>63.9</td>
<td>88.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>−20°C non-GM</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>GM</td>
<td>72.2</td>
<td>70.9</td>
<td>79.2</td>
<td>91.7</td>
<td>79.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Unit: %; n=2–6; w: weeks; m: months; −: not tested; 0 day: The result on the day we received the papayas.

**Table 2.** Result of Inter-laboratory Validation for GUS Method (5 Labs)

<table>
<thead>
<tr>
<th>Lab. No.</th>
<th>non-GM</th>
<th>GM-1</th>
<th>GM-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>58.3</td>
<td>66.7</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>75.0</td>
<td>50.0</td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>58.3</td>
<td>66.7</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>66.7</td>
<td>83.3</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>83.3</td>
<td>91.7</td>
</tr>
</tbody>
</table>

Average percentage of GUS-expressing embryos

<table>
<thead>
<tr>
<th>Lab. No.</th>
<th>The percentage of correct results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 (5/5)*</td>
</tr>
<tr>
<td>2</td>
<td>100 (5/5)*</td>
</tr>
<tr>
<td>3</td>
<td>100 (5/5)*</td>
</tr>
</tbody>
</table>

Asterisks indicate the ratio of the number of labs that submitted correct data to the number of participating labs.

In conclusion, the proposed method using the GUS assay is rapid, simple, and reproducible for the detection of GM papaya and does not require specialized equipment or complicated procedures. The method should be useful in monitoring imported papaya for the presence of GM fruits.

**Acknowledgments**

We are grateful to Dr. Akira Miki for providing the protocol suggested by the USDA. A part of this work was supported by a Grant for Scientific Research Expense for Health, Labour and Welfare Programs from the Japanese Government.

**References**


