

Study Title

Genetic Characterization of Maize Event 1507:
Southern Blot Analysis

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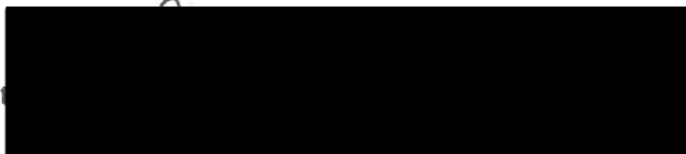
GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. FDA (21 CFR Part 58) Good Laboratory Practice Standards for Nonclinical Laboratory Studies and EPA FIFRA (40 CFR Part 160), EPA TSCA (40 CFR Part 792) Good Laboratory Practice Standards, which are consistent with OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM(98)17, and MAFF Japan Good Laboratory Practice Standards (59 NohSan No. 3850), except for the item documented below. The item listed does not impact the validity of the study.

The test substance was characterized by the sponsor prior to the initiation of this study. Although the characterization was performed at a non-GLP-compliant laboratory, the accuracy of the data is considered sufficient for the purposes of this study.

Submitter / Sponsor: Pioneer Hi-Bred International, Inc.
7250 NW 62nd Avenue
P. O. Box 552
Johnston, IA 50131

Study Director



Staff Scientist

09-Aug-2000
Date

QUALITY ASSURANCE STATEMENT

Haskell Sample Number(s):

24258
24259
24260
24261
24262
24263
24264
24265
24365

Dates of Inspections:

Conduct: October 18, 1999; December 15-16, 1999; March 3, 6-8, 2000

Records, Reports: August 2-4, 2000

Dates Findings Reported to:

Study Director: August 7, 2000

Management: August 9, 2000

Reported by



Quality Assurance Auditor

09-Aug-2000
Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Approved by Study

Sponsor:

[REDACTED]

08-22-2000

Date

Regulatory Science Coordinator

Issued by Study
Director

[REDACTED]

09-Aug-2000

Date

Staff Scientist

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STUDY INFORMATION

Substance Tested: Bt *cry1F* maize line 1507 DNA

Known Impurities: Not supplied by the sponsor

Physical Characteristics: Colorless liquid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Sponsor: Pioneer Hi-Bred International, Inc.
Johnston, IA 50131
U.S.A.

Study Initiated/Completed: October 18, 1999 / (see report cover page)

Haskell Number: 24258

Synonyms/Codes:

- H-24258
- Bt *cry1F* maize line 1507 DNA
- T1S1-1507-1A (Lot No.)

Composition: Bt *cry1F* maize line 1507 (T1S1-1507-1a) DNA

Haskell Number: 24259

Synonyms/Codes:

- H-24259
- Bt *cry1F* maize line 1507 DNA
- T1S1-1507-1B (Lot No.)

Composition: Bt *cry1F* maize line 1507 (T1S1-1507-1b) DNA

STUDY INFORMATION (CONTINUED)

Haskell Number: 24260

Synonyms/Codes:

- H-24260
- Bt *cry1F* maize line 1507 DNA
- T1S1-1507-1C (Lot No.)

Composition: Bt *cry1F* maize line 1507 (T1S1-1507-1c) DNA

Haskell Number: 24261

Synonyms/Codes:

- H-24261
- Bt *cry1F* maize line 1507 DNA
- T1S1-1507-1D (Lot No.)

Composition: Bt *cry1F* maize line 1507 (T1S1-1507-1d) DNA

Haskell Number: 24262

Synonyms/Codes:

- H-24262
- Bt *cry1F* maize line 1507 DNA
- BC4-1507-4A (Lot No.)

Composition: Bt *cry1F* maize line 1507 (BC4-1507-4a) DNA

Haskell Number: 24263

Synonyms/Codes:

- H-24263
- Bt *cry1F* maize line 1507 DNA
- BC4-1507-4B (Lot No.)

Composition: Bt *cry1F* maize line 1507 (BC4-1507-4b) DNA

STUDY INFORMATION (CONTINUED)

Haskell Number: 24264

Synonyms/Codes:

- H-24264
- *Bt cry1F* maize line 1507 DNA
- BC4-1507-4C (Lot No.)

Composition: *Bt cry1F* maize line 1507 (BC4-1507-4c) DNA

Haskell Number: 24265

Synonyms/Codes:

- H-24265
- *Bt cry1F* maize line 1507 DNA
- BC4-1507-4D (Lot No.)

Composition: *Bt cry1F* maize line 1507 (BC4-1507-4d) DNA

Haskell Number: 24365

Synonyms/Codes:

- H-24365
- GS3 non-transgenic control maize DNA
- Control DNA

Composition: GS3 non-transgenic control DNA

STUDY PERSONNEL

Study Director: [REDACTED]

Management: [REDACTED]

Primary Technician: [REDACTED]

Report Preparation: [REDACTED]
[REDACTED]

SUMMARY

Southern hybridization was used to determine the nature and number of *cry1F* and *pat* gene insertions which occur in maize transformation event 1507. Total genomic DNA from transgenic maize root samples (event 1507 transformation: T1S1 and BC4 generations) and from non-transgenic GS3 samples were digested with specific restriction endonucleases and electrophoretically separated on a 0.8% agarose gel to characterize the nature of the insertion of linear fragment PHI 8999 into the genome. The separated DNA was transferred and affixed to a positively charged nylon membrane and hybridized in the presence of a radiolabeled probe. The electrophoresis allowed resolution of fragments ranging in size from $\geq 20,000$ base pairs to approximately 300 base pairs. The location of the inserted transgene(s) was determined with standard mapping studies which analyzed autoradiographic signals from reporter probes that specifically hybridized to the size-separated restriction enzyme digested genomic DNA fragments. Reporter probes were generated by the use of specific primers to amplify five defined regions of the plasmid PHP 8999. These probes were subsequently used to separately identify specific regions of the inserted transgene. The probes that were used were: "ubiquitin," "*cry1F*," "*pat*," "CaMV35S," and "kanamycin." These probes were radiolabeled with ^{32}P using a random priming procedure. Resulting radioactive signals allowed for an analysis of the transgene's structure within the genome.

The results from this study indicate that T1S1 and BC4 generations of event 1507 contain one full-length insertion of the transgene. The data also suggest that a partial-length insert is present in both generations, and that this additional copy of the transgene contains all or part of the *cry1F* coding sequence. It is unclear from the data as to whether or not the additional copy contains the ubiquitin promoter region. The results from this study also indicate that neither generation contains the kanamycin resistance gene.

INTRODUCTION

Event 1507 transgenic maize was generated by isolating a 6235 base pair (bp) *Pme* I digestion fragment (PHI 8999) from the plasmid PHP 8999 (see Appendix 1), and inserting this linear fragment into the maize genome using particle bombardment. The intended fragment (also known as a transgene) contained regions for the expression of two genes: *cry1F* (conferring insect resistance) and *pat* (conferring glufosinate tolerance), as well as the necessary regulatory components for their expression (see Appendix 2). Glufosinate tolerance was used as a selectable marker for the transformation event. Selection procedures resulted in the two generations (T1S1 and BC4 generations) of maize that provided the DNA samples for this study. The objectives of this study were to characterize the insertion and stability of the exogenously integrated transgene and to demonstrate the absence of the gene encoding for kanamycin resistance. In this study genomic DNA samples from four replicates (labeled a, b, c, and d) each from event 1507 T1S1 and BC4 generations were pooled separately and analyzed by Southern blot.

MATERIALS AND METHODS

A. Test Substances

Two test substances were used in this study: genomic DNA obtained from the T1S1 and BC4 generations of maize event 1507. Each test substance consisted of a pooled sample of the four lot numbers (a, b, c, and d) for each generation (see Table 1).

Each test substance was supplied by the sponsor as a clear liquid. The DNA concentration for each sample was measured at Haskell Laboratory. Samples were in the 1.3-2.3 µg/µl concentration range. Equivalent amounts of DNA from each sample in each generation (T1S1 and BC4) were pooled together and the pools were labeled either as "T1S1" or "BC4," respectively. After combining the samples, the final concentration of each pool was measured and determined to be approximately 1.9 µg/µl for T1S1 and 1.5 µg/µl for BC4. Each test substance pool was briefly mixed before each aliquot was removed for restriction enzyme digestion. The test substances appeared to be stable under the storage conditions of the study. An aliquot of the original, undigested DNA was run on an agarose gel and stained with ethidium bromide. It was confirmed that upon receipt of the test substances, there was minor (not atypical) breakdown of the DNA, but that the amount of breakdown remained constant throughout the course of the study. No evidence of instability, such as a change in color or physical state, was observed.

B. Control Substances

Two control substances were used in this study: genomic DNA from GS3 maize and DNA from plasmid PHP 8999. GS3 is a non-transgenic maize line and genomic DNA from this line served as a negative control substance to: 1) demonstrate the absence of non-specific binding of the hybridization probes and 2) discriminate between endogenous and transgenic ubiquitin promoter regions. Plasmid PHP 8999 served as a positive control substance to demonstrate specificity of the hybridization probes used in the study. Lot numbers (where applicable) for the control substances are shown in Table 2.

Each control substance DNA was supplied by the sponsor as a clear liquid. The DNA concentration of each control was measured at Haskell Laboratory. PHP 8999 was found to be approximately 1.2 µg/µl and GS3 was found to be approximately 0.3 µg/µl. To increase its concentration, an aliquot of GS3 was ethanol-precipitated and resuspended in water. The resulting concentration was measured and found to be approximately 1.1 µg/µl. Each control substance was briefly mixed before each aliquot was removed for restriction enzyme digestion. The control substances appeared to be stable under the storage conditions of the study. No evidence of instability, such as a change in color or physical state, was observed.

C. DNA Probes

Five probes were employed to characterize the insertion in event 1507 T1S1 and BC4 generations. Each was generated by PCR-amplification of PHP 8999 and supplied by the sponsor. The probes were: ubiquitin (specific for the ubiZM (1) promoter), *cry1F* (specific for the *cry1F* gene), CaMV35S (specific for the CaMV35S promoter), *pat* (specific for the *pat* gene) and kanamycin (specific for *nptII* for kanamycin resistance). The probes were supplied as unlabeled, PCR-amplified DNA fragments. The location and size of each probe relative to the sequence of plasmid PHP 8999 is shown in Table 3. The physical map of PHP 8999 can be found in Appendix 1.

Each probe was supplied by the sponsor as a clear liquid and the DNA concentration of each was also supplied. Ubiquitin, *cry1F*, CaMV35S, *pat* and kanamycin were 74, 0.56, 75, 0.56, and 67 ng/µl, respectively. The probes appeared to be stable under the storage conditions of the study. No evidence of instability, such as a change in color or physical state, was observed.

D. Restriction Enzymes and Digestion

Plasmid PHP 8999 DNA, genomic DNA from GS3 maize, and genomic DNA from event 1507 T1S1 and BC4 generations was digested with the following restriction enzymes (or enzyme combinations as indicated with a "/" between the individual enzymes): *Pme* I, *Hind* III, *Pst* I, *Bam*HI, *Eco*R I and *Bam*HI/*Eco*R I. For each digestion reaction, three units of each enzyme per µg genomic DNA were used (five units of each were used for plasmid PHP8999), and the

reactions were carried out at 37°C for approximately 2.5 hours. They were then stored at -20°C until electrophoresis. Representative aliquots (see Table 4) of each digest and commercially available molecular weight markers were separated on five separate agarose (0.8%) gels, then transferred and fixed to five separate positively charged nylon membranes. Hence, the DNA content on all five gels/membranes was identical. Each membrane was assigned a specific probe as shown in Table 5.

Due to technical problems associated with membrane/hybridization solution incompatibility, the results for membrane #1 and #2 were unusable. Membrane #3 and #4 were subsequently hybridized with the ubiquitin and *cryIF* probes, respectively after confirming the complete removal of the CaMV35S and *pat* probes. Membrane #5 was also rehybridized with the ubiquitin probe after confirming the complete removal of the kanamycin probe in order to assist in the interpretation of the observed results.

E. Labeling and Hybridization

All probes were labeled with [α -³²P]dATP using a random priming procedure. The total amount of probe DNA that was labeled for each hybridization is shown in Table 6. The reaction consisted of the DNA to be labeled in the presence of 20 μ M dCTP, 20 μ M dGTP, 20 μ M dTTP, 50 μ Ci [α -³²P]dATP, random primers buffer mixture, and 3 units Klenow Fragment. Each reaction was carried out at 25°C for approximately one hour then quenched with 0.5 M EDTA pH 8.0. Non-incorporated deoxyribonucleoside triphosphates were removed from the labeled probes by extracting with phenol and chloroform and precipitating with ethanol. Standard TCA precipitation and scintillation counting determined the total amount of counts of the labeled probe. Before hybridization, the labeled probes were denatured under alkaline conditions.

To identify the molecular weight markers on a subsequent autoradiogram, 0.5 μ g of *Hind* III digested lambda DNA (Roche Molecular Biochemicals (Basel Switzerland)) was also labeled with [α -³²P]dATP using a nick translation procedure. The reaction consisted of the DNA to be labeled in the presence of 20 μ M dCTP, 20 μ M dGTP, 20 μ M dTTP, 2 μ Ci [α -³²P]dATP, nick translation buffer, and enzyme mixture (DNA-polymerase I and DNase I). The reaction was carried out at 15°C for approximately 35 minutes then quenched with 0.2 M EDTA pH 8.0 and heated to 65°C for approximately 10 minutes. Non-incorporated deoxyribonucleoside triphosphates were removed from the labeled probe by extracting with phenol and chloroform and precipitating with ethanol. Standard TCA precipitation and scintillation counting was used to determine the total counts of labeled probe. A proper dilution with water was subsequently made so that there would be approximately 40,000 counts for each hybridization. Before hybridization, the labeled probe was denatured under alkaline conditions.

For hybridization, each membrane was hybridized with its designated denatured probe and 40,000 counts of denatured *Hind* III digested lambda probe. The membranes and probes were incubated together overnight at 65 °C in 10% dextran sulfate, 1% SDS, 1 M NaCl, and 0.5 mg

sheared and denatured salmon sperm DNA. The following day, the membranes were washed two times at room temperature in 2X SSC for approximately 5 to 10 minutes each. Then they were washed two times at 60 °C in 2X SSC and 1% SDS for approximately 15 to 30 minutes each. Finally, they were washed at room temperature in 0.1X SSC for approximately 15 to 30 minutes. The membranes were then wrapped in Saran™ wrap and placed under a phosphor screen overnight. The phosphor screen was scanned with a Molecular Dynamics Phosphorimager™ and the resulting image was annotated and printed. Following this, the wrapped membranes were placed at -80°C under X-ray film in a cassette containing an intensifying screen. After exposure, the films were developed using a Kodak film developer.

Table 7 shows the fragment sizes that would be expected, using each of the five probes, if the transgene was inserted into the genome as one intact copy. It should be noted that the kanamycin fragments are shown to indicate the expected sizes if the kanamycin gene had contaminated the original transformation.

Cleavage with *Pme* I would theoretically generate a fragment of ≥ 6235 bp that would be detected with the ubiquitin, *cry1F*, CaMV35S, and *pat* probes, and a 3269 bp fragment that would be detected with the kanamycin probe.

Cleavage with *Hind* III would theoretically generate a 3890 bp fragment that would be detected with the ubiquitin and *cry1F* probes, a 2170 bp fragment that would be detected with the CaMV35S and *pat* probes, and a 3444 bp fragment that would be detected with the kanamycin probe.

Cleavage with *Pst* I would theoretically generate a 1986 bp fragment that would be detected with the ubiquitin probe, a 914 bp and a 944 bp fragment that would be detected with the *cry1F* probe, a 1916 bp fragment that would be detected with the CaMV35S and *pat* probes, and a 3488 bp fragment that would be detected with the kanamycin probe.

Cleavage with *Bam*H I would theoretically generate a >2080 bp fragment that would be detected with the ubiquitin probe, a 1828 bp fragment that would be detected with the *cry1F* probe, a 1361 bp fragment that would be detected with the CaMV35S probe, a 315 bp and a 490 bp fragment that would be detected with the *pat* probe and a 5510 bp fragment that would be detected with the kanamycin probe.

Cleavage with *Eco*R I would theoretically generate a >1467 bp fragment that would be detected with the ubiquitin probe, a 3202 bp fragment that would be detected with the *cry1F* probe, a 1329 bp fragment that would be detected with the CaMV35S and *pat* probes, and a 4919 bp fragment that would be detected with the kanamycin probe.

Cleavage with *Bam*H I/*Eco*R I would theoretically generate a >1467 bp fragment that would be detected with the ubiquitin probe, a 1828 bp fragment that would be detected with the *cry1F* probe, a 546 bp fragment that would be detected with the CaMV35S probe, a 315 bp and a 468

bp fragment that would be detected with the *pat* probe and a 4897 bp fragment that would be detected with the kanamycin probe.

The reader should note that the restriction digests using *Hind* III, *Pst* I, and *Bam*H I were conducted with the primary objective of characterizing the *cry*IF gene and its ubiquitin promoter. The *Hind* III restriction enzyme cuts upstream of the 5' end of the ubiquitin promoter and the 3' end of the *cry*IF coding sequence. The purpose of the *Hind* III digestion was to determine whether the full-length *cry*IF gene was present with its promoter intact. The *Pst* I digestion was intended to provide information about the presence of an intact ubiquitin promoter as this enzyme cuts essentially at both ends of this promoter. The purpose of the *Bam*H I digestion was intended to provide information about the presence of an intact *cry*IF coding sequence as this enzyme cuts at both the 5' and 3' end of this coding sequence.

The digestions with *Bam*H I, *Eco*R I, and the combination of *Bam*H I and *Eco*R I, were conducted with the primary objective of characterizing the *pat* gene and its CaMV35S promoter. The *Bam*H I enzyme cuts at the 5' end of the *pat* gene and within approximately 150 bp of the 3' end of this gene. The *Eco*R I enzyme cuts at the 5' end of the CaMV35S promoter and in the CaMV35S terminator for the *pat* gene. Both digestions were intended to provide information on the presence of an intact copy of the *pat* gene and its CaMV35S promoter. An additional digestion with *Bam*H I and *Eco*R I was conducted to determine whether a 546 bp fragment corresponding to the CaMV35S promoter could be detected after hybridization with the CaMV35S probe.

Pme I digestion was conducted with the objective of isolating the entire insert (PHI 8999) used to transform event 1507.

RESULTS

A summary of the observed fragment sizes for each probe is provided in Table 8. It is important to note that fragment bands which are identical in size to fragment bands in the negative control lanes (labeled "1" on the blots), are annotated with an "a". Fragments of the expected size are annotated with a "b".

A. Ubiquitin Probe

As shown in Figure 1, the ubiquitin probe detected a 23,000 bp *Pme* I fragment; 3890 and 6500 bp *Hind* III fragments; 1986 and 23,000 bp *Pst* I fragments; 9000, 15,000, and 20,000 bp *Bam*H I fragments; 1700, 3000, 3500, 4000, 4100, 9400, and 23,000 bp *Eco*R I fragments; and 1700, 3000, 4000, and 9000 bp *Bam*H I/*Eco*R I fragments in both the T1S1 and BC4 samples. The 23,000 *Pme* I fragment, 6500 bp *Hind* III fragment, 1986 and 23,000 bp *Pst* I fragments, 9000, 15,000, and 20,000 bp *Bam*H I fragments, 1700, 4000, and 4100 bp *Eco*R I fragments, and 1700 bp *Bam*H I/*Eco*R I fragment were also detected in the negative control GS3 DNA. The bands that are seen in the lanes marked "4" (plasmid control lanes) demonstrate the specificity of the ubiquitin probe used in the hybridization. In the plasmid control lanes, additional bands (below the expected band) were observed for the *Pst* I, *Eco*R I, and *Bam*H I/*Eco*R I digestions. A possibility for this could be due to a re-circularization of the fragment piece causing it to resolve lower than its linear counterpart in the gel.

B. *cry1F* Probe

As shown in Figure 2, the *cry1F* probe detected a 23,000 bp *Pme* I fragment; 1000, 2000, 3890 and 4000 bp *Hind* III fragments; 914, 944, 6500, and 23,000 bp *Pst* I fragments; 1828 and 8000 bp *Bam*H I fragments; 3000, 3202 and 23,000 bp *Eco*R I fragments; and 1828, 3000, 5000, and 8000 bp *Bam*H I/*Eco*R I fragments in both the T1S1 and BC4 samples. The 23,000 bp *Pme* I fragment, 1000 and 2000 bp *Hind* III fragment, 23,000 bp *Pst* I fragment, and 5000 bp *Bam*H I/*Eco*R I fragment were also detected in the negative control GS3 DNA. The bands that are seen in the lanes marked "4" demonstrate the specificity of the *cry1F* probe used in the hybridization. In the plasmid control lanes, an additional band (below the expected band) was observed for the *Pst* I, digestion. A possibility for this could be due to a re-circularization of the fragment piece causing it to resolve lower than its linear counterpart in the gel.

C. CaMV35S Probe

As shown in Figure 3, the CaMV35S probe detected a 23,000 bp *Pme* I fragment; 2170 bp *Hind* III fragment; 1916 bp *Pst* I fragment; 1361 bp *Bam*H I fragment; 1329 bp *Eco*R I fragment; and 546 bp *Bam*H I/*Eco*R I fragment in both the T1S1 and BC4 samples. The bands that are seen in

the lanes marked "4" demonstrate the specificity of the CaMV35S probe used in the hybridization. In the plasmid control lanes, an additional band (below the expected band) was observed for the *Pst* I digestion. A possibility for this could be due to a re-circularization of the fragment piece causing it to resolve lower than its linear counterpart in the gel.

D. *pat* Probe

As shown in Figure 4, the *pat* probe detected a 23,000 bp *Pme* I fragment; 2170 bp *Hind* III fragment; 1916 bp *Pst* I fragment; 315 and 490 bp *Bam*H I fragments; 1329 bp *Eco*R I fragment; and 315 and 468 bp *Bam*H I/*Eco*R I fragments in both the T1S1 and BC4 samples. The bands that are seen in the lanes marked "4" demonstrate the specificity of the *pat* probe used in the hybridization. In the plasmid control lanes, an additional band (below the expected band) was observed for the *Pst* I digestion. A possibility for this could be due to a re-circularization of the fragment piece causing it to resolve lower than its linear counterpart in the gel.

E. Kanamycin Probe

As shown in Figure 5, the kanamycin probe did not detect any bands from any digestion in both the T1S1 and BC4 samples. The bands that are seen in the lanes marked "4" demonstrate the specificity of the kanamycin probe used in the hybridization, and that the hybridization was successfully performed. In the plasmid control lanes, an additional band (below the expected band) was observed for the *Pst* I digestion. A possibility for this could be due to a re-circularization of the fragment piece causing it to resolve lower than its linear counterpart in the gel.

DISCUSSION

The results of this study support the conclusion that event 1507 contains one full-length copy of the DNA insert PHI 8999 used to transform this maize line and one additional copy of the *cry1F* gene. The data support the conclusion that the kanamycin resistance gene, *nptII* is not present in event 1507.

The conclusion that a full-length copy of the insert PHI 8999 is present in event 1507 is based on the results of several hybridization experiments. *Hind* III digestion produced an expected 3890 bp fragment after hybridization with probes specific for the ubiquitin promoter and *cry1F*. The ubiquitin promoter was considered to be intact based on the *Pst* I digestion which resulted in an expected 1986 bp fragment following hybridization with the ubiquitin probe. The expected 1828 bp fragment was present when genomic DNA was digested with *Bam*H I and hybridized with the *cry1F* probe, indicating the presence of an intact *cry1F* coding sequence. The *Eco*R I enzyme cuts at the 5' end of the CaMV35S promoter and in the CaMV35S terminator for the *pat* gene and is expected to result in a 1329 bp fragment if an intact copy of the *pat* gene and CaMV35S promoter is present in event 1507. The 1329 bp fragment was observed after hybridization with the *pat* and CaMV35S probes. The presence of an intact *pat* gene was confirmed because the expected fragments were observed after *Bam*H I digestion followed by hybridization with the *pat* probe. The presence of an intact CaMV35S promoter was confirmed because the expected 546 bp fragment was observed following the combined *Bam*H I and *Eco*R I digestion and hybridization with the CaMV35S probe. In addition, *Hind* III digestion would be expected to produce a 2170 bp fragment containing the CaMV35S promoter and *pat* genes, if present as full-length copies; this expected fragment size was observed after hybridization with the CaMV35S and *pat* probes.

Digestion with *Pme* I was also conducted in an attempt to release the entire sequence for insert PHI 8999. However, no bands of the expected 6235 bp size were observed, most likely as a result of the loss of the *Pme* I sites either at the 3' end, 5' end, or both ends of the PHI 8999 insert.

Evidence for an additional copy of the *cry1F* sequence is based on the results of the *Hind* III and *Pst* I digestions followed by hybridization with the *cry1F* and ubiquitin probes. *Hind* III digestion and hybridization with the *cry1F* probe resulted in two bands: one of the expected 3890 bp size and a second, representing an additional copy, that is larger and estimated at ~4000 bp in size. Hybridization of the *Hind* III digest with the ubiquitin probe resulted in one band of the expected size and failed to reveal the ~4000 bp fragment. This suggests that the promoter region is either absent in this additional copy or it is not intact. A small portion of the ubiquitin promoter could not be detected with the ubiquitin DNA probe used in this study because the probe was prepared from a fragment of the ubiquitin promoter extending from 120 bp to 1707 bp. In other words, an approximately 300 bp region of the ubiquitin promoter that is 5' to the *cry1F* gene could not be detected with this probe. None of the other digestions were specifically

designed to provide evidence for the presence or absence of the ubiquitin promoter on the additional *cryIF* gene. Additional fragments were observed with the ubiquitin probe, particularly following digests with *EcoR* I and/or *BamH* I. However, these were considered to result from the presence of the endogenous maize ubiquitin promoter. Additionally, larger fragments (~23,000 kb) were observed with the ubiquitin and *cryIF* probes following digests with *Pme* I and *Pst* I. The predominant fragment size that these two particular enzymes generated was approximately 23,000 kb as was detected with ethidium bromide staining for DNA in general (data not shown). Furthermore, a greater overall level of non-specific background on the ubiquitin and *cryIF* blots was observed when compared to the CaMV35S, *pat*, and kanamycin blots. This was possibly due to the fact that both the ubiquitin and *cryIF* probes were approximately twice as long as the other probes making them more likely to bind nonspecifically. Hence, in addition to the presence of the endogenous maize ubiquitin promoter, the additional large fragments that were observed with the ubiquitin and *cryIF* probes was considered to possibly result from non-specific binding.

CONCLUSIONS

The following conclusions were derived from the results of this study:

- Event 1507 T1S1 and BC4 generations contain one full-length insertion of the PHI 8999 transgene. This is supported by confirmation of the predicted fragment sizes based on the restriction enzyme/probe combinations employed in the study. Additional fragments were observed with the ubiquitin and *cryIF* probes; however, with one exception (see below) these were concluded to be the result of the presence of the endogenous maize ubiquitin promoter or possibly due to nonspecific binding of the ubiquitin and *cryIF* probes.
- Event 1507 T1S1 and BC4 generations contain one additional copy of the *cryIF* gene; a fragment of approximately 4000 bp was observed following digestion with *Hind* III and hybridization with the *cryIF* probe. The location and size of the additional copy of *cryIF* in the event 1507 genome is unknown. It is unclear from the data as to whether or not the additional copy contains the ubiquitin promoter region.
- Event 1507 T1S1 and BC4 generations do not contain the kanamycin resistance gene *nptII*.

RECORDS AND SAMPLE STORAGE

Specimens (if applicable), raw data, and the final report will be retained at DuPont Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware, 19802.

TABLES

Table 1: Lot Numbers of Maize Event 1507 Genomic DNA

Maize Event 1507 Genomic DNA			
T1S1 Generation		BC4 Generation	
Lot numbers	Haskell numbers	Lot numbers	Haskell numbers
T1S1-1507-1A	H-24258	BC4-1507-4A	H-24262
T1S1-1507-1B	H-24259	BC4-1507-4B	H-24263
T1S1-1507-1C	H-24260	BC4-1507-4C	H-24264
T1S1-1507-1D	H-24261	BC4-1507-4D	H-24265

Table 2: Lot Numbers of Control Substance DNA

Non-Transgenic Maize (GS3; Negative Control)		Plasmid PHP 8999 (Positive Control)	
Lot number	Haskell number	Lot number	Haskell number
Not applicable	H-24365	15472	Not applicable

Table 3: Summary of Probe Locations Relative to Plasmid PHP 8999

Hybridization Probe	Probe size (kb)	Location on plasmid PHP 8999 (bp to bp)	Comments
Ubiquitin	1587	120-1707	Hybridizes to ubiquitin promoter region for <i>cryIF</i>
<i>cryIF</i>	979	2548-3527	Hybridizes to coding region for <i>cryIF</i>
CaMV35S	438	4791-5229	Hybridizes to CaMV35S promoter region for <i>pat</i>
<i>pat</i>	309	5537-5846	Hybridizes to coding region for <i>pat</i>
Kanamycin	536	7497-8033	Hybridizes to coding region for Kanamycin

Table 4: Amount of Digested DNA Per Aliquot and Molecular Weight Markers Amount

DNA Digest	Amount Per Aliquot
T1S1	5 µg
BC4	5 µg
GS3	5 µg
PHP 8999	9.5 pg (1 copy equivalent)
Lambda (<i>Hind</i> III digest)	1 µg

Table 5: Membrane Assignment for Hybridization Probes

Hybridization Probe	Membrane (gel) Number Assignment
Ubiquitin	1
<i>cry</i> 1F	2
CaMV35S	3
<i>pat</i>	4
Kanamycin	5

Table 6: Amount of Hybridization Probe in Labeling Reaction

Hybridization Probe	Amount in Labeling Reaction
Ubiquitin	37 ng
<i>cry</i> 1F	10 ng
CaMV35S	37.5 ng
<i>pat</i>	10 ng
Kanamycin	33.5 ng

Table 7: Summary of Expected Hybridizing Fragment Sizes (bp)

Restriction Enzyme	Hybridization Probe Name				
	Ubiquitin	<i>cry 1F</i>	CaMV35S	<i>pat</i>	Kanamycin
<i>Pme</i> I	≥6235	≥6235	≥6235	≥6235	3269
<i>Hind</i> III	3890	3890	2170	2170	3444
<i>Pst</i> I	1986	914 944	1916	1916	3488
<i>Bam</i> H I	>2080	1828	1361	315 490	5510
<i>Eco</i> R I	>1467	3202	1329	1329	4919
<i>Bam</i> H I/ <i>Eco</i> R I	>1467	1828	546	315 468	4897

Table 8: Summary of Observed Fragments in T1S1 and BC4 samples (Estimated Sizes (bp))

Restriction Enzyme	Hybridization Probe Name				
	Ubiquitin	<i>cry</i> 1F	CaMV35S	<i>pat</i>	Kanamycin
<i>Pme</i> I	23,000 ^a	23,000 ^a	23,000	23,000	No fragments observed
<i>Hind</i> III	3890 ^b 6500 ^a 20,000 ^c	1000 ^a 2000 ^a 3890 ^b 4000	2170 ^b	2170 ^b	No fragments observed
<i>Pst</i> I	1986 ^{a,b} 23,000 ^a	914 ^b 944 ^b 6500 23,000 ^a	1916 ^b	1916 ^b	No fragments observed
<i>Bam</i> H I	9000 ^a 15,000 ^a 20,000 ^a	1828 ^b 8000	1361 ^b	315 ^b 490 ^b	No fragments observed
<i>Eco</i> R I	1700 ^a 3000 3500 4000 4100 ^a 6500 ^c 9400 23,000	3000 3202 ^b 23,000	1329 ^b	1329 ^b	No fragments observed
<i>Bam</i> H I/ <i>Eco</i> R I	1700 ^a 3000 4000 ^a 6500 ^c 9000	1828 ^b 3000 5000 ^a 8000	546 ^b	315 ^b 468 ^b	No fragments observed

^a Also detected in negative control

^b Expected fragments

^c Detected in negative control only

FIGURES

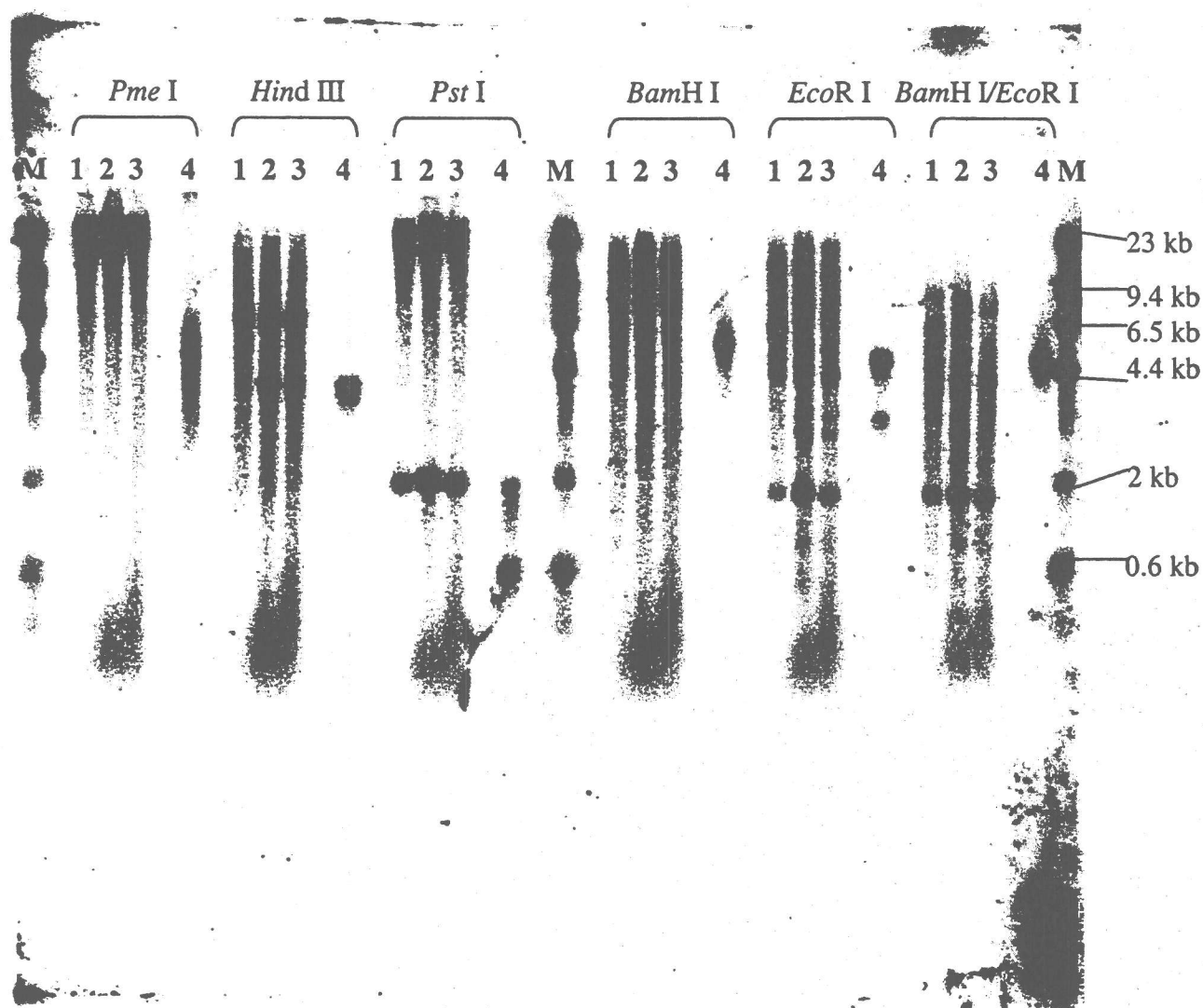


FIG. 1. Gel electrophoresis and Southern blot analysis of event 1507 – Ubiquitin probe. DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*H I, *Eco*R I, and *Bam*H I/*Eco*R I. The lanes contain:

- 1: GS3 non-transgenic control (5 µg)
- 2: pooled T1S1 DNA (5 µg)
- 3: pooled BC4 DNA (5 µg)
- 4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
- M: molecular weight marker (*Hind* III digest of lambda DNA) (1 µg). MW of lambda DNA fragments is given in kilobases (kb).

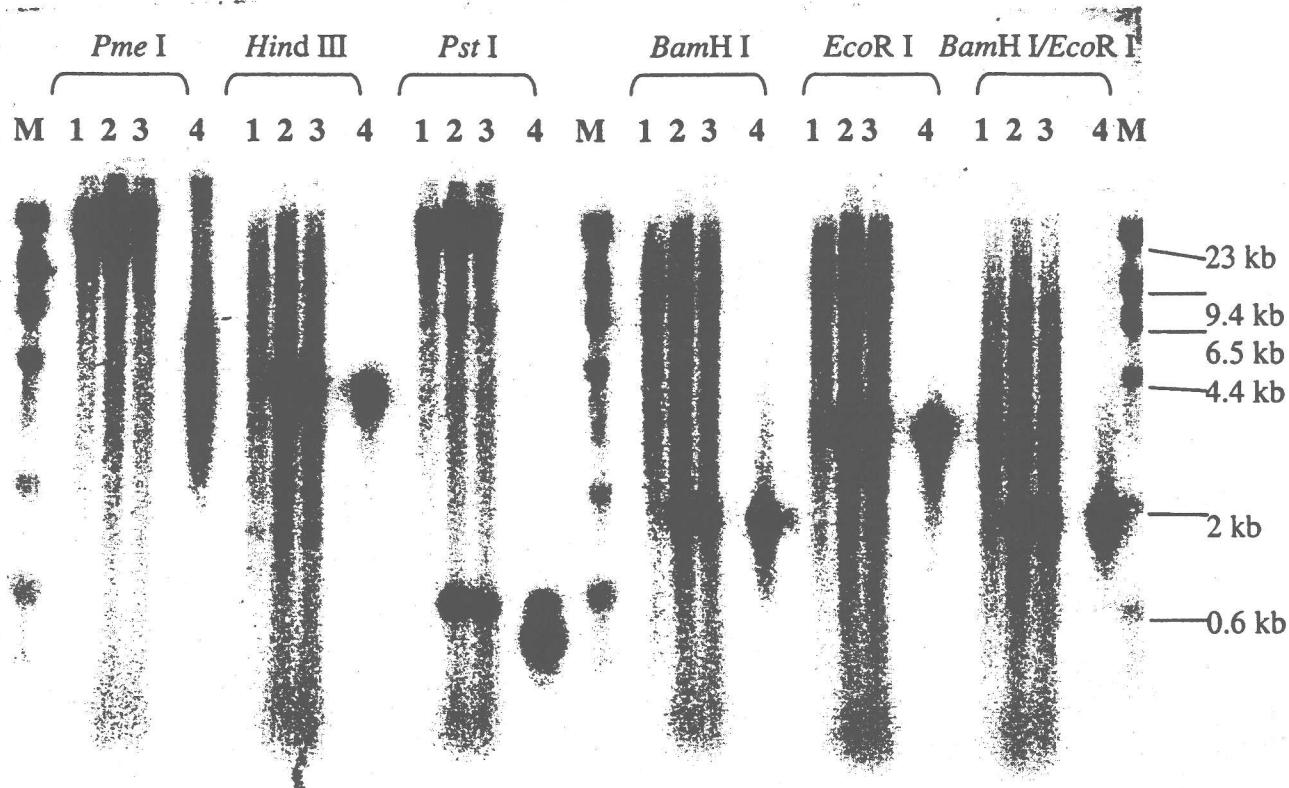


FIG. 2. Gel electrophoresis and Southern blot analysis of event 1507 – *cryIF* probe.

DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*H I, *Eco*R I, and *Bam*H I/*Eco*R I. The lanes contain:

- 1: GS3 non-transgenic control (5 µg)
- 2: pooled T1S1 DNA (5 µg)
- 3: pooled BC4 DNA (5 µg)
- 4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
- M: molecular weight marker (*Hind* III digest of lambda DNA) (1 µg). MW of lambda DNA fragments is given in kilobases (kb).

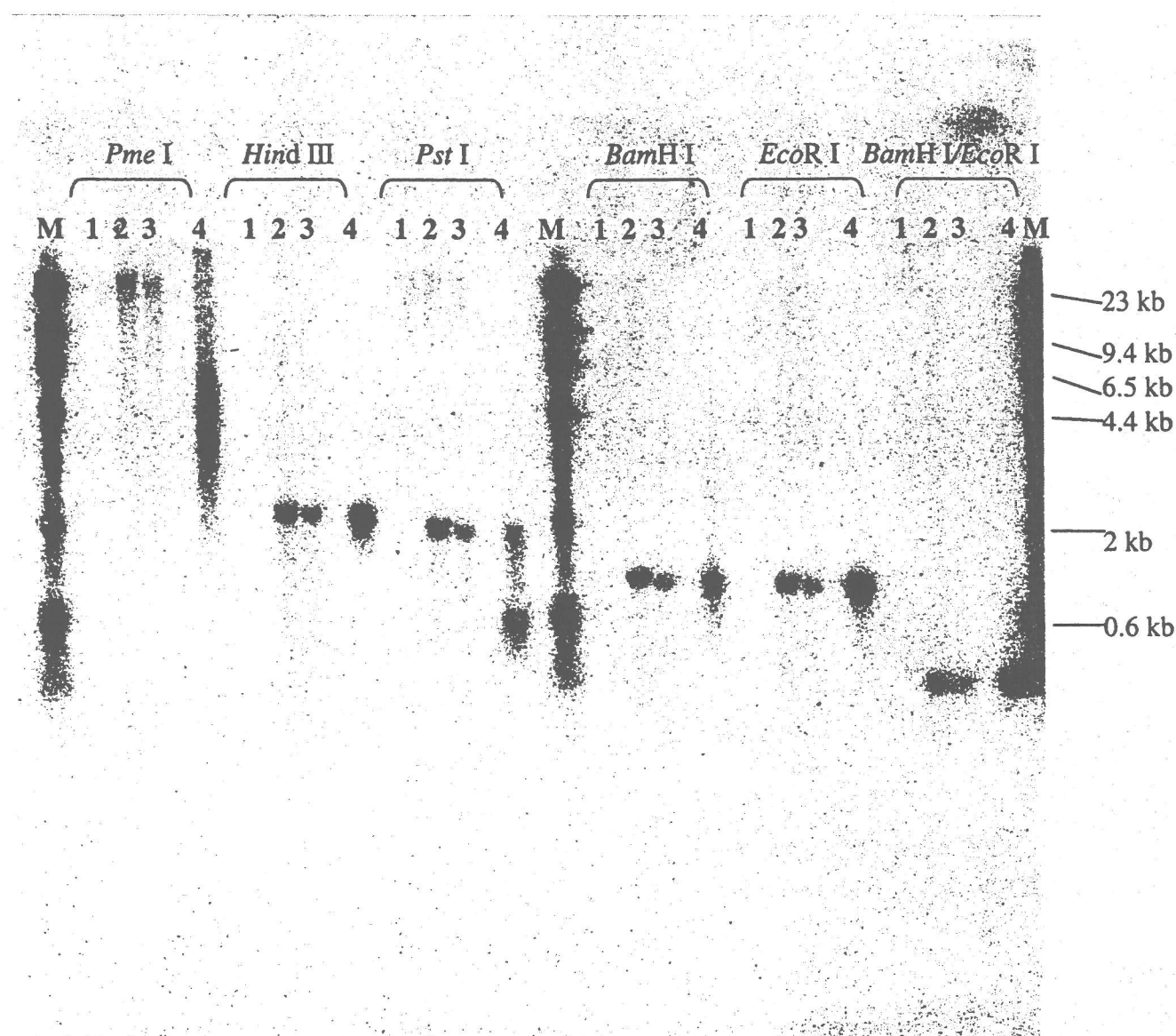


FIG. 3. Gel electrophoresis and Southern blot analysis of event 1507 – CaMV35S probe.

DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*HI, *Eco*R I, and *Bam*HI/*Eco*R I. The lanes contain:

- 1: GS3 non-transgenic control (5 µg)
- 2: pooled T1S1 DNA (5 µg)
- 3: pooled BC4 DNA (5 µg)
- 4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
- M: molecular weight marker (*Hind* III digest of lambda DNA) (1 µg). MW of lambda DNA fragments is given in kilobases (kb).

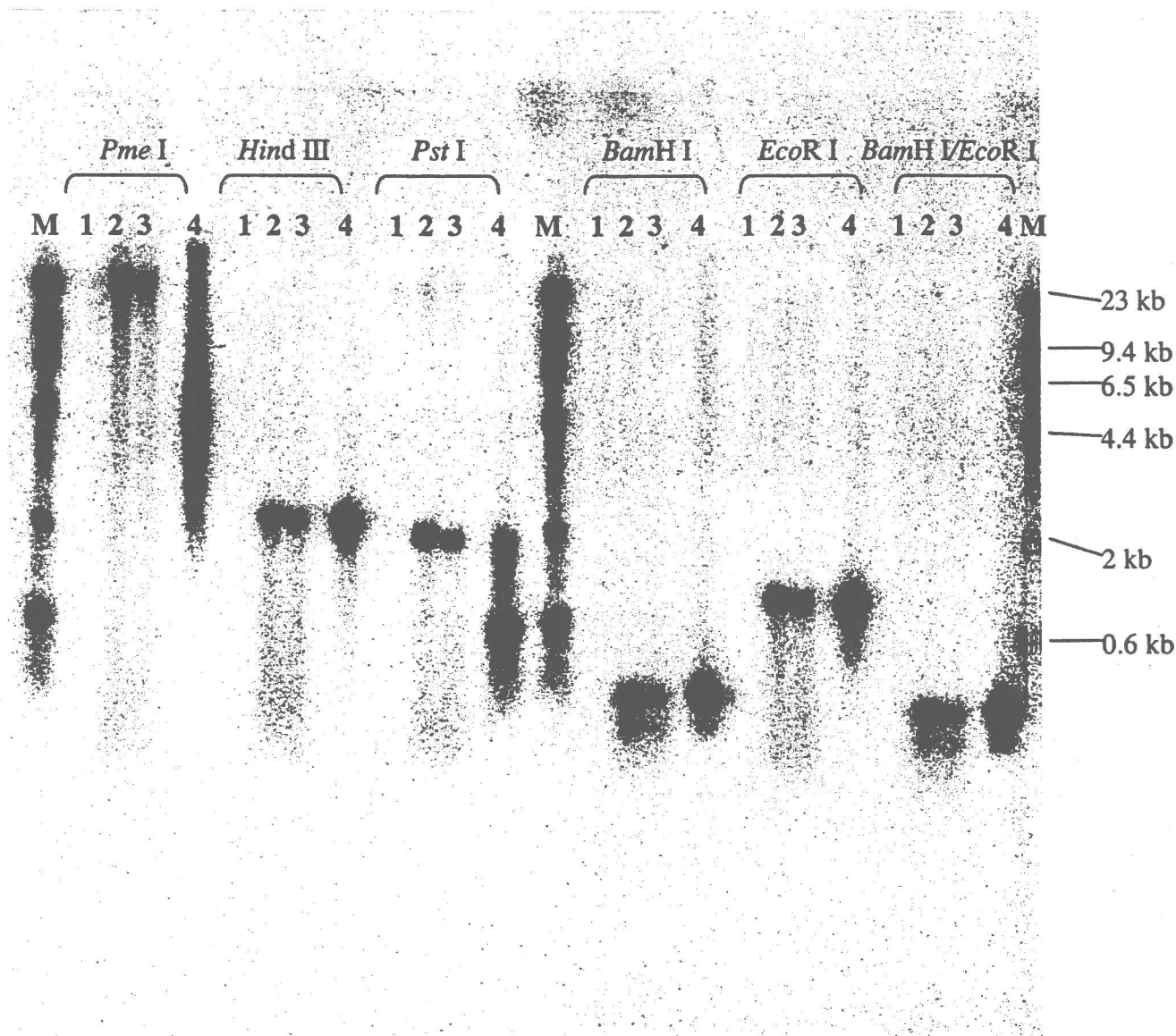


FIG. 4. Gel electrophoresis and Southern blot analysis of event 1507 – *pat* probe.

DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*H I, *Eco*R I, and *Bam*H I/*Eco*R I. The lanes contain:

- 1: GS3 non-transgenic control (5 µg)
- 2: pooled T1S1 DNA (5 µg)
- 3: pooled BC4 DNA (5 µg)
- 4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
- M: molecular weight marker (*Hind* III digest of lambda DNA) (1 µg). MW of lambda DNA fragments is given in kilobases (kb).

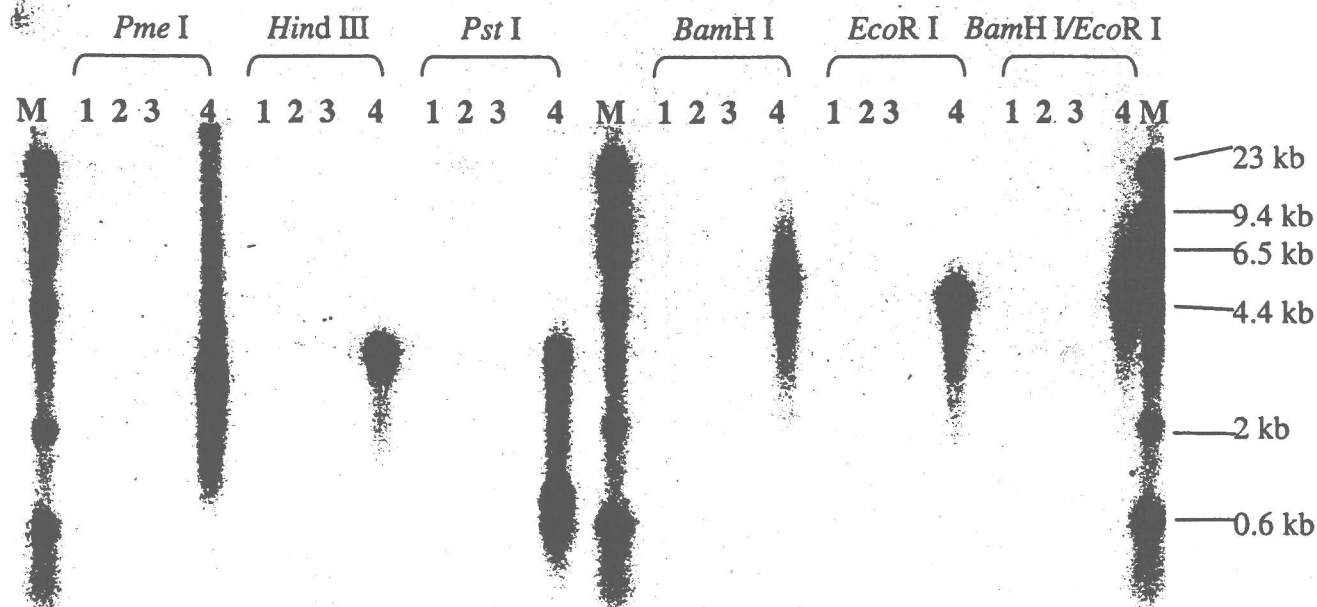


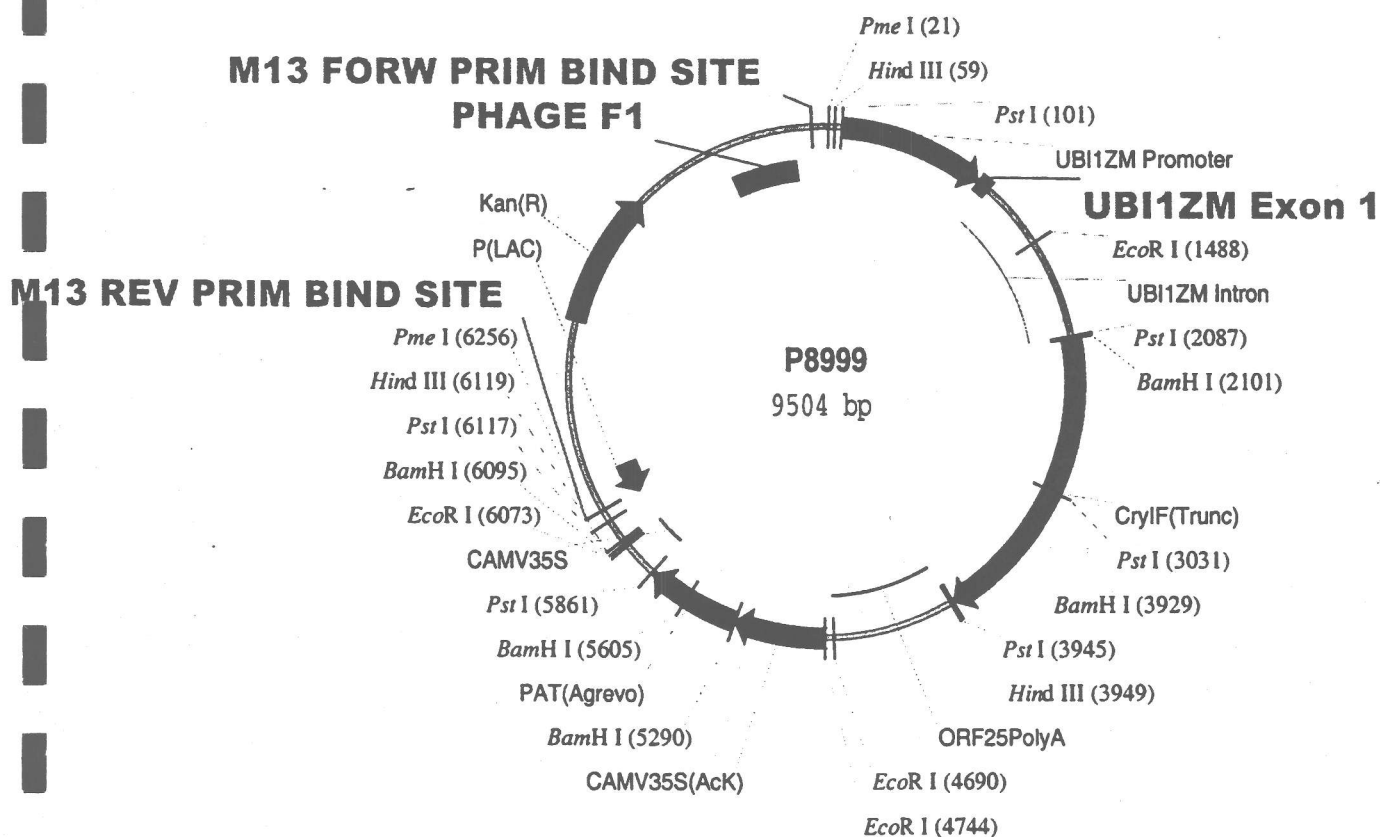
FIG. 5. Gel electrophoresis and Southern blot analysis of event 1507 – Kanamycin probe. DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*H I, *Eco*R I, and *Bam*H I/*Eco*R I. The lanes contain:

- 1: GS3 non-transgenic control (5 µg)
- 2: pooled T1S1 DNA (5 µg)
- 3: pooled BC4 DNA (5 µg)
- 4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
- M: molecular weight marker (*Hind* III digest of lambda DNA) (1 µg). MW of lambda DNA fragments is given in kilobases (kb).

APPENDIX

Appendix 1

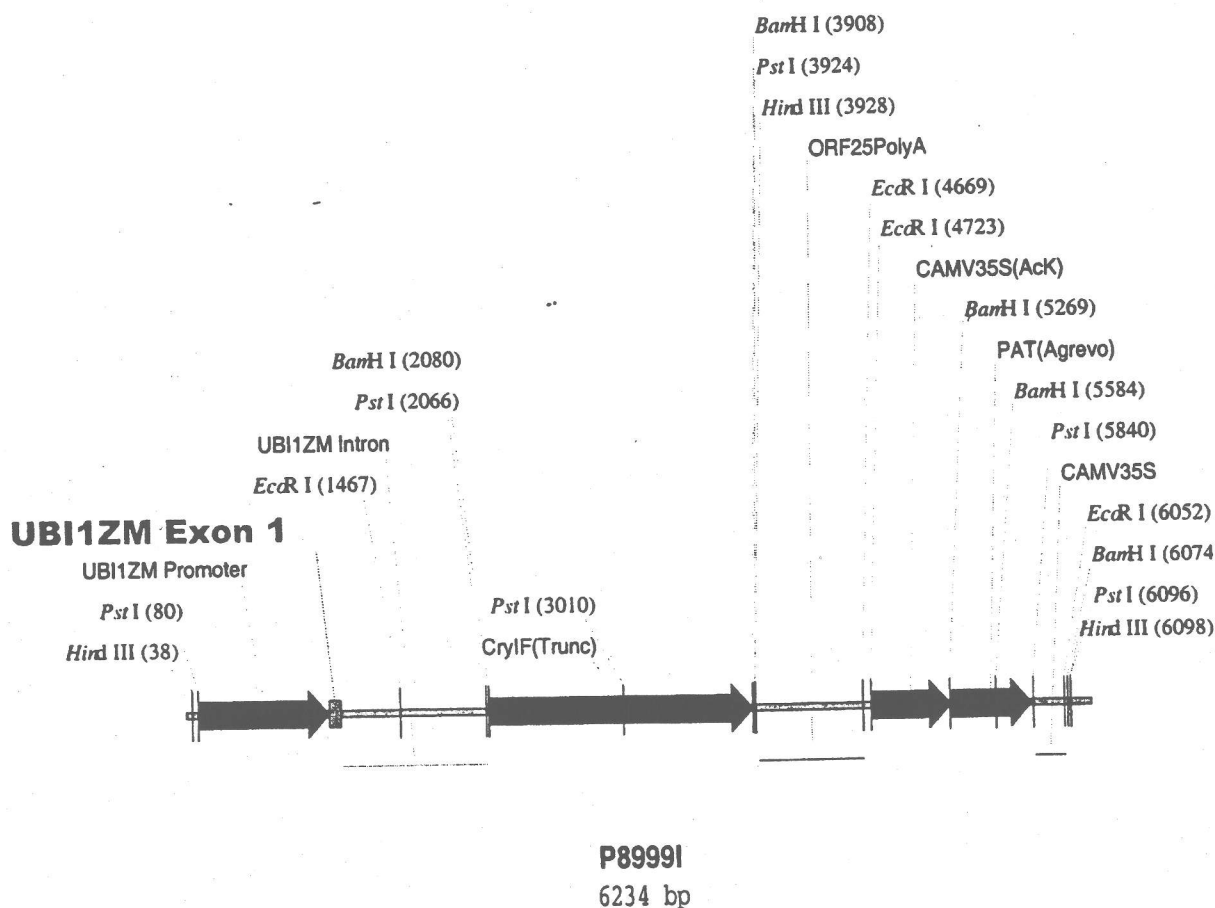
PLASMID PHP 8999



PP. 1. Physical map of the plasmid PHP 8999. The restriction enzyme sites *Pme* I, *Hind* III, *Pst* I, *Bam*H I, and *Eco*R I are identified on the map. The construction of the original clone and the procedures for its insertion into the genome are described elsewhere. Briefly, the 6.2 kb *Pme* I fragment, called PHI 8999, was isolated from the complete plasmid (PHP 8999) and was inserted into the maize genome using particle bombardment. The positions of the restriction enzymes are located in parenthesis.

Appendix 2

INSERT PHI 8999



APP. 2. Physical map of the insert PHI 8999. The restriction enzyme sites *Hind* III, *Pst* I, *Ban* H I, and *Eco* R I are identified on the map. The 6.2 kb insert resulted from the digestion of the complete plasmid (PHP 8999) with *Pme* I. This insert was isolated from PHP 8999 and inserted into the maize genome by particle bombardment. The positions of the restriction enzymes are located in parenthesis.