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ADDITIONAL INFORMATION FOR SECTION 2.3(d) OF THE APPLICATION TO AMEND THE FOOD STANDARDS CODE - FOODS PRODUCED USING GENE TECHNOLOGY

***B.t.* Cry1F-Insect-resistant, Glufosinate-tolerant Maize Line 1507**

ANZFA Application Number A446

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January 2002

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MOLECULAR CHARACTERIZATION OF *B.T.* CRY1F MAIZE LINE 1507

Introduction

Application Number A446 from Dow AgroSciences Australia Ltd. referred to ongoing molecular characterisation of maize line 1507.

This summary contains new and additional information on the sequences at the 5' and 3' ends of the full-length insert in *B.t.* Cry1F maize line 1507. The 5' sequence includes 2498 base pairs 5' to the inserted DNA in transformation event TC1507 and the majority of this 5' sequence has been reconfirmed. Additionally, DNA in *B.t.* Cry1F maize line 1507 corresponding to the full-length insert of PHI8999A has been sequenced in its entirety and compared to the original fragment used for transformation. Sequence on the 3' end of the full-length insert is also provided, including a complete characterization of the extent of the ORF25 terminator sequence and 3' border. This border region is currently being analyzed to confirm that it is of maize genomic origin. Southern and Northern data are also provided to complete the characterization of the insert in *B.t.* Cry1F maize line 1507.

Analysis of the sequence data and the Southern data supports the conclusion that only two *cry*1F hybridizing bands are present in the insert. One band is the complete *cry*1F coding sequence in the full-length insert. The second hybridizing band is a 335 bp fragment of *cry*1F present in the sequence 5' to the full-length insert in event TC1507. Southern analysis of the insert using two different *pat* gene probes, a 310 bp probe from the 3' end of the *pat* coding sequence and a 548 bp probe covering the complete *pat* coding sequence, revealed the presence of an additional *pat* gene fragment containing only the 5' portion of the *pat* gene. A restriction fragment map of the insert that corresponds with both the sequence data and the Southern data is included. The northern blot results clearly show that any partial copies of *cry*1F, *pat,* or the ORF25 terminator are not expressed as unique RNA transcripts in *B.t.* Cry1F maize line 1507.

Northern results also demonstrated that two open reading frames (ORFs) located in the sequence 5' to the full-length insert in event TC1507 and novel to *B.t.* Cry1F maize line 1507 do not produce any RNA transcripts. No open reading frames are present in the sequence 3' to the full-length insert.

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I. Border sequence 5' to inserted DNA in *B.t.* Cry1F maize line 1507

Identification of a clone containing border sequence 5' to the insert in *B.t.* Cry1F maize line 1507

To identify a DNA fragment that included sequence 5' to the insert in *B.t.* Cry1F maize line 1507, *Spe* I restriction enzyme fragments from *B.t.* Cry1F maize line 1507 genomic DNA were size selected on agarose gels, purified, and screened by Southern analysis to confirm hybridization to the *cry*1F probe. Following confirmation of hybridization and fragment size, the fragments of interest were cloned into a pBluescript II SK (+) cloning vector to prepare an enriched size selected plasmid based genomic DNA library. A probe homologous to a portion of the *cry*1F gene was used to screen the plasmid library for positive clones. A positive clone was identified, purified by additional screening, and confirmed to result in a positive signal when hybridized to the *cry*1F probe. Nearly 3 kb of the *Spe* I fragment contained in the isolated positive clone were sequenced using a primer walking approach. To initiate the first sequencing run, a primer that binds to a known sequence in the cloning vector DNA was designed to sequence a portion of the DNA of interest. A second sequencing run over the same region using another primer oriented in the reverse direction provided second strand coverage. Primer walking was accomplished by repeatedly using sequence data from previous runs to design new primers that were then used to extend the next round of sequencing further into the DNA of interest until the sequence provided in this supplement was obtained.

Description of border sequence 5' to the *B.t.* Cry1F maize line 1507 insert

In order to more fully describe the *B.t.* Cry1F maize line 1507 5' border sequence, homology searching was done against the GenBank public databases (release 122, 2/01) using the Basic Local Alignment Search Tool (BLAST). The BLAST program performs sequence similarity searching and is particularly useful for identifying homologs to an unknown sequence. In addition to searching the public databases, pairwise alignments were performed using AlignX (InforMax Inc.) to look for homology between the maize line 1507 border sequence and the PHI8999A insert. The results of the homology searches are presented in Table 1. The *B.t.* Cry1F maize line 1507 5' border sequence is numbered with base 1 being the furthest 5' to the insert and base 2499 at the starting point of the full-length PHI8999A insert (Figure 1). The percent identity values indicate the percentage of identical matches across the length of the sequences analyzed.

In most cases, similarity searching with the *B.t.* Cry1F maize line 1507 5' border sequence resulted in a match to one unique sequence based on a very high percent identity value. Those sequences are identified in Table 1. In addition, there are two regions in the *B.t.* Cry1F maize line 1507 5' DNA border sequence with high similarity to more than one known sequence. In regions 870-1681 and 2338-2354, the percent identity scores with both sequence fragments are sufficiently high that a single match (homolog) cannot be determined. The two possible homologs for each of these regions are indicated in Table 1.

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Highly similar sequences were identified for all but the first 669 base pairs of sequence. Generally, the results of similarity searching indicate high homology with maize genomic sequences 5' to base 1681. The region from base 1682 to the start of the PHI8999A insert at position 2499 appears to contain some fragments associated with the transformation event.

Analysis of potential ORFs in the border sequence 5' to the *B.t.* Cry1F maize line 1507 insert

In addition to homology searching, the *B.t.* Cry1F maize line 1507 5' border sequence was analyzed for potential open reading frames (ORFs) in the border region. In the maize genome, smaller ORFs in the alternate frames of a known gene sequence are common. Typically, ORFs that could potentially code for proteins of less than 150 amino acids are quite numerous in maize. However, it is less common to find ORFs in maize that could potentially result in expression of proteins of greater than 200 amino acid residues. Therefore, in searching for potential ORFs in the border sequence 5' to the *B.t.* Cry1F maize line 1507 insert, no significant concern would be associated with ORFs of less than 100 amino acids (300 bp) since these are commonly found in maize. Three potential ORFs longer than 300 bp (100 amino acid residues) were identified (Figure 2). Two of the ORFs are in the undescribed region upstream of the start of the maize genomic sequence. ORF1 spans bases 362-691 (330 bp total), while ORF2 spans bases 433-780 (348 bp total) in an alternate reading frame. Although similarity searching against public databases failed to identify homologs for this region, the presence of these two ORFs 5' to regions of maize homology is support for their existence in the native maize genome.

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PCR analysis was used to determine if the undescribed region of sequence is present in the unmodified corn line GS3 used for transformation to produce *B.t.* Cry1F maize line 1507. Presence of the ORF1 and ORF2 in unmodified GS3 lines demonstrates that these ORFs are not novel to *B.t.* Cry1F maize line 1507. Five different PCR analyses were carried out on genomic DNA prepared from *B.t.* Cry1F maize line 1507 and the unmodified control corn line GS3 as outlined in Table 2 with results shown in Figure 3. Two reactions were designed to amplify DNA within Region 1 of the 5'border from bp 25 to 324 (Reaction A - 300 bp amplicon) and from bp 25 to 480 (Reaction B - 456 bp amplicon). The expected amplicons were present in both the GS3 unmodified corn line and in *B.t.* Cry1F maize line 1507 as shown in lanes 2-7 of Figure 3. One PCR primer pair, Reaction C, spanned Region 2 to Region 3 of the 5' border from bp 759 to 1182 (424 bp amplicon) and again produced PCR products of the expected size in both GS3 and *B.t.* Cry1F maize line 1507 (lanes 8 – 10 of Figure 3). Reactions D and E were designed as specific primer pairs for the *pat* gene region of the full-length insert of PHI8999A in *B.t.* Cry1F maize line 1507 and should not produce an amplicon in the unmodified GS3 corn line. The results, shown in lanes 12 – 17 of Figure 3, indicate that both Reactions D and E are specific for *B.t.* Cry1F maize line 1507 and produce the expected amplicon.

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The PCR results show that the undescribed sequence (Region 1) in which ORF1 and ORF2 initiate is present in the unmodified corn line GS3 and that Regions 2 and 3, including a portion of ORF2, are contiguous in the unmodified corn line GS3. The DNA sequences amplified in Reactions A, B, and C are not unique to the 5' border of *B.t.* Cry1F maize line 1507 but are also present in the unmodified corn line GS3.

[redacted] ORF3 spans bases 1896-2576 (680 bp total), extending from near the end of the 5' *cry1F* fragment to the start of the *ubi*ZM1(2) promoter in the PHI8999A full-length insert (Figure 2). This ORF is an artifact of the insertion of PHI8999A into the genome, it is a randomly generated sequence without many of the critical gene expression elements known to be associated with expression of stable proteins. For example, searches conducted in the sequence upstream of ORF3 identified no consensus promoter elements, such as TATA-like sequences. In addition, the G/C content of ORF3 is 46% compared to an average of 56% for maize genes (Codon Usage Database, http://www.kazusa.or.jp/codon/). Low G/C content in maize is an indication of poor codon usage relative to native maize coding sequences, and poor codon usage is known to adversely affect the translation of proteins. While these factors provide a basis to question the potential for expression of ORF3, northern analysis results shown later in this supplement clearly show that ORF3 sequence is not present as an RNA transcript in *B.t.* Cry1F maize line 1507.

The putative amino acid sequence of ORF3 was analyzed for homology with known allergenic proteins. No significant homology was found based on the criteria for a minimal domain size of identity across 8 contiguous amino acids.

Confirmation of border sequence 5' to the *B.t.* Cry1F maize line 1507 insert

To confirm the 5' border sequence of the *B.t.* Cry1F maize line 1507 insert, PCR primer pairs were designed to obtain overlapping PCR products. PCR products were successfully amplified, isolated, and sequenced for the regions from bp 1 to bp 2311 (Figure 1, Table 1) and confirmed to match the previously determined sequence for this region. However, the region from bp 2312 to bp 2498, immediately adjacent and 5' to the start of the full-length insert was recalcitrant to PCR amplification and thus the sequence of this region has not been confirmed. The tentative sequence in this region indicates 19 bp of *pat* gene sequence, however Southern data shown later in this supplement (see Figures 8 and 9) indicates that the *pat* fragment is potentially larger than 19 bp as evidenced by a hybridizing fragment detected on Southern blots with a full-length *pat* gene probe.

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Confirmation of the presence of ORF1 and ORF2 in untransformed maize genomic sequence

PCR analysis was repeated and used to re-confirm that the undescribed region in the 5' border sequence is present in the unmodified corn line GS3 used for transformation to produce *B.t.* Cry1F maize line 1507. This analysis confirmed that the presence of the ORF1 and ORF2 in this region is not novel to *B.t.* Cry1F maize line 1507. Five different PCR analyses were carried out on genomic DNA prepared from *B.t.* Cry1F maize line 1507 and the unmodified control corn line GS3 as outlined in Table 3 with results shown in Figure 4. Three reactions were designed to amplify DNA within Regions 1 through 3 (for details on regions see Table 1). The first reaction was entirely within Region 1 of the 5' border from bp 25 to 324 (Reaction A - 300 bp amplicon), the second reaction spanned Region 1 to Region 3 from bp 415 to 1182 (Reaction B - 768 bp amplicon) and the third spanned Region 2 to Region 3 from bp 759 to 1182 (Reaction C - 424 bp amplicon). The expected amplicons were present in both the GS3 unmodified corn line and in *B.t.* Cry1F maize line 1507 as shown in Figure 4. Reactions D and E were designed as specific primer pairs for the *B.t.* Cry1F maize line 1507 insert and should not produce an amplicon in the unmodified GS3 corn line. The results, shown in lanes 12 – 17 of Figure 4, indicate that both Reactions D and E are specific for *B.t.* Cry1F maize line 1507 and produce an amplicon of the expected size.

The PCR results show that the undescribed sequence (Region 1 and Region 2), which contains ORF1 and ORF2, is present in the unmodified corn line GS3. These results also show that Regions 2 and 3, including a portion of ORF2, are contiguous in the unmodified corn line GS3. The DNA sequences amplified in Reactions A, B, and C are not unique to the 5' border of *B.t.* Cry1F maize line 1507 but are also present in the unmodified corn line GS3.

II. Confirmation of the full-length insert sequence of PHI8999A in *B.t.* Cry1F maize line 1507

In order to sequence the full-length insert, primer pairs were designed based on the sequence of PHI8999A to produce overlapping PCR products to cover the entire full-length insert region. Amplification reactions were performed with multiple primer pairs on genomic DNA prepared from two to three individual plants of *B.t.* Cry1F maize line 1507 and on DNA prepared from an unmodified control corn line (GS3 or GP24). Prior to PCR amplification the DNA was confirmed to contain the Southern blot pattern descriptive of *B.t.* Cry1F maize line 1507. Fragments unique to *B.t.* Cry1F maize line 1507 were gel isolated and sequenced directly or subcloned into pGEM®-T Easy followed by sequencing using standard vector primers. The sequence of the full-length insert in *B.t.* Cry1F maize line 1507 was determined by the PCR method. The sequence obtained from the cloned *Spe* I fragment described earlier in this supplement matched the corresponding sequence of the PHI8999A insert.

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Analysis of the full-length insert sequence of *B.t.* Cry1F maize line 1507 identified three open reading frames. Two of the ORFs are the expected full length *cry*1F and *pat* gene sequences. The third ORF, identified as ORF4, spans the 3' end of the ORF25 terminator sequence and the 5' end of the 35S promoter (Figure 2.) This potential ORF is 630 bp (210 amino acid residues) in length. Northern analysis results shown later in this supplement clearly show the presence of full length transcripts of *cry*1F and *pat* and no transcript of any length with ORF4 sequence probes.

The putative amino acid sequences of *cry*1F, *pat*, and ORF4 were analyzed for homology with known allergenic proteins. No significant homologies were found based on the criteria for a minimal domain size of identity across 8 contiguous amino acids.

III. Border sequence 3' to the full-length insert in *B.t.* Cry1F maize line 1507

Two separate PCR approaches were used to extend the length of the sequence information 3' to the full-length insert in *B.t.* Cry1F maize line 1507. In the first approach PCR primer pairs were designed to amplify a product that spanned the junction between the full-length insert and the inverted ORF25 terminator. A forward primer was located at the end of the full-length insert and a series of reverse primers were located at 100 bp intervals in the inverted sequence. In this manner the length of the inverted fragment present in the *B.t.* Cry1F maize line 1507 insert could be determined within a 100 bp region based on the successful PCR reactions. This method indicated the inverted fragment contained the majority of the ORF25 terminator but no *cry*1F sequence. PCR fragments were isolated and sequenced from this region.

In the second approach PCR primers were designed to walk out into the flanking DNA sequence from the inverted ORF25 terminator region as determined in PCR experiment described above. Genomic DNA isolated from two to three individual plants of *B.t.* Cry1F maize line 1507 and unmodified control corn line was digested with various restriction enzymes and then ligated to adaptors specific for the restriction enzyme used for digestion (Universal Genome Walker™ Kit, Clontech Laboratories, Inc.). Primary PCR was carried out using an ORF25 terminator specific primer and a primer homologous to the adaptor sequence ligated onto the digested DNA. In order to increase the specificity of the reaction a nested secondary PCR was performed again with another ORF25 terminator specific primer and a secondary primer homologous to the adaptor sequence with the secondary primers being internal to the respective primers used in the primary PCR. Products produced by the nested PCR were analyzed by agarose gel electrophoresis and fragments unique to the *B.t.* Cry1F maize line 1507 DNA samples were isolated and sequenced. Fragments were amplified from both the ORF25 terminator contained within the full-length insert and from the targeted (inverted) ORF25 terminator on the 3' end of the full-length insert. Fragments from the full-length insert were of a predicted size based on the knowledge of the restriction enzyme sites located in the full-length insert. Fragments produced from the 3' inverted ORF25 terminator appeared as fragments of unexpected size. Sequence analysis of amplified fragments from the 3' ORF25 terminator resulted in bordering DNA sequence of 1064 bp.

In order to describe the *B.t.* Cry1F maize line 1507 3' border sequence, homology searching was done against the GenBank public databases using the Basic Local Alignment Search Tool (BLAST). The BLAST program performs sequence similarity searching and is particularly useful for identifying homologs to an unknown sequence. In addition to searching the public databases, alignments were performed using SeqMan 4.05©, Martinez and Needleman-Wunsch alignment algorithms (DNASTAR Inc.) to look for homology between the *B.t.* Cry1F maize line 1507 3' border sequence and the PHI8999A insert. The results of the homology searches are presented in Table 4. The percent identity values indicate the percentage of identical matches across the length of the sequences analyzed. The results of similarity searching indicate high homology with three regions of maize chloroplast DNA, a 188 bp fragment of the *pat* gene, and 271 bp of DNA with no significant homology on the end of 3' border sequence. PCR analysis on control and *B.t.* Cry1F maize line 1507 genomic DNA is in progress to determine if the 271 bp sequence is present in the maize genome. The location of the *pat* gene fragment is consistent with Southern blot hybridization results. The *B.t.* Cry1F maize line 1507 3' border sequence is presented in Figure 5 and diagrammed in Figure 6.

No open reading frames longer than 300 bp (100 amino acid residues) are present in the region at the 3' end of the *B.t.* Cry1F maize line 1507 insert.

IV. Southern blot analysis of *B.t.* Cry1F maize line 1507

In order to compare the sequence and Southern data, a Southern blot loaded with *BamH* I/*EcoR* I digest of DNA isolated from *B.t.* Cry1F maize line 1507, was re-probed with two different *pat* gene probes. The 3' *pat* probe used in the original submission is a 310 bp probe from bp 5517 to bp 5826 of PHI8999A. The full-length *pat* probe is a 548 bp probe containing the majority of the *pat* coding region from bp 5265 to bp 5812 of PHI8999A (Figure 7). Table 5 contains a summary of the hybridizing bands for the *cry*1F probe (submitted previously as part of the original application), the 3' *pat* probe, and the full-length *pat* probe. Southern blots for the two *pat* gene probes are shown in Figure 8 and Figure 9. Hybridization with the full-length *pat* probe revealed the expected hybridizing fragments plus two additional hybridizing bands of lower intensity. The 3' *pat* probe produced only bands of the expected size and no additional bands. These results suggest that two additional fragments of the *pat* gene are present in the *B.t.* Cry1F maize line 1507 insert and these two fragments contain sequence homologous to the 5' end of the *pat* gene in the region where the two probes do not overlap (bp 5265 to bp 5516 of PHI8999A; Figure 7).

When *B.t.* Cry1F maize line 1507 genomic DNA is digested with *Hind* III or *BamH* I and probed with the *cry*1F probe only two hybridizing bands are seen on Southern blots suggesting that only two *cry*1F fragments are present in the insert. A restriction map of the insert that is compatible with both the sequence and the Southern data is presented in Figure 10. In contrast, the *EcoR* I digest consistently produces three hybridizing bands. The extra band in the *EcoR* I digest is believed to be due to partial digestion of the *EcoR* I site located in the highly repetitive and GC rich area in Region 3 (Huck-1

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retrotransposon) of the 5' border sequence (Figure 10). Thus, two hybridizing bands on the Southern blots are in actuality the result of the probe hybridizing to one *cry*1F fragment in the 5' border. One hybridizing band is from complete digestion giving the expected 2.8 kb *EcoR* I fragment containing the 335 bp *cry*1F fragment and the second band of approximately 20 kb is due to partial digestion. The partial digestion at the *EcoR* I site is also evident in the double digest with *BamH* I and *EcoR* I.

The restriction map in Figure 10 also accounts for the two additional *pat* hybridizing bands. One *pat* fragment is contained within the same *EcoR* I fragment on the 5' border as the 335 bp *cry*1F fragment giving both the complete and partial digest hybridizing bands of 2.8 kb and approximately 20 kb, respectively. While the unconfirmed sequence in Region 7 (Table 1) contains 19 bp of *pat* homologous DNA, the Southern analysis results presented here indicate that more *pat* sequence is present. Additional analysis is in progress to confirm the sequence in the region immediately adjacent to the 5' end of the full-length insert. The second *pat* fragment located in the 3' border sequence as discussed above and shown in Figures 2 and 6 is present on a band that only hybridizes to the *pat* probes and not to the *cry*1F probe. The restriction map in Figure 10 locates the second *pat* homologous fragment on the 3' border of the full-length insert 3' to the inverted ORF25 terminator sequence.

V. Northern blot analysis of open reading frames in *B.t.* Cry1F maize line 1507

In order to determine if there is any expression resulting from partial sequences of *cry*1F, *pat*, or the ORF25 terminator in *B.t.* Cry1F maize line 1507, RNA was analyzed by Northern blot for the presence of corresponding transcripts. Prior to initiation of the Northern analysis, Cry1F lateral flow strips (Strategic Diagnostics, Inc., Newark, DE) were used to confirm Cry1F protein expression in test plants and absence of the protein in non-transgenic control plants. Total RNA from leaf tissue of *B.t.* Cry1F maize line 1507 (4 plants) and non-transgenic maize line 4QK414 (5 plants) was extracted and blotted onto nitrocellulose membranes. Three blots containing the same set of samples were produced (Figure 11a, Figure 12a, Figure 13a). These membranes were hybridized with full-length probes identical to the 1817 bp *cry*1F coding sequence, 723 bp ORF25 terminator, and the 552 bp *pat* gene in pHI8999A, respectively (Table 6). The membranes were stripped and re-hybridized with a native maize alcohol dehydrogenase gene probe (GenBank Accession # X04050) as a positive Northern control (data not shown).

When hybridized with the full-length *cry*1F probe, no hybridization signal was visible in the non-transgenic maize line, but a strong hybridization band (~1800 bp) appeared in the *B.t.* Cry1F maize line 1507 (Figure 11b). A hybridization pattern identical to *cry*1F was observed when the full-length ORF25 terminator was used as a probe (Figure 12b). As expected, both the *cry*1F gene and ORF25 terminator probes hybridized to the same transcript since part of the ORF25 terminator sequence serves as a 3' untranslated region in the *cry*1F transcript. There were no visible hybridization signals in the non-transgenic maize line using the full-length *pat* probe, but a strong hybridization band (~600 bp) was observed in the *B.t.* Cry1F maize line 1507 (Figure 13b). No bands other than the

expected full-length transcripts were observed with any of the probes tested. These Northern blot results clearly show that any partial copies of *cry*1F, *pat* or the ORF25 terminator are not expressed as unique RNA transcripts in *B.t.* Cry1F maize line 1507.

In the border sequence region 5' to the insert of *B.t.* Cry1F maize line 1507, we have identified a potential open reading frame, ORF3, that extends from base 1896 to 2576 (681 bp, 227 amino acid residues) (Figure 2). Sequence homology results from the 5' border region indicate that this ORF includes 121 bp of the partial *cry*1F gene, a 320 bp partial maize chloroplast *rpo*C2 gene, and the adjoining sequence up to and including the first 72 bp of the *ubi*ZM1 promoter from the full insert of PHI8999A. In order to determine if this potential ORF is expressed, *B.t.* Cry1F maize line 1507 was analyzed by Northern blot for the presence of ORF3 transcript. Total RNA from leaf tissue of *B.t.* Cry1F maize line 1507 (9 plants) and non-transgenic maize line 4QK414 (5 plants) was extracted and blotted onto a nitrocellulose membrane (Figure 14a). The membrane was hybridized with a probe identical to the 320 bp fragment of maize chloroplast *rpo*C2 gene in ORF3 (Table 6, Figure 14b). The membrane was stripped and re-hybridized with a rice 18S ribosomal RNA gene probe (GenBank Accession # AF06218, 1754 bp) to verify that similar amounts of RNA from each sample were loaded (Figure 14c). No hybridization signal was visible in either the *B.t.* Cry1F maize line 1507 or the non-transgenic maize line with the *rpo*C2 probe, but a strong signal was seen in all samples with the 18S control probe. These Northern blot results clearly show that the potential ORF3 sequence is not present as an RNA transcript in *B.t.* Cry1F maize line 1507.

A potential open reading frame, ORF4, was identified in the full insert of *B.t.* Cry1F maize line 1507 partially spanning the ORF25 terminator and the 35S promoter sequences (Figure 2). This potential ORF of 630 bp (210 amino acid residues) is present in the PHI8999A sequence used in the transformation of *B.t.* Cry1F maize line 1507. To determine if a transcript is produced from ORF4, Northern blot analysis was performed using the sequence of ORF4 as a probe (Table 6). The same RNA sample set that was generated for Northern analysis of ORF3 was used for hybridization with ORF4 (Figure 15a). No hybridization signal was visible with the ORF4 probe (Figure 15b), but the rice 18S ribosomal RNA gene gave a strong signal (Figure 15c). Therefore, there is no evidence of expression of ORF4 in the *B.t.* Cry1F maize line 1507.

[redacted]

Table 1. Summary of sequence for *B.t.* Cry1F maize line 1507 insert
Regions 6, 7, 8, 14, and 15 are currently under sequence evaluation. All other listed regions have been confirmed (see Figure 2.)

Region	Location in 1507	Size bp	% Identity	Homolog	Location in homologous sequence	Description
1	1-669	669	N/A[1]	N/A	N/A	No significant homology detected
2	670-869	200	90.5	AF123535	52432-52632 (complement)	Undescribed maize genomic sequence
3	870-1681	812	89.4	AF050439	1-801	Fragment of maize Huck-1 retrotransposon 5' LTR[2]
			86.6	AF050438	1-797	Fragment of maize Huck-1 retrotransposon 3' LTR
4	1682-2016	335	100.0	PHI8999A	3149-3483	Fragment of *cry*1F gene
5	2017-2337	321	100.0	X86563	29429-29749	Fragment of maize chloroplast *rpoC2* gene (RNA polymerase beta-2 subunit)
6	2338-2354	17	100.0	X86563	97643-97659	Fragment of maize chloroplast *trnI* gene (tRNA-Ile)
			82.4	PHI8999A	182-197	Fragment of maize *ubi*ZM1(2) promoter
7	2358-2376	19	100.0	PHI8999A	5320-5338	Fragment of *pat* gene
8	2381-2498	118	100.0	PHI8999A	36-153	Fragment of polylinker region (bases 36-80) and *ubi*ZM1(2) promoter (bases 81-153)
9	2499 -8684	6186	100.0	PHI8999A	11 – 6196	Full-length insert of PHI8999A
10	8685-9234	550	100.0	PHI8999A	3906-4456 (complement)	Inverted ORF25 terminator
11	9235-9362	128	100.0	NC_001666	121851-121978 (complement) & 100759-100886	Fragment of maize chloroplast *rps12* rRNA (23S ribosomal RNA)
12	9365-9756	392	99	NC_001666	-17091-17483 (complement)	Fragment of maize chloroplast genome
13	9757-9944	188	99	PHI8999A	5333-5520 (complement)	Fragment of *pat* gene
14	9947-10027	81	100	NC_001666	137122-137202 (complement)	Fragment of maize chloroplast "ORF241" – hypothetical protein gene
15	10028-10298	271	N/A[1]	N/A	N/A	No significant homology detected

[1] N/A; not applicable
[2] LTR; long terminal repeat

Table 2. PCR primers for sequence 5' to the PHI8999A insert in *B.t.* Cry1F maize line 1507 and for the *pat* gene within the full-length insert of PHI8999A in *B.t.* Cry1F maize line 1507

Reaction	PCR Amplicon Location	Amplicon Size (bp)	Region in 1507 border sequence or insert PHI8999	Amplicon present in GS3	Amplicon present in maize line 1507
A	25 – 324 bp in 1507 border sequence	300	Region 1	Yes	Yes
B	25 – 480 bp in 1507 border sequence	456	Region 1	Yes	Yes
C	759 – 1182 bp in 1507 border sequence	424	Region 2 to Region 3	Yes	Yes
D	4750 – 5794 bp in PHI8999A	1045	Region 9 [In full-length insert of PHI8999A 35S pro to *pat* gene]	No	Yes
E	4827 - 5308 bp in PHI8999A	482	Region 9 [In full-length insert of PHI8999A 35S pro to *pat* gene]	No	Yes

[redacted]

Table 3. PCR primers for sequence 5' to the full-length insert in *B.t.* Cry1F maize line 1507 and for two regions unique to the insert in *B.t.* Cry1F maize line 1507

Reaction	PCR Amplicon Location	Amplicon Size (bp)	Region in 1507 border sequence or insert PHI8999	Amplicon present in GS3	Amplicon Present in maize line 1507
A	In 1507 5' border sequence	300	Region 1	Yes	Yes
B	In 1507 5' border sequence	768	Region 1 to Region3	Yes	Yes
C	In 1507 5' border sequence	424	Region 2 to Region 3	Yes	Yes
D	PHI8999A sequence in insert	194	Spans 35S term to inverted ORF25 terminator on 3' end	No	Yes
E	*cry*1F sequence in 5' border sequence	366	Spans 335 bp *cry*1F sequence in 5' border sequence	No	Yes

[redacted]

Table 4. Summary of the 3' end region of the *B.t.* Cry1F maize line 1507 insert

Location in the 3'end junction sequence	Size (bp)	% identity	Homolog	Location in homologous sequence	Description
8425-8538	114	100.0	PHI8999A	5937-6050	CaMV 35S terminator
8539-8684	146	100.0	PHI8999A	6051-6196	Polylinker region
8685-9234	550	100.0	PHI8999A	3906-4456 (complement)	Inverted ORF25 terminator
9235-9362	128	100.0	NC_001666	121851-121978 (complement) & 100759-100886	Fragment of maize chloroplast *rps12* rRNA (23S ribosomal RNA)
9365-9756	392	99	NC_001666	17091-17483 (complement)	Fragment of maize chloroplast genome
9757-9944	188	99	PHI8999A	5333-5520 (complement)	Fragment of *pat* gene
9947-10027	81	100	NC_001666	137122-137202 (complement)	Fragment of maize chloroplast "ORF241" – hypothetical protein gene
10028-10298	271	N/A[1]	N/A	N/A	No significant homology detected

Table 5. Summary of expected and observed hybridizing fragments for the *cry*1F and *pat* probes. Expected bands from digestion of the full-length insert are in bold numbers and bands resulting from partial digestion are underlined.

Restriction Enzyme	*cry*1F Probe (bp)	3' *pat* Probe (bp)	Full-length *pat* Probe (bp)
EcoR I	~23000 **3202** 2800	**1329**	~23000 9400 2800 **1329**
BamH I + *EcoR* I	7100 2800 **1828**	**468**	7100 3000 2800 **468** **315**
BamH I	7400 **1828**	**490**	7400 3000 **490** **315**
Hind III	4100 **3890**	**2170**	4100 2300 **2170**

[redacted]

Table 6. Summary of Northern blot analysis for transcripts of *cry*1F, *pat*, and ORF25, and novel open reading frames, ORF3 and ORF4, in the *B.t.* Cry1F maize line 1507

Target transcripts	Location in sequence	Minimum size of potential transcripts (bp)	Northern probe	Hybridization signal
*cry*1F	4578-6395	1818	1818 bp full-length *cry*1F coding region	~1800 bp positive signal of full length *cry*1F gene
ORF25 terminator	6433-7156	723	724 bp full-length ORF25 terminator	~1800 bp positive signal of full length *cry*1F gene
pat	7766-8317	552	552 bp full-length *pat* coding region	~600 bp positive signal of full length *pat* gene
ORF3	1896-2576	681	320 bp maize chloroplast *rpo*C2 gene fragment	negative
ORF4	7015-7644	630	630 bp ORF4 fragment	negative

[redacted]

Figure 2. Diagram of the *B.t.* Cry1F maize line 1507 insert

The diagram on the *B.t.* Cry1F maize line 1507 insert is divided into three separate major sections as referred to in the text; the 5' region that includes the border with corn genomic DNA and other DNA segments as described in Table 1, the full-length insert of PHI8999A fragment in the center of the diagram, and the 3' region that includes the inverted ORF25 terminator and additional sequence as described in the Table 4. Arrows beneath the diagram of the insert indicate the open reading frames. At the bottom of the diagram the sections of the *B.t.* Cry1F maize line 1507 insert that have been sequenced are indicated as well as the region identified for re-confirmation.

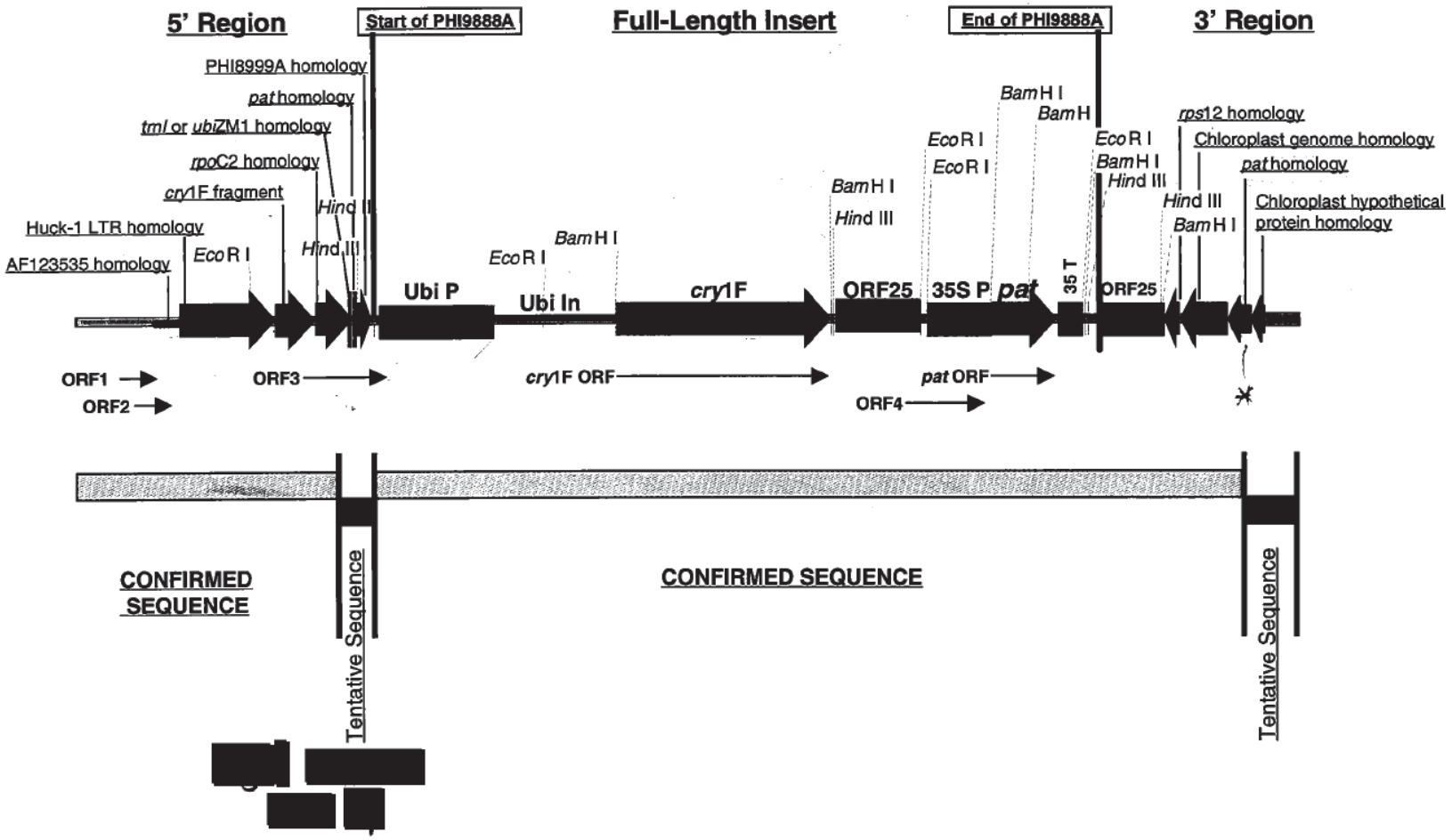
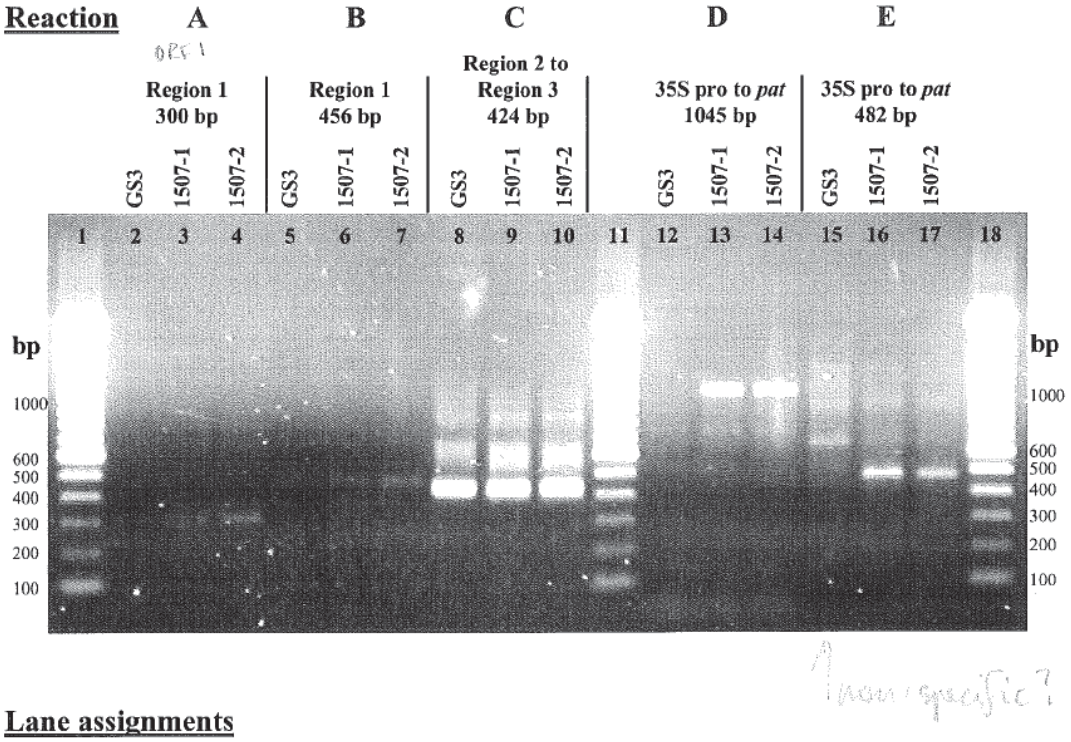


Figure 3. Results of PCR on genomic DNA for sequence 5' to the PHI8999A insert in *B.t.* Cry1F maize line 1507 and for the *pat* gene within the full-length insert of PHI8999A in *B.t.* Cry1F maize line 1507

GS3 was used as the unmodified control. The expected amplicon size is indicated above the lanes and the regions refer to those listed in Table 1. Genomic DNA samples prepared from two different plants of *B.t.* Cry1F maize line 1507 are designated as 1507-1 and 1507-2.



Lane assignments

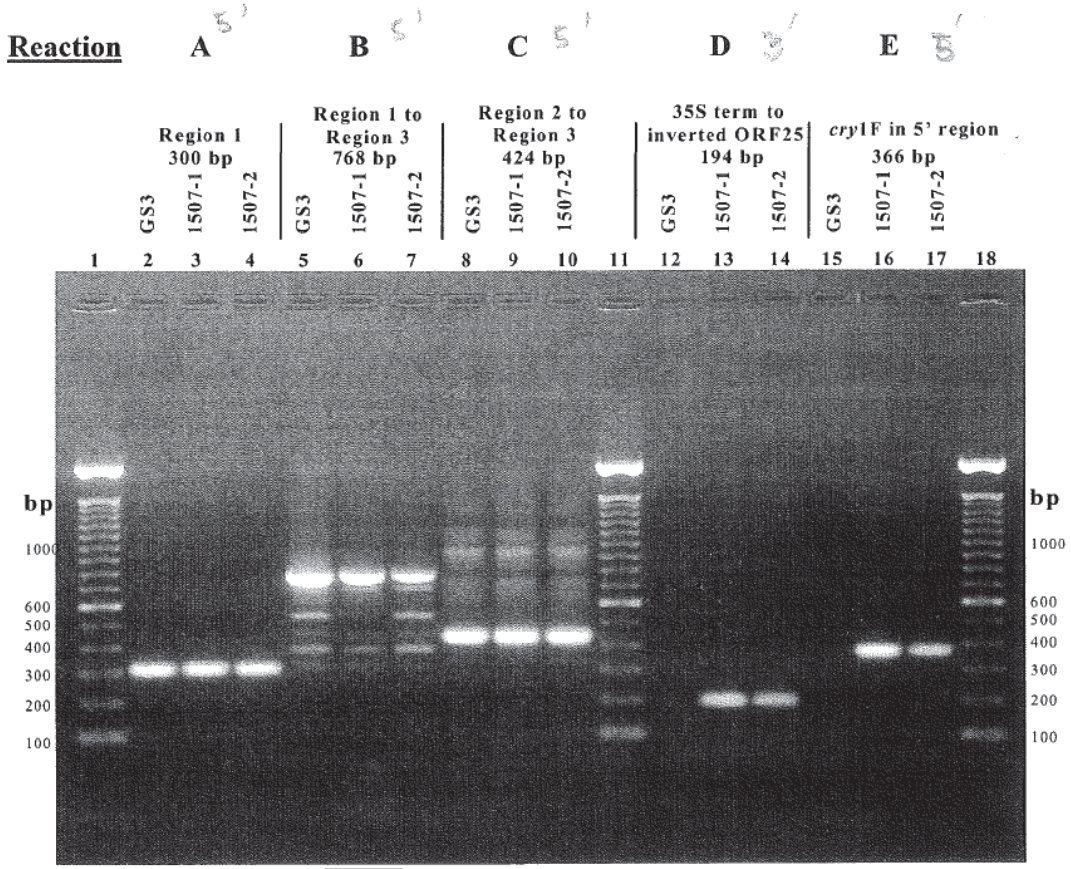
Lane	DNA Sample	Primer Region
1	100 bp ladder	
2	GS3	Region 1
3	1507-1	Region 1
4	1507-2	Region 1
5	GS3	Region 1
6	1507-1	Region 1
7	1507-2	Region 1
8	GS3	Region 2-3
9	1507-1	Region 2-3

Lane	Sample	Primer Region
10	1507-2	Region 2-3
11	100 bp ladder	
12	GS3	35S to *pat*
13	1507-1	35S to *pat*
14	1507-2	35S to *pat*
15	GS3	35S to *pat*
16	1507-1	35S to *pat*
17	1507-2	35S to *pat*
18	100 bp ladder	

[redacted]

Figure 4. Results of PCR on genomic DNA for sequence 5' to the full-length insert in *B.t.* Cry1F maize line 1507 and for two regions unique to the *B.t.* Cry1F maize line 1507 insert

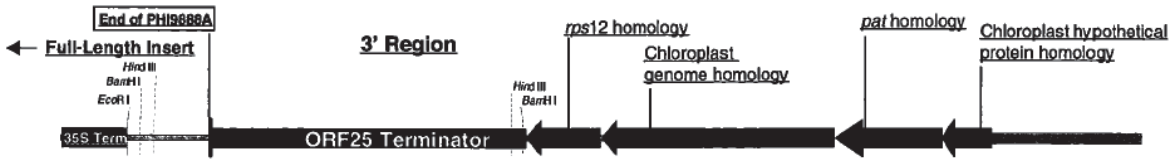
GS3 was used as the unmodified control. The expected amplicon size is indicated above the lanes and the regions refer to those listed in Table 1. Genomic DNA samples prepared from two different plants of *B.t.* Cry1F maize line 1507 are designated as 1507-1 and 1507-2.



[redacted]

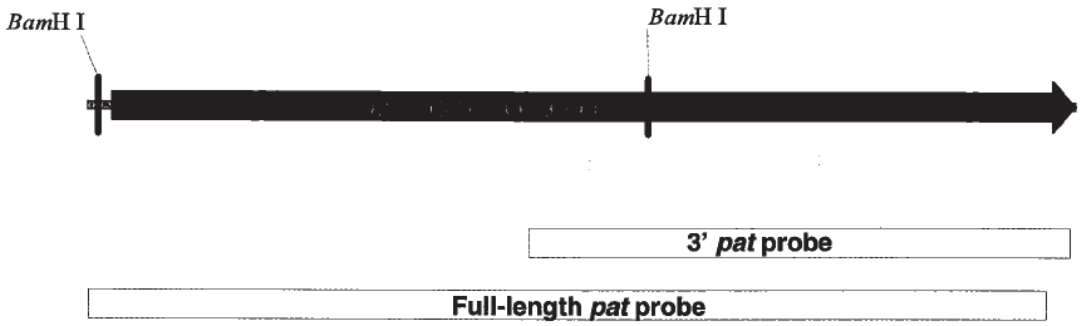
[redacted]

Figure 6. Diagram of the region 3' to the end of the full-length insert in *B.t.* Cry1F maize line 1507. This region is also depicted in Figure 2.



[redacted]

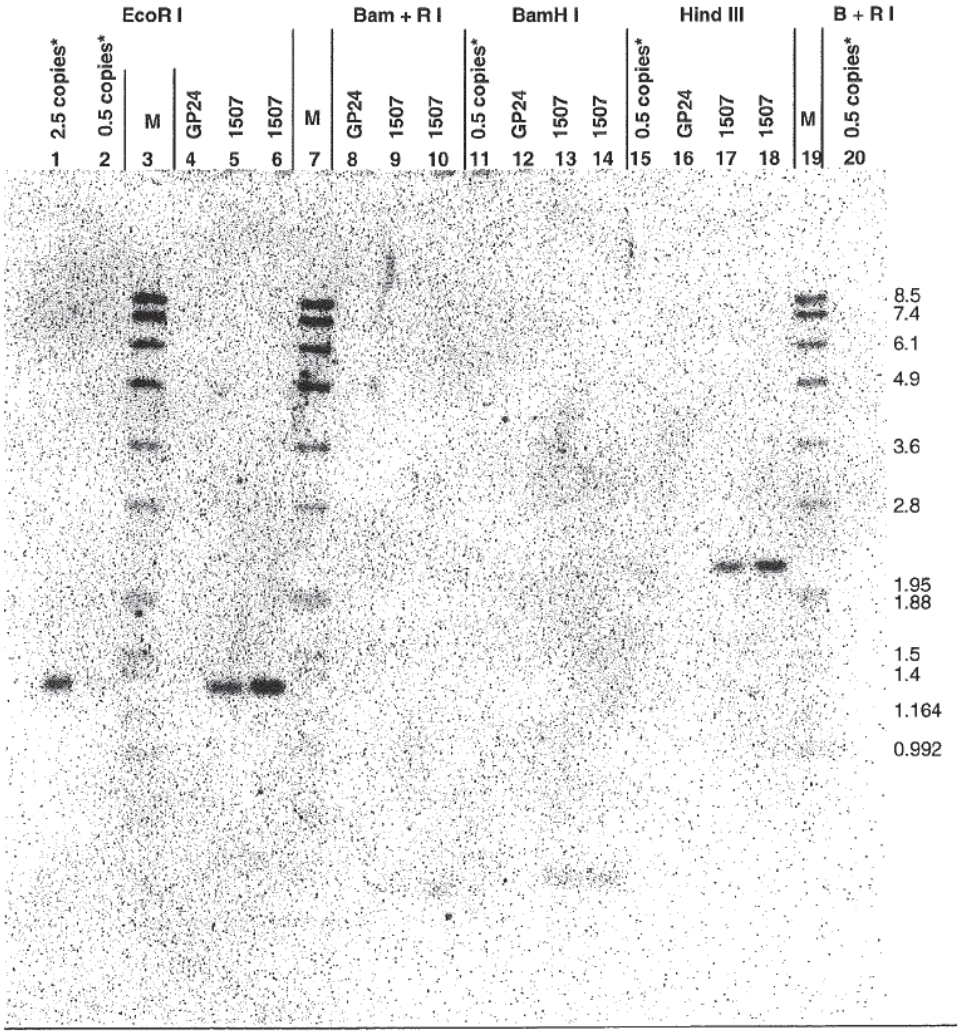
Figure 7. Diagram indicating the location of the two *pat* probes relative to the *pat* coding sequence.



[redacted]

Figure 8. Southern blot analysis of the *B.t* Cry1F maize line 1507 insert with the 3' *pat* probe

DNA isolated from *B.t.* Cry1F maize line 1507 (DNA isolated from two individual plants) and GP24 unmodified control corn was digested with the indicated enzymes and probed with the 3' *pat* probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP8999 at the indicated approximate gene copy number equivalents and 7 µg of GP24 genomic DNA.



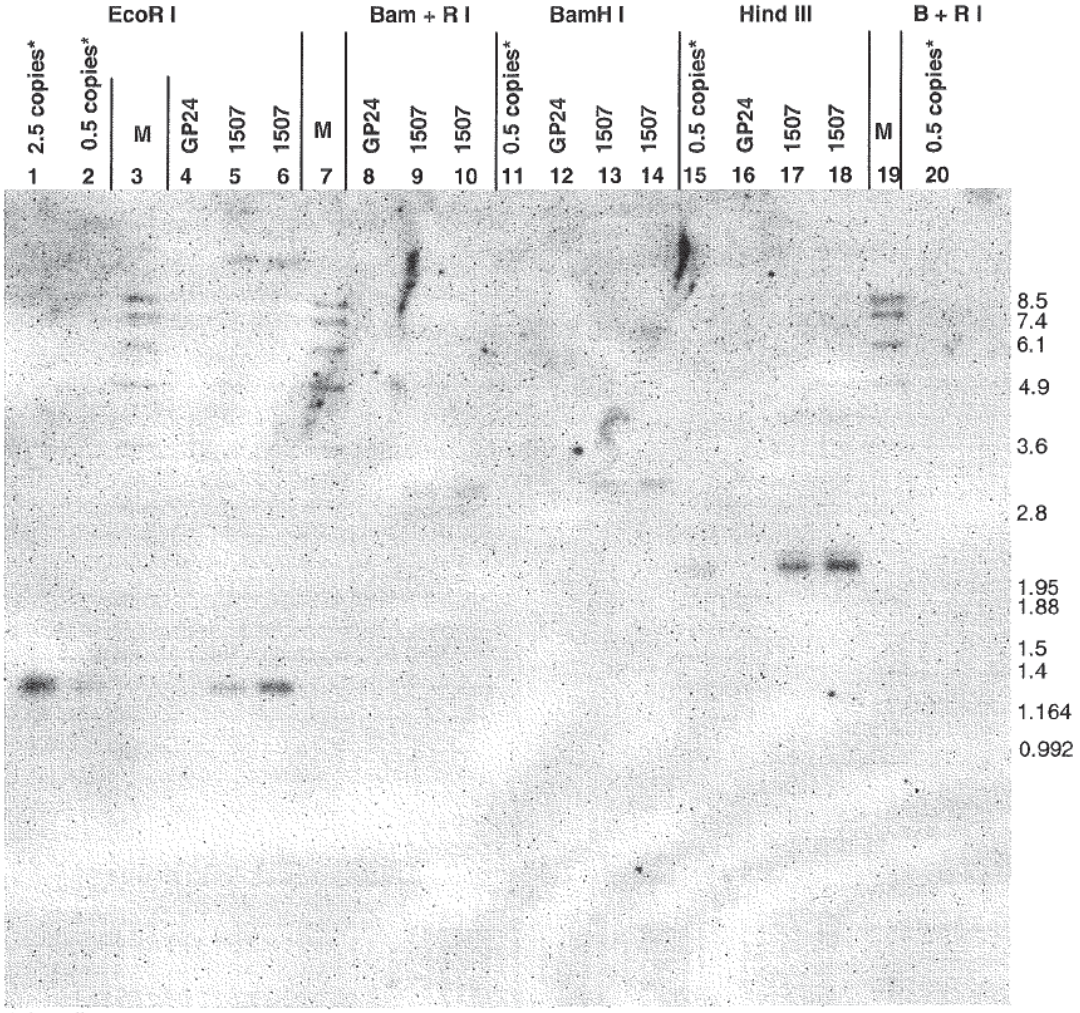
Gel Loading:

Lane 1 - GP24 (5 µg) + 2.5 copies PHP8999 *EcoR* I
Lane 2 - GP24 (5 µg) + 0.5 copies PHP8999 *EcoR* I
Lane 3 – Dig VII Marker (Roche)
Lane 4 – GP24 *EcoR* I
Lane 5 – TC1507 *EcoR* I
Lane 6 – TC1507 *EcoR* I
Lane 7 – Dig VII Marker (Roche)
Lane 8 – GP24 *BamH* I/*EcoR* I
Lane 9 - TC1507 *BamH* I/*EcoR* I
Lane 10 – TC1507 *BamH* I/*EcoR* I
Lane 11 - GP24 + 0.5 copies PHP8999 *BamH* I
Lane 12 - GP24 *BamH* I
Lane 13 - TC1507 *BamH* I
Lane 14 - TC1507 *BamH* I
Lane 15 - GP24 + 0.5 copies PHP8999 *Hind* III
Lane 16 - GP24 *Hind* III
Lane 17 - TC1507 *Hind* III
Lane 18 - TC1507 *Hind* III
Lane 19 - Dig VII Marker (Roche)
Lane 20 - GP24 + 0.5 copies PHP8999 *BamH* I/*EcoR* I

[redacted]

Figure 9. Southern blot analysis of *B.t* Cry1F maize line 1507 insert with the full-length *pat* probe

DNA isolated from *B.t.* Cry1F maize line 1507 (DNA isolated from two individual plants) and GP24 unmodified control corn was digested with the indicated enzymes and probed with the full-length *pat* probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP8999 at the indicated approximate gene copy number equivalents and 7 µg of GP24 genomic DNA.



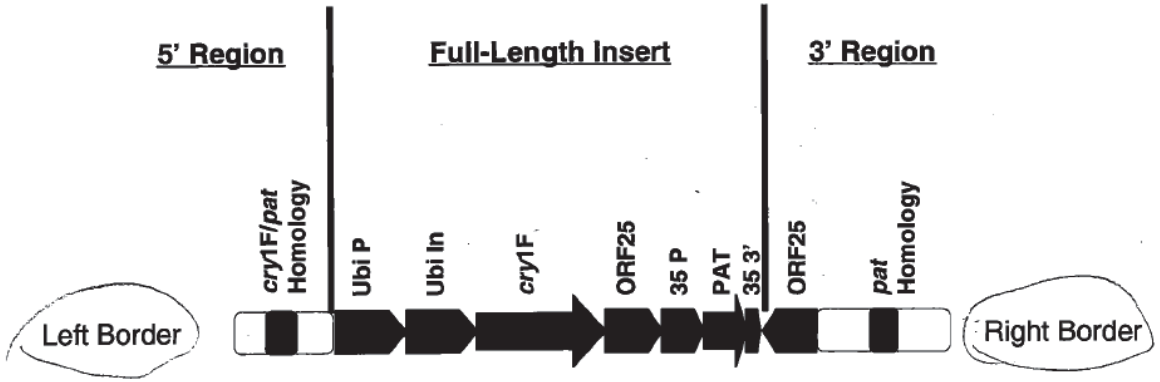
Gel Loading:

Lane 1 - GP24 (5 µg) + 2.5 copies PHP8999 *EcoR* I
Lane 2 - GP24 (5 µg) + 0.5 copies PHP8999 *EcoR* I
Lane 3 – Dig VII Marker (Roche)
Lane 4 – GP24 *EcoR* I
Lane 5 – TC1507 *EcoR* I
Lane 6 – TC1507 *EcoR* I
Lane 7 – Dig VII Marker (Roche)
Lane 8 – GP24 *BamH* I/*EcoR* I
Lane 9 - TC1507 *BamH* I/*EcoR* I
Lane 10 – TC1507 *BamH* I/*EcoR* I
Lane 11 - GP24 + 0.5 copies PHP8999 *BamH* I
Lane 12 - GP24 *BamH* I
Lane 13 - TC1507 *BamH* I
Lane 14 - TC1507 *BamH* I
Lane 15 - GP24 + 0.5 copies PHP8999 *Hind* III
Lane 16 - GP24 *Hind* III
Lane 17 - TC1507 *Hind* III
Lane 18 - TC1507 *Hind* III
Lane 19 - Dig VII Marker (Roche)
Lane 20 - GP24 + 0.5 copies PHP8999 *BamH* I/*EcoR*

[redacted]

Figure 10. Restriction fragment map of the *B.t* Cry1F maize line 1507 insert

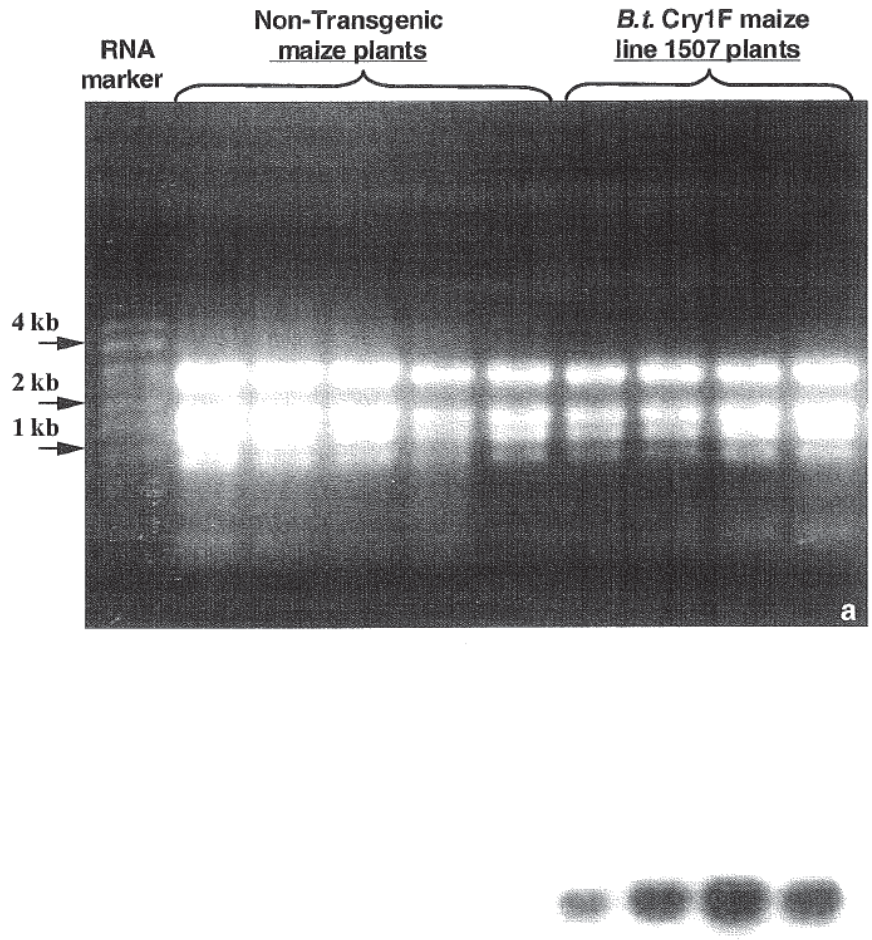
The dotted vertical line indicates the *EcoR* I site located within the GC rich repetitive sequence that is only partially digested by the *EcoR* I restriction enzyme. The horizontal dotted lines delineate two fragments that appear as separate hybridizing bands on Southern blots but are the result of hybridization to the same region due to partial digestion. The sequence and Southern data suggests that the *B.t.* Cry1F maize line 1507 insert contains one full-length copy of PHI8999A, one partial copy of cry1F in the 5' region, and two partial copies of pat; one in the 5' region and one in the 3' region.



[redacted]

Figure 11. Northern blot analysis with a full-length *cry*1F coding sequence as a probe in the *B.t.* Cry1F maize line 1507

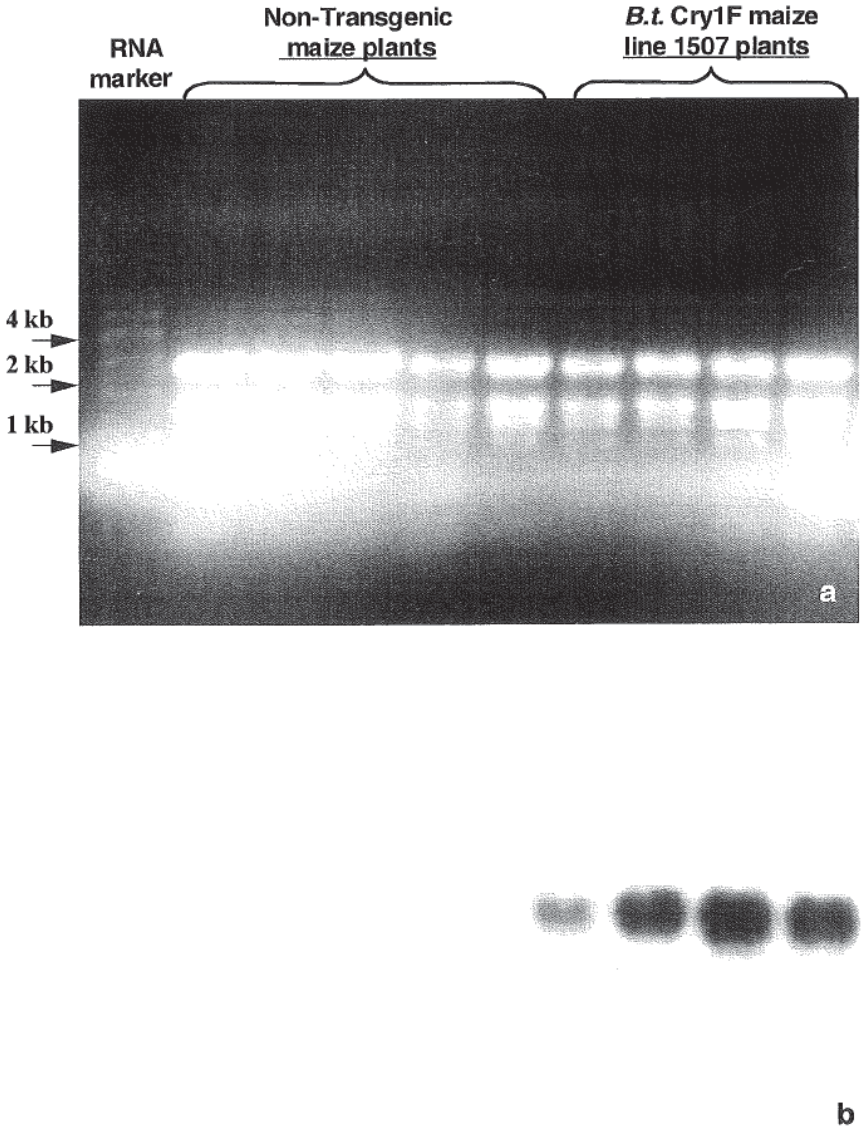
a. Total RNA samples on a 1% formaldehyde-agarose gel.
b. Hybridization with the *cry*1F probe



[redacted]

Figure 12. Northern blot analysis with a full-length ORF25 terminator sequence as a probe in the *B.t.* Cry1F maize line 1507

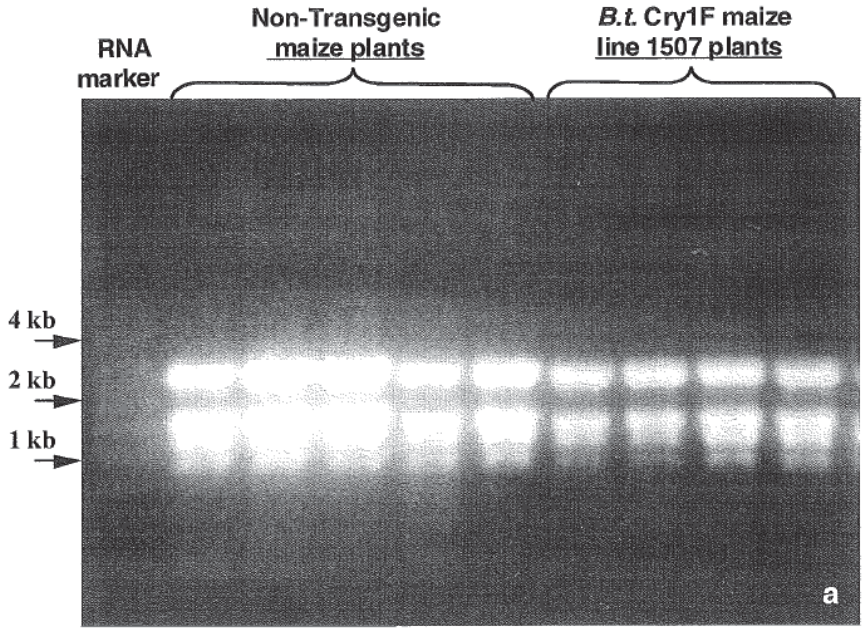
a. Total RNA samples on a 1% formaldehyde-agarose gel.
b. Hybridization with the ORF25 probe

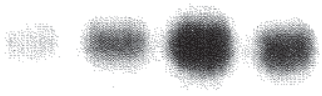


[redacted]

Figure 13. Northern blot analysis with a full-length *pat* coding sequence as a probe in the *B.t.* Cry1F maize line 1507

a. Total RNA samples on a 1% formaldehyde-agarose gel.
b. Hybridization with the *pat* probe





b

[redacted]

Figure 14. Northern blot analysis of ORF3 in the Full Insert of *B.t.* Cry1F Maize line 1507

a. Total RNA samples on a 1% formaldehyde-agarose gel
b. Hybridization with the 320 bp fragment of maize chloroplast *rpo*C2 gene in ORF3
c. Hybridization with a rice 18S rRNA gene as a probe

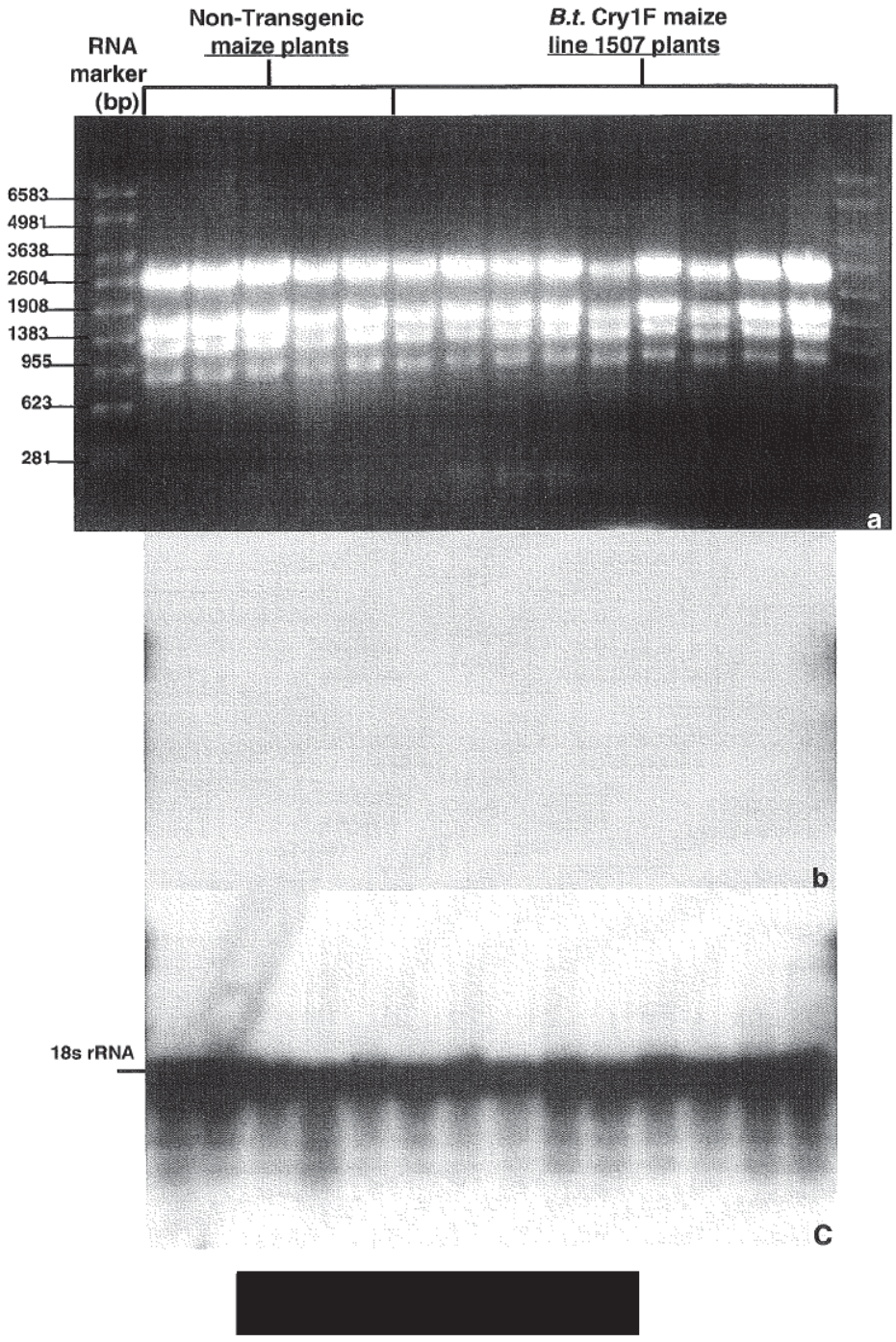
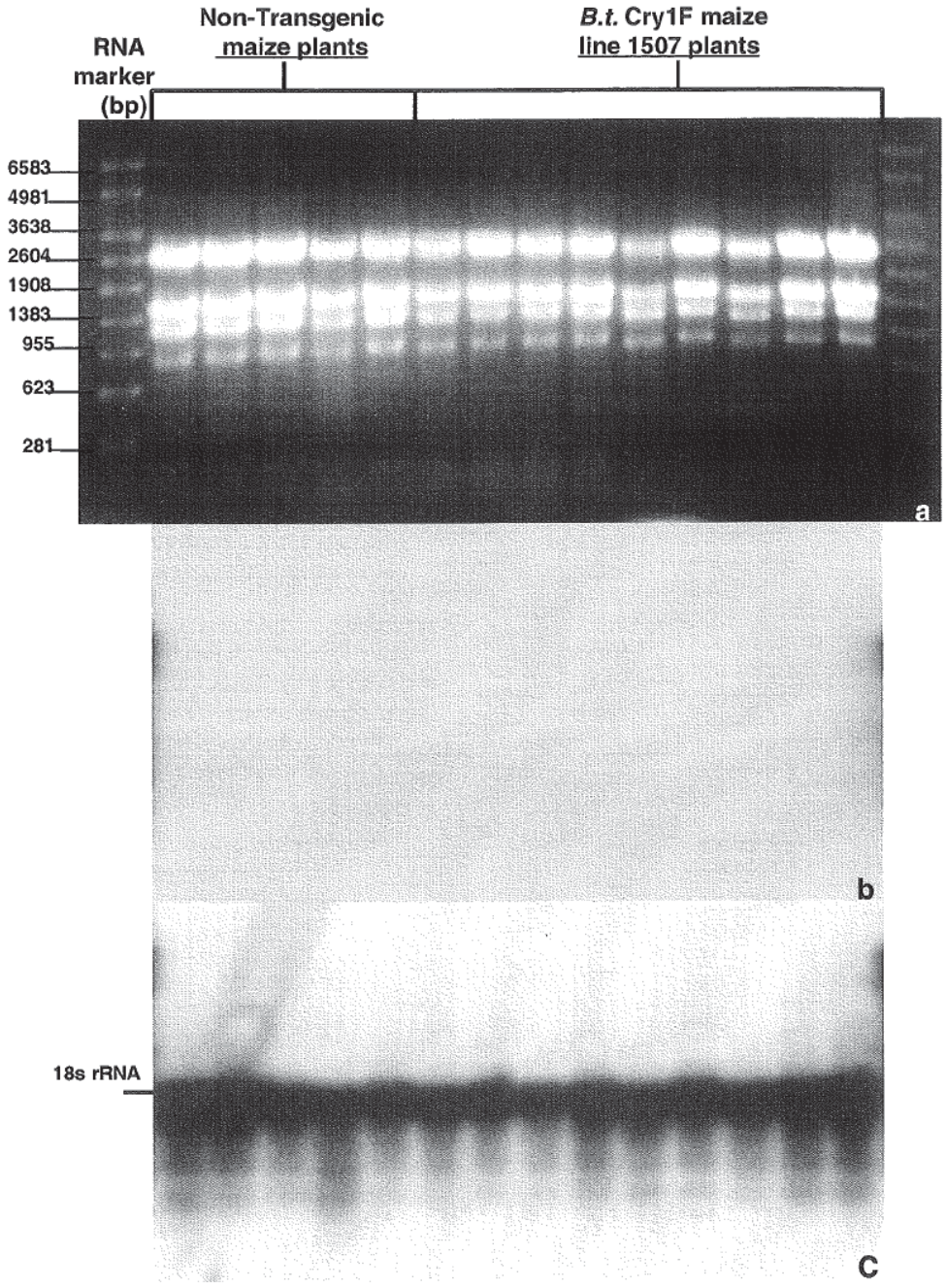


Figure 15. Northern blot analysis of ORF4 in the Full Insert of *B.t.* Cry1F Maize line 1507

a. Total RNA samples on a 1% formaldehyde-agarose gel
b. Hybridization with the 630 bp ORF4 sequence
c. Hybridization with a rice 18S rRNA gene as a probe



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