99-173-01p

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MONSANTO COMPANY
700 CHESTERFIELD PARKWAY NORTH
ST. LOUIS, MISSOURI 63198
PHONE (314) 694-1000
http://www.monsanto.com

June 21, 1999

Coordination, and Technical Assistance,
Biotechnology, and Scientific Services,
Plant, Protection, and Quarantine,
Animal and Plant Health Inspection Service,
U.S. Department of Agriculture
4700 River Road
Riverdale, MD 20737

Attention: Dr. James White

Subject: Application for Extension of a Determination of Nonregulated Status - NewLeaf Plus Potatoes: Document 99-246-U (revised)

Enclosed is a revised USDA application for determination on the regulatory status of *Solanum tuberosum* cultivar Russet Burbank, line RBMT22-82. The application is revised to address the requests in the USDA letter dated June 4, 1999.

Based on the data and information contained in the enclosed application, we believe that there is no longer "reason to believe" that the modified potato plants should be deemed to be regulated articles. The modified potato plants do not present a plant pest risk and are not otherwise deleterious to human health or the environment. The enclosed revised application does not contain confidential business information.

The undersigned certifies that, to the best of his/her knowledge and belief, this petition includes all data, information, and views relevant to the matter, whether favorable or unfavorable to the position of the undersigned, which is the subject of the application.

Please phone me or email me if you have questions on this request.

Signature

Elizabeth D. Owens, Ph.D.
Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway North
BB1K
St. Louis, MO 63198
Phone: 314-737-5721
FAX: 314-737-7085
Email: Elizabeth.D.Owens@monsanto.com

6/22/99

99-173-01p

APPLICATION FOR AN EXTENSION OF THE DETERMINATION OF NONREGULATED STATUS FOR NEWLEAF® PLUS POTATO Line RBMT22-82

Document No. 99-246-U
Revised

June 18, 1999

Submitted by

Elizabeth D. Owens, Ph.D.
Monsanto Company

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6/22/99

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ABBREVIATIONS

aad	adenylyltransferase
AlMV	Alfalfa Mosaic Virus
BCTV	Beet Curly Top Virus
BMV	Brome Mosaic Virus
BWYV	Beet Western Yellows Virus
bp	Basepairs
B.t.t.	*Bacillus thuringiensis* subsp. *tenebrionis* bacterium
ca.	About
cm	Centimeters
CaMV	Cauliflower Mosaic Virus
CMV	Cucumber Mosaic Virus
CP	Coat Protein
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. CP4
CPB	Colorado Potato Beetle
cry3A	Gene which confers resistance to CPB
cvs.	Cultivars
cwt	Centiweight
EBN	Endosperm Balance Number
ELISA	Enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FDA	Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMV	Figwort Mosaic Virus
g, ng, μg	Grams, nanograms, micrograms
GS	Glutamine Synthetase gene
HSPs	High-Scoring Segment Pairs
kb	Kilobases
kD	Kilodaltons
lb.	Pound
LR7	Isolate of Potato Leafroll Luteovirus
mg	Milligrams
nt	Nucleotides
ORF	Open Reading Frames
PCR	Polymerase chain reaction
PLRV	Potato Leaf Roll Virus
PLRVrep	PLRV replicase gene
PV-STMT21	Plant vector
PV-STMT22	Plant vector
PVA, PVX, PVY PVM, PVS	Potato viruses
reps.	Replicates
RBMT22, etc.	Plant lines
spp.	Species
T-DNA	Transfer DNA
TGMV	Tomato Golden Mosaic Virus
TRV	Tobacco Rattle Virus
USDA	United States Department of Agriculture

I. RATIONALE FOR THE SUBMISSION OF A REQUEST FOR EXTENSION FOR NEWLEAF® PLUS POTATOES

There are no changes in the rationale from the previously approved petition number 97-204-01p, used to support deregulation of two other lines (USDA 1998). The specific genetic elements of the new line are summarized below and discussed in the appropriate sections of this application. The only difference between NewLeaf Plus line RBMT22-82 and the previously approved lines is that the plasmid used to transform this line contains the chimeric gene for selection of transformed plant cells on media containing a herbicide, glyphosate.

Information on the potato plant line derived from one specific independent transformation event is presented in this petition. The parental potato line RB1, of the Russet Burbank variety, was transformed with PV-STMT22 to insert the DNA between the left and right border of the *Agrobacterium tumefaciens* Ti plasmid (Klee and Rogers, 1989).

This plant line (Line No. RBMT22-82) contains the following genetic elements:

- The chimeric gene for selection of transformed plant cells on glyphosate (FMV/*CP4 EPSPS*/E9 3') which consists of the 35S promoter region of the Figwort mosaic virus (FMV) (Richins *et al.*,1987), the *CP4 EPSPS* gene which encodes the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (Barry *et al.*, 1992) and the nontranslated 3' region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) referred to as E9 3' (Coruzzi *et al.*, 1984).. Results from Southern blot analysis indicate that two copies of T-DNA contain an intact FMV/*CTP2-CP4 EPSPS* coding region, and that insertion of the third copy of T-DNA resulted in a less than full length copy of the FMV/*CTP2-CP4 EPSPS* coding region in which the FMV promoter and *CTP2* coding region are intact, and that a predicted truncation occurs somewhere downstream in the *CP4 EPSPS* coding region
- The chimeric gene responsible for the control of CPB (AraSSU1A/*cry3A*/nos 3') which consists of the *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase small subunit ats1A promoter (Almeida *et al.*, 1989; Wong *et al.*, 1992), the *cry3A* gene which encodes the Cry3A protein (McPherson *et al.*, 1988; Perlak *et al.*, 1993) and the nontranslated 3' region of the nopaline synthase gene referred to as NOS 3' (Depicker *et al.* 1982; Bevan *et al.*, 1983).
- The chimeric gene responsible for control of PLRV (FMV/*PLRVrep*/E9 3') which consists of the 35S promoter region of FMV (Richins *et al.*,1987), the full-length ORF1 and ORF2 (hereafter referred to collectively as *PLRVrep)* from a naturally occurring PLRV isolate (Miller *et al.*, 1995; Murphy *et al.*, 1995; Van der Wilk *et al.*, 1989), the PLRV intergenic region and the nontranslated 3' region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) referred to as E9 3' (Coruzzi *et al.*, 1984).
- Backbone sequence inserted in this line includes the *aad* gene (bacterial selectable marker), and the bacterial origin of replication, *ori*-322. The inserted *aad* gene is the coding region for TN7 adenylyltransferase conferring spectinomycin or streptomycin resistance in *E. coli* (Fling *et al.*, 1985). The *ori*-322 segment (rop) is a segment of pBR322 which provides the origin of replication for maintenance of the PV-STMT22 plasmid in *E.coli* and *bom* site for conjugational transfer into *Agrobacterium* (Bolivar *et al.*, 1977; Sutcliffe, 1978). Because the *aad* gene is under the control of a bacterial promoter, no AAD protein was expected to be produced in the line. This expectation was confirmed from results using an AAD specific ELISA.

Field experiments with this transgenic potato line were conducted beginning in 1994 under permits/notifications from the USDA (93-362-01r, 94-217-02R, 94-342-01r, 96-277-01r, 97-017-03r, 98-06801n, 98-06808n, 98-6809n, 98-06810n, 98-12108n, and 98-13209n). The final reports for these permits/notifications are on file with USDA. This petition provides information to demonstrate that the modified potato plants do not present a plant pest risk and are not otherwise deleterious to human health or the environment.

II. THE POTATO FAMILY

OECD compiled a report on the biology of the potato (OECD 1997). This is a Consensus Document on the Biology of *Solanum tuberosum* subsp. *tuberosum* in the OECD Series on the Harmonization of Regulatory

Oversight in Biotechnology. The document, prepared by the Netherlands as the lead country in collaboration with the United Kingdom, addresses the biology of the crop plant *Solanum tuberosum* subsp. *tuberosum* and contains information for use during the regulatory assessment of potato products. This supersedes the information provided in the petition submission. The cultivar, Russet Burbank, is the same as in the petition submission.

III. TRANSFORMATION SYSTEM

The line RBMT22-82 was transformed with a different plasmid than the previously approved lines. The transgenic Russet Burbank potato line was developed by transforming one Russet Burbank parental line (RB1) with the PV-STMT22 vector using the *Agrobacterium tumefaciens* transformation system.

The transformation vector PV-STMT22 was created and used in transformation of potato using the methods as described in the submitted petition with the plant selection media used being one containing glyphosate instead of kanamycin.

IV. THE DONOR GENES AND REGULATORY SEQUENCES

The line RBMT22-82 was transformed with a different plasmid than the previously approved lines. Genetic elements contained in the vector PV-STMT22 (Figure IV.1) are summarized in Table IV.1. For clarity, please note that when reference is made to "replicase" in the context of the transgene or its product(s), this refers to PLRV ORF1 and ORF2 which are thought to encode a fusion protein having both helicase and a RNA-dependent RNA polymerase activity, respectively.

A. Construction of the Plant Vector

The plant transformation vector used in the generation of transformed Russet Burbank potatoes was PV-STMT22. Plant vector PV-STMT22 employs the *CP4 EPSPS* gene which allows selection of transformed plants in a medium containing glyphosate.

PV-STMT22 is a double border binary transformation vector, which is described in the submitted petition. The genes contained in the vector are described below.

Three chimeric genes, with signals for plant expression, were introduced between the right and left border regions of the plant transformation vector. For vector PV-STMT22, the chimeric genes and their components are as follows. The chimeric gene for selection of transformed plant cells on glyphosate (FMV/*CP4 EPSPS*/E9 3') which consists of the 35S promoter region of the Figwort mosaic virus (Richins *et al.*, 1987), the *CP4 EPSPS* gene which encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (Barry *et al.*, 1992) and the nontranslated 3' region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) referred to as E9 3' (Coruzzi *et al.*, 1984). The chimeric gene responsible for the control of CPB (AraSSU1A/*cry3A*/NOS 3') which consists of the *A. thaliana* ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit ats1A promoter (Almeida *et al.*, 1989; Wong *et al.*, 1992), the *cry3A* gene which encodes the Cry3A protein (McPherson *et al.*, 1988; Perlak *et al.*, 1993), and the nontranslated region of the 3' region of the nopaline synthase gene referred to as NOS 3' (Depicker *et al.*, 1982; Bevan *et al.*, 1983). The chimeric gene responsible for control of PLRV (FMV/*PLRVrep*/E9 3') which consists of the 35S promoter region of the Figwort mosaic virus (Richins *et al.*, 1987), the full length *PLRVrep* gene from a naturally occurring PLRV isolate (Miller *et al.*, 1995; Murphy *et al.*, 1995; van der Wilk *et al.*, 1989) and the nontranslated 3' region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) referred to as E9 3' (Coruzzi *et al.*, 1984).

B. *Description of the Inserted Genes*

1. *Cry3A* gene and its encoded Cry3A *protein*

This gene is the same as described in the petition submission.

2. *PLRVrep* gene

This gene is the same as described in the petition submission.

3. *CP4 EPSPS* gene and its encoded CP4 EPSPS protein

The *CP4 EPSPS* gene, present in plant vector PV-STMT22, was isolated from *Agrobacterium* sp. strain CP4 (Barry *et al.*, 1992). The *CP4 EPSPS* gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation (Howe *et al.*, 1992). The *CP4 EPSPS* gene encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in all plants, bacteria and fungi (Levin and Sprinson, 1964). The aromatic amino acid pathway is not present in mammalian metabolic pathways (Cole, 1985). EPSPS enzyme is inhibited by the herbicide glyphosate (N-phosphonomethylglycine). Plant cells treated with glyphosate are unable to synthesize the aromatic amino acids essential for protein synthesis which result in death of the plant cells (Barry *et al.*, 1992). In contrast, CP4 EPSPS enzyme is not inhibited by glyphosate. Thus, insertion of the *CP4 EPSPS* gene in a plant cell results in plant cells which are tolerant to glyphosate. The purpose of inserting the *CP4 EPSPS* gene into potato cells with the *cry3A* and *PLRVrep* genes is to have an effective method of selecting cells that contain the insect and viral resistant genes using glyphosate as the selective agent. Consequently, cells selected for plant regeneration contain the *CP4 EPSPS*, *cry3A* and *PLRVrep* genes.

4. *AAD* and *ORI-322* genes

Backbone sequence in this T-DNA insertion includes the *aad* gene (bacterial selectable marker), and the bacterial origin of replication, *ori*-322. The inserted *aad* gene is the coding region for TN7 adenylyltransferase conferring spectinomycin or streptomycin resistance in *E. coli* (Fling *et al.*, 1985). The *ori*-322 segment (rop) is a segment of pBR322 which provides the origin of replication for maintenance of the PV-STMT22 plasmid in *E.coli* and *bom* site for conjugational transfer into *Agrobacterium* (Bolivar *et al.*, 1977; Sutcliffe, 1978). Because the *aad* gene is under the control of a bacterial promoter, no AAD protein was expected to be produced in the line. This expectation was confirmed from results using an AAD specific ELISA.

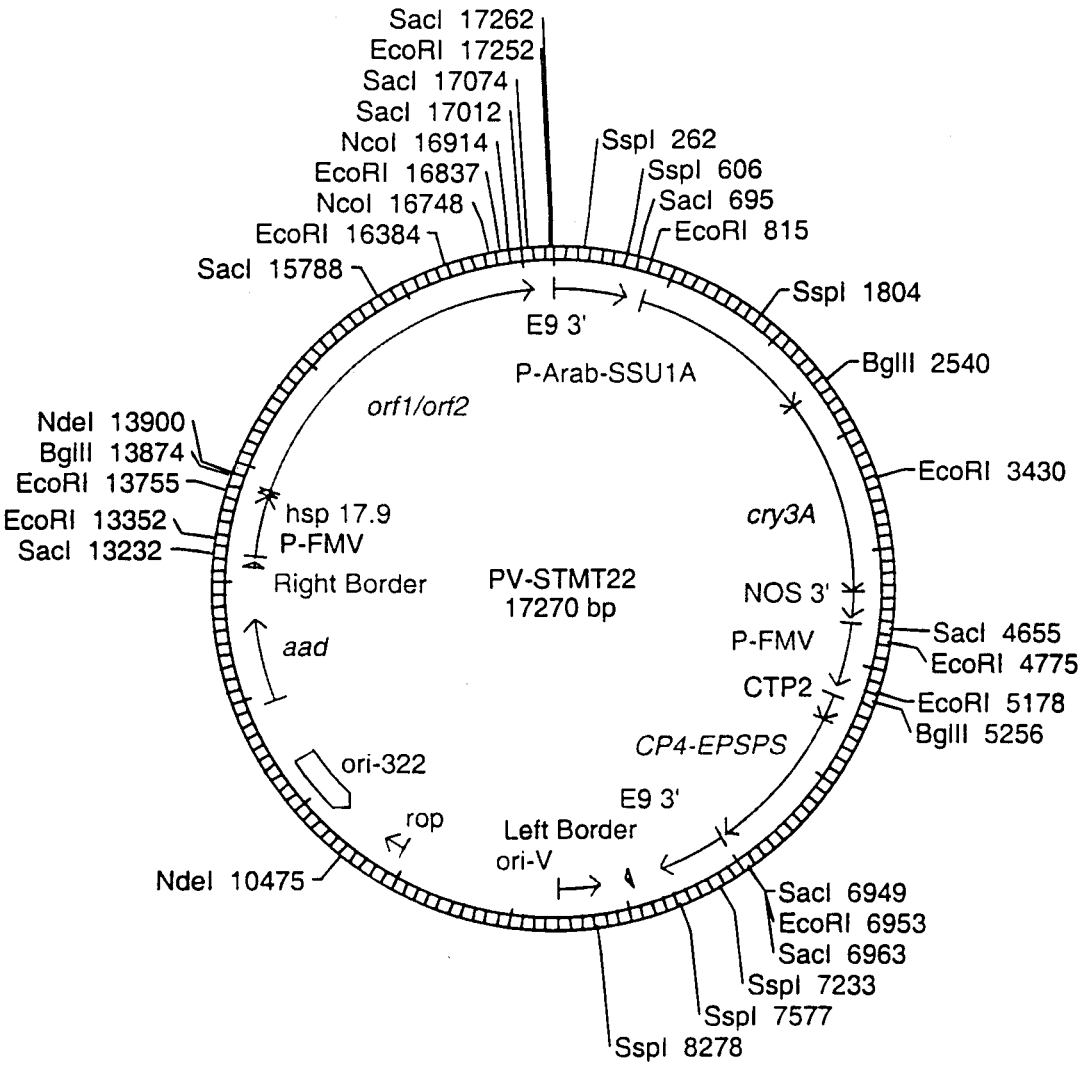

Figure IV.1. Plasmid map of PV-STMT22.
See text in Section IV for details. The expected base pair location where the enzyme is expected to cleave the plasmid is listed next to the restriction enzyme. Plasmid elements are located within the circle.

TABLE IV.1. SUMMARY OF DNA COMPONENTS IN PV-STMT22

Genetic Element	Size, kb	Function and Source
aad	0.8	Coding region for TN7 adenylyltransferase conferring spectinomycin or streptomycin resistance in *E. coli* (Fling *et al.*, 1985).
RB	0.36	*Pvu*I to *Bcl*I restriction fragment from the pTiT37 plasmid containing the nopaline-type T-DNA right border region (Depicker *et al.*, 1982)
P-FMV	0.57	The 35S promoter region of the Figwort mosaic virus (FMV) (Richins *et al.*, 1987)
Hsp	0.077	The soybean heatshock protein 17.9 kD, 5' 77-nucleotide leader sequence (Raschke *et al.*, 1988).
PLRVrep	3.4	The gene which confers resistance to PLRV. This gene is identical to the PLRV ORF1/ORF2 (referred to collectively as replicase gene) present in PLRV (Kaniewski *et al.*, 1994).
E9 3'	0.63	A 3' nontranslated region of the pea ribulose-1,5-bisphosphate carboxylase, small subunit (Rubisco) E9 gene (Coruzzi *et al.*, 1984), which functions to terminate transcription and direct polyadenylation of the *PLRVrep* mRNA.
P-Arab-SSU1A	1.7	The *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit *ats*1A promoter (Almeida *et al.*, 1989; Wong *et al.*, 1992).
cry3A	1.8	The gene which confers resistance to CPB. The gene encodes an amino acid sequence identical to the CPB control protein (referred to as the *B.t.t.* Band 3 protein) found in *B.t.t.* as described by Perlak *et al.* (1993).
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which functions to terminate transcription and direct polyadenylation of the *cry3A* mRNA (Depicker *et al.*, 1982; Bevan *et al.*, 1983).
P-NOS	0.3	The promoter region of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens* (Fraley *et al.*, 1983).
CTP2	0.23	The chloroplast transit peptide leader sequence from the *Arabidopsis thaliana* EPSPS gene (Klee *et al.* 1987).
CP4	1.4	The gene isolated from *Agrobacterium* sp. (Barry *et al.*, 1992) which encodes 5-enolpyruvylshikimate-3-phosphate synthase. Expression of this gene in plant cells confers glyphosate resistance and serves as a selectable marker for transformation.
LB	0.45	A restriction fragment from the octopine Ti plasmid, pTI15955, containing the 24 bp T-DNA left border used to terminate the transfer of the DNA from *Agrobacterium tumefaciens* to the plant genome (Barker *et al.*, 1983).
oriV	1.3	Origin of replication for the ABI *Agrobacterium* derived from the broad host range plasmid RK2 (Stalker *et al.*, 1981).
ori-322/rop	1.8	A segment of pBR322 which provides the origin of replication for maintenance of the PV-STMT22 plasmid in *E.coli* and *bom* site for conjugational transfer into *Agrobacterium* (Bolivar *et al.*, 1977; Sutcliffe, 1978).

V. GENETIC ANALYSIS, AGRONOMIC PERFORMANCE AND COMPOSITIONAL ANALYSIS

A. Genetic Analysis

Product characterization studies were carried out on the transgenic Russet Burbank potato plant line RBMT22-82. The studies included characterization of the DNA inserted, protein or RNA, and levels of proteins expressed in the recipient plants. These data demonstrate that, with the exception of the *CP4 EPSPS* gene and some inserted plasmid sequence, the inserted genes are the same and are expressing in potato as would be expected from data submitted on the previously approved lines.

1. Characterization of inserted DNA

Genomic DNA was isolated from young leaf tissue from NewLeaf Plus Line No. RBMT22-82 and control Russet Burbank. The isolated DNA was digested with restriction enzymes that cleaved the DNA into smaller fragments which were subjected to Southern blot analysis. The analysis defined the genetic elements which were transferred from plasmid PV-STMT22 to the genome of the potato lines. In addition, based on the number and sizes of DNA fragments generated from the restriction enzyme cleavage reactions and the known locations of the restriction sites within the plant vector PV-STMT22 (Figure IV.1), the analysis provided information on the number of loci and insert integrity of the DNA introduced in these NewLeaf Plus potato lines.

Southern blot analyses indicate that for NewLeaf Plus Line No. RBMT22-82, insertion of the T-DNA occurred at three loci. All three copies of T-DNA contain intact coding regions for both the *PLRVrep orf1/orf2* gene and the *cry3A* gene. Two copies of T-DNA contain an intact coding region for the *CP4 EPSPS* gene. However, at one locus, T-DNA insertion did not occur as expected, resulting in insertion of a less than full length copy of the *CTP2-CP4 EPSPS* gene. Characterization of the CTP2-CP4 EPSPS mRNA produced by this line using northern blot analysis showed that only the expected CTP2-CP4 EPSPS mRNA is produced. Therefore, insertion of the T-DNA did not result in production of any gene product other than the expected CTP2-CP4 EPSPS mRNA. This suggests that for the third locus the less than full length gene produces a CTP2-CP4 EPSPS mRNA that does not accumulate to detectable levels. For another T-DNA, plasmid backbone sequence beyond the right border was also inserted. This sequence is adjoined to the right border of the T-DNA. Backbone sequence in this T-DNA insertion includes the *aad* gene (bacterial selectable marker), and the bacterial origin of replication, *ori*-322. Because the *aad* gene is under the control of a bacterial promoter, no AAD protein was expected to be produced in the line. This expectation was confirmed from results using an AAD specific ELISA. Tuber and leaf tissues from this line were assayed for the presence of the AAD protein using an ELISA as described by Rogan *et al.*, (1997). No AAD protein was detected at the detection limit of the ELISA assay. The coding regions of all other genetic elements are intact. A summary of the elements present in this line, the number of loci and the integrity of the coding regions of the genes transferred to this line is presented in Table V.1. A description of the analysis of NewLeaf Plus Line No. RBMT22-82 by Southern blot is summarized below.

TABLE V.1. ESTIMATION OF THE NUMBER OF LOCI AND ASSESSMENT OF GENE INTEGRITY IN NEWLEAF PLUS LINE NO. RBMT22-82

Conclusions	Digest/Probe	Size (kb) of restriction fragments
Three loci	*NcoI/orf1/orf2*	5.8[2], 7.0[2], 14.0[2]
	BglII/CP4 EPSPS	8.0[2], 8.5[2], 13.0[2]
	SspI/orf1/orf2	8.7[2], 9.0[2], 9.6[2]
Intact *orf1/orf2* coding region	*Eco*RI/ $orf1/orf2_{ft}$	2.6[1]
	*Eco*RI/E9	0.8[1]
Intact E9 terminator	*Eco*RI/E9	0.8[1]
E9 border fragments		1.2[2], 1.7[2]
Intact *cry3A* coding region	*SacI/cry*3A	4.0[1]
	*Eco*RI/*cry*3A	1.3[1], 2.6[1]
Truncation in *CTP2-CP4 EPSPS* coding region	*SspI/CP4 EPSPS*	6.2[2]
	SacI/CP4 EPSPS	24.0[2]
	*Eco*RI/*CP4 EPSPS*	13.0[2]
Intact *CTP2-CP4 EPSPS* coding region	*SspI/CP4 EPSPS*	5.4[1]
	SacI/CP4 EPSPS	2.3[1]
	*Eco*RI/*CP4 EPSPS*	1.8[1]
Presence of backbone	*Eco*RI/*ori*V, *ori*322, *aad*	23.0[2]
	*NcoI/ ori*V, *ori*322, *aad*	14.0[1]
Absence of *ori*V	*NdeI/ori*V	No hybridization
Presence of *aad*	*NcoI/aad*	14.0[1]
Presence of *ori*322	*NcoI/ori*322	14.0[1]

[1]Restriction fragment is the size predicted from digestion of the plasmid or linked copies of the T-DNA.
[2]Restriction fragment produced as a result of insertion of T-DNA into genomic (border fragment) or truncation within gene.

Estimation of loci number. Restriction enzymes that cleave near the right border were used to estimate the number of inserts. This strategy is consistent with the most common mechanism known for insertion of DNA using *Agrobacterium* based plant transformation which suggests that the right border remains intact during insertion of the T-DNA (Tinland, 1996). The use of a restriction enzyme that cleaves near the right border would therefore be the best method to generate border fragments (plant DNA to T-DNA junctions) which could be used to predict the number of inserts. Three different restriction enzymes were used: *Nco*I, *Ssp*I and *Bgl*II.

To estimate the number of loci in NewLeaf Plus Line No. RBMT22-82 via right border analysis, genomic DNA was digested using *Nco*I and probed with ^{32}P labeled *orf1/orf2*. Both *Nco*I sites of the plasmid PV-STMT22 are located in close proximity near the 3' end of the *orf1/orf2* open reading frame. This restriction enzyme cuts twice in the 3' portion of the *orf1/orf2* gene. The results from this digest using this probe were expected to yield border fragments of unpredictable size which could be used to estimate the number of loci present in this line. Southern blot data for Line No. RBMT22-82 using *Nco*I and the *orf1/orf2* probe are presented in Figure V.2, Lane 5. Restriction fragments of approximately 5.8, 7.0, and 14.0 kb were observed that hybridized to the *orf1/orf2* probe. The fragments observed were produced as a result of border fragments associated with three independent loci. *Nco*I also generates an internal *orf1/orf2* fragment of approximately 0.2 kb. A fragment of this size would not be observed on this Southern blot. Additionally, digestion of a T-DNA insertion containing a complete *orf1/orf2* cassette with *Nco*I would be predicted to give a left border fragment of unpredictable size. However, because *Nco*I cuts very close to the 3'end of the *orf1/orf2* gene, there would be insufficient sequence available for hybridization with the *orf1/orf2* probe to generate a detectable signal. Based on these observations, we conclude that the three signals observed in Figure V.2, Lane 5 represent three right border junctions, indicating three insertion events.

This finding was confirmed by digesting the genomic DNA of NewLeaf Plus line RBMT22-82 with the *Ssp*I restriction endonuclease and probing with the *orf1/orf2* coding region. The results of this experiment are presented in Figure V.3, Lane 4. Three fragments of approximately 9.6, 9.0 and 8.7 kb were observed. Each of these fragments represents a border fragment, indicating three insertion events, which is consistent with the results of the *Nco*I blot probed with the *orf1/orf2* gene.

The number of loci in Line No. RBMT22-82 was further confirmed via left border analysis, in which genomic DNA was digested using *Bgl*II and probed with ^{32}P labeled *CP4 EPSPS*. A *Bgl*II site is located at the beginning of the *CTP2-CP4 EPSPS* open reading frame in the plasmid PV-STMT22. Therefore, the results from this digest using this probe were also expected to yield border fragments which could be used to estimate the number of loci present in this line since each T-DNA insertion should produce a single fragment of unpredictable size. Southern blot data for Line No. RBMT22-82 using *Bgl*II and the *CP4 EPSPS* probe are presented in Figure V.4, Lane 3. Restriction fragments of approximately 8.0, 8.5 and 13.0 kb were observed that hybridized to the *CP4 EPSPS* probe. The fragments observed were produced as a result of border fragments associated with three independent loci. This result corroborates the presence of three independent loci determined by Southern blot using both *Nco*I and *Ssp*I restriction endonucleases and the *orf1/orf2* probe.

Characterization of the orf1/orf2 gene cassette. The integrity of the *orf1/orf2* coding region was assessed using the restriction enzymes *Eco*RI and probing with the ^{32}P labeled *orf1/orf2*$_{ft}$. The probe, *orf1/orf2*$_{ft}$ was produced by PCR amplifying the first third of the *orf1/orf2* coding region. The probe has been given a different name for the purposes of this report by attaching the subscript "ft" standing for "first third". This is in order to avoid confusing it with the full length *orf1/orf2* coding region. The reason for using this particular sequence as a probe was to avoid the possibility of confusion resulting from the three *Eco*RI sites present in the 3' end of the *orf1/orf2* coding region.

*Eco*RI restriction sites of the plasmid vector PV-STMT22 are located at the beginning and in the last one-third of the *orf1/orf2* gene (Figure IV.1). Digestion of genomic and plasmid DNA using *Eco*RI was expected to excise a 2.6 kb restriction fragment, which corresponds to the first four-fifths (80%) of the coding region of the *orf1/orf2* gene. **Note**: PV-STMT21 (Figure V.1) was used as the plasmid control for this Southern blot. Since this plasmid contains an identical *orf1/orf2* gene, digestion of the plasmid using *Eco*RI is expected to release a 2.6 kb fragment which was used for a control on this blot. The results from the Southern blot are presented in Figure V.5, Lane 6 for RBMT22-82. Only one restriction fragment of approximately 2.6 kb hybridized to the *orf1/orf2* probe. The size of the 2.6 kb fragment corresponds to the predicted size produced by digestion of plasmid PV-STMT21 using

*Eco*RI. The production of the 2.6 kb restriction fragment strongly suggests that all three copies of T-DNA contain an intact *orf1/orf2* coding region.

To confirm that all three copies of T-DNA contain an intact *orf1/orf2* coding region, the integrity of the E9 terminator used for transcriptional termination of the *orf1/orf2* open reading frame was assessed using the restriction enzyme *Eco*RI and probing with ^{32}P labeled E9 terminator. In the plasmid PV-STMT22, *Eco*RI restriction sites flank the 5 and 3-prime regions of the coding sequence for the E9 terminator in the *orf1/orf2* gene cassette, while a single *Eco*RI site is located at the beginning of the E9 terminator element in the *CTP2-CP4 EPSPS* gene cassette. The results from this digest using this probe were expected to excise the complete E9 terminator region from the *orf1/orf2* gene cassette, and to produce E9 border fragments derived from the *CTP2-CP4 EPSPS* cassette which could be used to estimate the number of loci present in this line. The results from this Southern blot are presented in Figure V.6, Lane 6. PV-STMT21 (Figure V.1) was used as the plasmid control for this Southern blot. Three restriction fragments of approximately 1.7 , 1.2 and 0.8 kb hybridized to the E9 probe. The size of the 0.8 kb fragment corresponds to the predicted size produced by digestion of plasmid PV-STMT22 using *Eco*RI. The 1.2 and 1.7 kb restriction fragments observed were produced as a result of E9 border fragments associated with two independent loci. While this appears to be inconsistent with three independent loci determined by Southern blot using *Bgl*II and the *CP4-EPSPS* probe (see above), this is consistent with insertion of two complete T-DNAs plus a third copy of T-DNA which resulted in a less than full length copy of the *CTP-CP4 EPSPS* gene and absence of its E9 terminator (see below). Therefore, on this Southern blot the 0.8 kb restriction fragment represents three copies of the E9 terminator element from the *orf1/orf2* cassette. On the basis of the production of the 0.8 kb restriction fragment, all three copies of T-DNA contain an intact E9 terminator in the *orf1/orf2* gene cassette, which also strongly supports that the 3' region of the *orf1/orf2* open reading frame is intact in all three copies of T-DNA. The intactness of the E9 terminator used for transcriptional termination of the *orf1/orf2* gene also provides a linkage between the *orf1/orf2* gene cassette and the *cry3A* gene cassette.

Characterization of the cry3A gene cassette. The integrity of the *cry3A* gene cassette was evaluated using the endonuclease *Sac*I and probing with ^{32}P labeled *cry3A*. Digestion of genomic and plasmid DNA using *Sac*I was expected to excise the entire *cry3A* gene cassette as a 4.0 kb fragment. The results from this Southern blot are presented in Figure V.7, Lane 5. The *cry3A* probe hybridized to a single 4.0 kb restriction fragment. The 4.0 kb fragment is the size predicted and observed for digestion of the plasmid with *Sac*I and probing with *cry3A* (Figure V.7, Lane 2). It is concluded that all three copies of the T-DNA in Line No. RBMT22-82 contain intact *cry3A* gene cassettes.

Confirmation that all three copies of T-DNA in Line No. RBMT22-82 contain an intact *cry3A* gene cassette was achieved by digestion of genomic and plasmid DNA with the restriction enzyme *Eco*RI and probing with ^{32}P labeled *cry3A*. *Eco*RI restriction sites of the plasmid PV-STMT22 are located at the beginning of the SSU1A promoter used to drive expression of the *cry3A* gene, at the middle of the *cry3A* open reading frame and at the end of the NOS 3' terminator used for transcriptional termination of the *cry3A* gene. Digestion of genomic and plasmid DNA using *Eco*RI was expected to excise the *cry3A* gene cassette as two restriction fragments, a 1.3 kb fragment and a 2.6 kb fragment. The results from this Southern blot are presented in Figure V.8, Lane 6. Two restriction fragments of approximately 1.3 and 2.6 kb hybridized to the *cry3A* probe. The size of the 1.3 and 2.6 kb fragments correspond to the predicted size produced by digestion of plasmid PV-STMT22 using *Eco*RI. It should be noted that Figure V.8, Lanes 2 and 8 contain the plasmid control PV-STMT21. This plasmid contains the *cry3A* coding region, but only two of the three *Eco*RI sites are in the same positions as plasmid PV-STMT22. Therefore, only one of the *cry3A* containing fragments produced from the digestion of the plasmid PV-STMT21 migrates at the same molecular weight (2.6 kb) as the fragment predicted from plasmid PV-STMT22. The other fragment has a predicted molecular weight of approximately 1.9 kb and therefore, would not migrate at the same rate as the fragment generated from the plasmid PV-STMT22. We have indicated the proper molecular fragments for plasmid PV-STMT22 in the text above, and the observation of the 1.3 and 2.6 kb restriction fragments confirms that all three copies of T-DNA contain an intact *cry3A* gene cassette.

Characterization of the CP4 EPSPS gene cassette. The integrity of the *CP4 EPSPS* gene cassette was evaluated using the restriction enzyme *Ssp*I for digestion of genomic and plasmid DNA and probing with ^{32}P labeled *CP4 EPSPS*. *Ssp*I cuts in the SSU1A promoter of the adjacent *cry*3A cassette and also in the middle of the E9 terminator of the *CP4 EPSPS* cassette. Digestion of genomic and plasmid DNA using *Ssp*I was expected to excise most of the *cry3A* gene cassette, plus the *CP4 EPSPS* gene cassette through the 5' half of its E9 terminator, as a 5.4

kb restriction fragment. Results from this Southern blot are presented in Figure V.9, Lane 4. The *CP4 EPSPS* probe hybridized to two restriction fragments of approximately 5.4 and 6.2 kb. The 5.4 kb fragment corresponds to the size predicted and observed for digestion of the plasmid PV-STMT22 using *Ssp*I (Figure V.9, Lane 2). It is believed that the *Ssp*I restriction fragment observed for the plasmid control (Figure V.9, Lane 2) migrated at a slightly lower position than 5.4 kb due to excessive salt contamination in the DNA preparation as evidenced by curvature of the band, and that the 5.4 kb restriction fragment from the genomic digest is accurate in size. However the size of the 6.2 kb *Ssp*I restriction fragment exceeds the predicted and observed size of 5.4 kb. Therefore, this band represents a border fragment resulting from the elimination of the *Ssp*I site in the E9 portion of the *CP4 EPSPS* gene cassette. Results from this Southern blot indicate that two copies of T-DNA contain an intact *CP4 EPSPS* gene cassette with at least the 5' half of the E9 terminator, and that insertion of the third copy of T-DNA resulted in a less than full length copy of the *CP4 EPSPS* gene cassette in which the FMV promoter and *CTP2* coding region of the *CP4 EPSPS* gene cassette are intact, and that a predicted truncation occurs somewhere downstream in the *CP4 EPSPS* or E9 coding region.

To further evaluate the integrity of the FMV promoter and *CTP2-CP4 EPSPS* coding region, genomic and plasmid DNA were digested using the restriction enzyme *Sac*I and probed with ^{32}P labeled *CP4 EPSPS*. The *Sac*I digest was expected to excise the FMV promoter and *CTP2-CP4 EPSPS* open reading frame as a 2.3 kb restriction fragment. Results from this Southern blot are presented in Figure V.10, Lane 5. The *CP4 EPSPS* probe hybridized to two restriction fragments of approximately 2.3 and 24.0 kb. The 2.3 kb fragment corresponds to the size predicted and observed for digestion of the plasmid PV-STMT22 using *Sac*I (Figure V.10, Lane 2). However the size of the 24.0 kb *Sac*I restriction fragment exceeds the predicted and observed size of 2.3 kb. Therefore, this band represents a border fragment in which the *Sac*I site in the E9 terminator is eliminated. Results from this Southern blot indicate that two copies of T-DNA contain an intact FMV/*CTP2-CP4 EPSPS* coding region, and that insertion of the third copy of T-DNA resulted in a less than full length copy of the FMV/*CTP2-CP4 EPSPS* coding region in which the FMV promoter and *CTP2* coding region are intact, and that a predicted truncation occurs somewhere downstream in the *CP4 EPSPS* coding region. This result is consistent with the results from the *Ssp*I digestion.

To more precisely define the truncation in the *CP4 EPSPS* coding region, the integrity of the *CTP2-CP4 EPSPS* open reading frame was evaluated using the restriction enzyme *Eco*RI for digestion of genomic and plasmid DNA and probing with ^{32}P labeled *CP4 EPSPS*. *Eco*RI restriction sites of the plasmid PV-STMT22 are located at the beginning and end of the *CTP2-CP4 EPSPS* open reading frame. Digestion of genomic and plasmid DNA using *Eco*RI was expected to excise the *CTP2-CP4 EPSPS* open reading frame as a 1.8 kb restriction fragment. The results from this Southern blot are presented in Figure V.11, Lane 4. The *CP4 ESPS* probe hybridized to two restriction fragments of approximately 1.8 and 13.0 kb. The 1.8 kb fragment corresponds to the size predicted and observed for digestion of the plasmid using this restriction enzyme (Figure V.11, Lane 2). It is believed that the *Eco*RI restriction fragment observed for the plasmid digest (Figure V. 11, Lane 2) migrated at a slightly lower position than 1.8 kb due to excessive salt contamination in the DNA preparation as evidenced by curvature of the band, and that the 1.8 kb restriction fragment from the genomic digest is accurate in size. However, the size of the 13.0 kb restriction fragment exceeds the predicted and observed size of 1.8 kb. This confirms that two copies of T-DNA contain intact *CTP2-CP4 EPSPS* coding regions, and that insertion of one copy of T-DNA resulted in incomplete resolution of the left border resulting in insertion of a less than full length copy of the *CTP2-CP4 EPSPS* gene in which a truncation occurred in the *CP4 EPSPS* coding region.

For the T-DNA locus which contains a less than full length copy of the *CTP2-CP4 EPSPS* coding sequence, the possibility exists at the border junction for a gene fusion between the truncated *CTP2-CP4 EPSPS* open reading frame and genomic sequence. To assess whether mRNA is produced from this sequence, poly-A mRNA from the *CTP2-CP4 EPSPS* coding region was characterized by northern blot analysis using ^{32}P labeled *CP4 EPSPS*. The northern blot demonstrates that a single mRNA species of approximately 1.8 kb was produced in line RBMT22-82 (Figure V. 12, Lane 6, Panel B), which was the size for the expected CTP2- CP4 EPSPS mRNA. It is concluded that no gene product other than the expected CTP2- CP4 EPSPS mRNA was produced from insertion of the T-DNAs. Therefore, for the third insert of T-DNA with the less than full length copy of the *CTP2-CP4 EPSPS* cassette, there is no gene fusion that creates a mRNA that accumulates to a detectable level.

Characterization of backbone elements. The presence of sequences beyond the right and left borders (plasmid backbone sequence) was evaluated by digestion of genomic DNA using *Eco*RI or *Nco*I and probing with a mixture

of the individually ^{32}P labeled backbone elements. These elements consist of the individual coding regions *oriV*, *ori322* and *aad* combined in equal amounts based on their specific activities. The mixture of these ^{32}P labeled elements was then used as a probe against Southern blots possessing DNA digested with *Eco*RI and *Nco*I restriction enzymes. The results from the *Eco*RI experiment are presented in Figure V. 13, Lane 5. **Note**: PV-STMT21 (Figure V.1) was used as the plasmid control for this Southern blot. A single restriction fragment of approximately 23.0 kb was observed indicating the presence of some or all of the backbone elements used as a probe and that these elements are together on a single T-DNA.

These findings were confirmed by digesting the genomic DNA with the *Nco*I restriction endonuclease and probing with the backbone elements. Since both *Nco*I sites of plasmid PV-STMT22 are located near the end of the *orf1/orf2* coding region, digestion of genomic DNA was expected to excise three border fragments associated with three independent loci. The results from this Southern blot are presented in Figure V.14, Lane 5 in which the combined *ori*V, *ori*322 and *aad* probe hybridized to a single genomic restriction fragment of approximately 14.0 kb. Specific hybridization to *Nco*I linearized PV-STMT22 plasmid DNA that contains complete backbone sequence was observed (Figure V. 14, Lane 4) as the predicted 17.1 kb fragment. Therefore, we conclude that two copies of T-DNA inserted without sequences beyond the right and left borders, and that insertion of the third copy of T-DNA resulted in incomplete resolution of either the right or left border, in which complete or partial backbone sequence is adjoined to one of the borders within a 14.0 kb border fragment.

To ascertain which border and which backbone elements are present, genomic and plasmid DNA were digested with the *Nde*I restriction enzyme and probed with ^{32}P labeled *ori*V. In plasmid PV-STMT22 an *Nde*I site is located between the *ori*V and *ori*322 elements, and at the beginning of the *orf1/orf2* gene. If the backbone sequences are associated with the left border, digestion with *Nde*I was expected to release a 13.8 kb restriction fragment which contained the *ori*V gene. The results from this Southern blot are presented in Figure V. 15, Lane 4, in which the *ori*V probe showed no hybridization to any genomic restriction fragment. The *ori*V probe did hybridize to an approximate 13.8 kb restriction fragment in the plasmid control, which is the size predicted and observed for digestion of the plasmid with *Nde*I and probing with *ori*V (Figure V.15, Lane 2). From the absence of the *ori*V element in the genomic digest, it is concluded that a less than full length backbone sequence is associated with the right border of the T-DNA, and this sequence lacks the *ori*V element.

To assess where the truncation occurred in the less than full length backbone sequence, genomic and plasmid DNA were digested with the restriction enzyme *Nco*I and probed separately with either ^{32}P labeled *aad* or *ori*322. Digestion of genomic and plasmid DNA using *Nco*I was expected to excise three border fragments associated with three independent loci. The results from this Southern blot are presented in Figure V.16, Lane 5 in which the *aad* probe (panel A) and the *ori*322 probe (panel B) both individually hybridized to a single genomic restriction fragment of approximately 14.0 kb. This fragment is the same size as the fragment observed on the identical *Nco*I blot probed with the *orf1/orf2* gene. Specific hybridization by the *aad* probe or the *ori*322 probe, respectively, to *Nco*I linearized PV-STMT22 plasmid DNA that contains complete backbone sequence was observed (Figure V.16, Lane 4, panels A & B) as the predicted 17.1 kb fragment. Therefore, we conclude that two copies of T-DNA inserted without adjoinment to sequences beyond the right and left borders, and that insertion of the third copy of T-DNA resulted in incomplete resolution of the right border, in which partial backbone sequence adjoined to the right border contains both the *aad* gene and the *ori*322 region, but not the *ori*V region, within a 14.0 kb border fragment.

In plasmid PV-STMT22 the *aad* gene is under the control of a bacterial promoter which is not expected to function in plants. Therefore, no AAD protein was expected to be produced in this line. This expectation was confirmed from results using an AAD specific ELISA. Tuber and leaf tissues from this line were assayed for the presence of the AAD protein using an ELISA as described by Rogan *et al.*, (1997). No AAD protein was detected at the detection limit of the ELISA assay (the detection limit of the assay was estimated to be 6.7 and 5.3 ng AAD protein per gram tissue fresh weight for tuber and leaf respectively).

2. Characterization of Expressed Proteins

Cry3A and CP4 EPSPS

The results of the protein characterization study established that the *Cry3A* and CP4 EPSPS proteins produced in the transformed Russet Burbank lines are equivalent to the previously characterized *E. coli*-produced reference standards and produced in previously registered plant lines.

PLRV Replicase

RNA Analysis
For line RBMT22-82 and the Russet Burbank control, expression of the *PLRVrep* gene was analyzed using Northern hybridization for characterization and RNA dot-blot analysis for relative quantification. To obtain RNA for Northern analysis, plants were grown in a test tube in sterile plant culture medium. After ten weeks, tissue was harvested, frozen in dry ice and ground. Two hundred milligrams of the ground tissue was suspended in 4 ml of TRIZOL® Reagent (Gibco BRL, Life Technologies, Gaithersburg, MD) and total RNA was isolated using the standard TRIZOL protocol. For Northern analysis, approximately 20 µg of total RNA was separated on an agarose/formaldehyde gel and transferred to Hybond N (Amersham, Arlington Heights, IL) according to standard protocol. A random-labeled ^{32}P-dCTP probe was made from PLRV ORF2 using the RTS RadPrime DNA Labeling system (Gibco BRL, Life Technologies, Gaithersburg, MD). After hybridization, the membrane was washed in 0.5X SSC, 0.1% SDS at 65°C for 1 h. The membrane was then exposed to film for 41 h.

The ORF2 DNA probe hybridized to an RNA band having a maximum size of approximately 3.8-3.9 kb, the expected size for the full-length replicase mRNA (Figure V.17.). No hybridization was seen with the RNA from the Russet Burbank control line. This data demonstrates that the full-length mRNA is synthesized and the *PLRVrep* expression cassette is intact in each transgenic line. Assuming translational frameshifting occurs in the transgene as shown for the wild-type virus, a full-length replicase protein having both helicase and RNA-dependent-RNA polymerase function could be produced.

In a separate experiment, RNA dot hybridization analysis was used to estimate the amount of PLRVrep mRNA in leaf and tuber tissues. These data are shown in Figures V.18 and V.19, respectively. *PLRVrep* RNA transcript was not detected by dot hybridization analysis in either leaf or tuber tissue of transgenic line RBMT22-82 in this assay. Based on the amount of *PLRVrep* RNA standard spotted (Lane 1, Figure V.18 and V.19), the limits of detection of the RNA dot hybridization was estimated to be in the range of 0.05 ng of *PLRVrep* mRNA per µg of total leaf RNA and 0.002 ng of *PLRVrep* mRNA per µg of total tuber RNA tuber. This experiment used a fluorescein-labelled RNA probe and therefore does not have the sensitivity of the 32P-labelled probe used to detect the full-length PLRV ORF1/2 mRNA (Figure V.17.). Since the average amount of RNA isolated from leaf and tuber of the RBMT22-82 line corresponded to 840 ng/mg leaf fresh weight and 190 ng/mg tuber fresh weight, the limit of detection was estimated to be approximately 0.04 ng and 0.0004 ng of *PLRVrep* mRNA per mg of leaf and tuber tissue fresh weight, respectively. The RNA dot hybridization analysis indicates that the amount of PLRV viral RNA in infected non-transgenic Russet Burbank is approximately 0.4 ng/mg of leaf tissue and 0.002 ng/mg of tuber tissue. These results indicate that the level of viral *PLRVrep* RNA in naturally infected Russet Burbank potato plants is 5 to 10 fold higher than the mRNA expressed in whole leaf and tuber tissues of the RBMT22-82 transgenic line.

Protein Analysis
The assay for the presence of the PLRV replicase (combined helicase and polymerase) protein was completed as described in the submitted petition. The results for line RBMT22-82, no immunoreactive bands of expected molecular weight, were the same as for the previously approved lines. Since the purified protein standard was detected, as well as mRNA in the Northern analysis, it can be assumed that for this line, as it was reported for previously approved lines, replicase proteins are not produced in detectable levels.

3. Levels of Expressed Proteins

The level of *Cry3A* and CP4 EPSPS proteins expressed in leaf and tuber tissues of the transformed Russet Burbank plant line RBMT22-82 were determined using enzyme-linked immunosorbent assays. Potatoes of this line were sampled and analyzed as described in the submitted petition. The Cry3A protein level in RBMT22-82 was found to be at levels similar to those of the previously approved lines (Lavrik and Grace, 1996).

The mean Cry3A protein expression level in leaf tissue of the line collected across three sites was estimated to be in the range of 14.97 - 29.44 μg/g tissue fresh weight (Table V.2.). The mean Cry3A protein expression level in tuber was estimated to be in the range of 0.49 - 0.79μg/g tissue fresh weight. The Cry3A protein expression levels correspond to a range of 0.09 to 0.17% of total foliage protein and 0.002 to 0.004% of total tuber protein, using total protein levels of 1.6 and 2.0% for foliage and tuber fresh weight, respectively (Lavrik and Grace, 1996).

In 1998, a season long expression study for Cry3A protein was conducted (Bookout and Dodson 1998). In this study, leaf tissue from NewLeaf Plus potato plants was collected from two sites, one in Quebec and one in New Brunswick, at three time points after planting, 6, 10 and 14 weeks for the Quebec site and two time points (10 and 14 weeks) for the New Brunswick site. Each sample was a composite of one compound leaf from six plants in the plot. Samples were analyzed in triplicate using a quantitative ELISA method for Cry3A protein. For RBMT22-82, expression levels averaged, over both sites, all five collection points, 18.1 μg/g tissue fresh weight. The results from the NewLeaf Plus lines, including RBMT22-82, are consistent with Cry3A protein expression levels observed in previously deregulated NewLeaf lines.

The mean CP4 EPSPS protein expression level in leaf tissue of this line transformed with PV-STMT22 collected across three sites was estimated to be in the range of 17.36 - 35.66 μg/g tissue fresh weight (Table V.3.). The mean tuber CP4 EPSPS protein expression level was estimated to be in the range of 0.21 to 0.78 μg/g tissue fresh weight. The CP4 EPSPS protein expression levels correspond to a range of 0.11 to 0.22% of total foliage protein and 0.001 to 0.004% of total tuber protein, using total protein levels of 1.6 and 2.0% for foliage and tuber fresh weight, respectively (Lavrik and Grace, 1996).

As discussed above in Section V.A.2., the PLRV replicase has not been detected in any of the transgenic plant lines. The purified protein standard, which was purified from recombinant *E. coli* and used to produce anti-PLRV ORF1/ORF2 protein antiserum, was detected.

TABLE V.2. CRY3A PROTEIN EXPRESSION IN TISSUE OF RUSSET BURBANK LINE TRANSFORMED WITH PV-STMT22: COMBINED MULTI-SITE DATA

Line	Tissue[2]	Number of Sites[1] (Reps)	Mean[3]	Standard Error[4]	Range[5] µg/g tissue fresh wt.
RBMT22-82	Leaf	3 (18)	20.97	2.061	14.97 - 29.44
	Tuber	3 (12)	0.63	0.058	0.49 - 0.79
RB-Control	Leaf	3 (18)	0.01[6]	0.007	0.00 - 0.07
	Tuber	3 (12)	0.03[6]	0.013	0.00 - 0.06

[1]Combined data from tissue collected during 1995 field trials at Echo, OR; Ephrata, WA; and Pasco, WA.
[2]Leaf tissues were collected approximately ten weeks post-planting. Tubers were collected at harvest. All field trials consisted of eight replicates per line. Leaf expression was determined on five replicates from Echo, OR; six replicates from Pasco, WA; and seven replicates from Ephrata, WA. Tuber expression was determined on four replicates from each site.
[3]Overall mean for each line is the simple mean of the site means.
[4]Standard error of the overall mean was estimated as the standard deviation of the site means divided by the square root of the number of sites. These standard errors reflect the total precision of the mean and cannot be used to compare lines.
[5]"Range" denotes the highest and lowest individual assay sample for each line across sites.
[6]Value is within the background level of the assay.

TABLE V.3. CP4 EPSPS PROTEIN EXPRESSION IN TISSUE OF THE RUSSET BURBANK LINE TRANSFORMED WITH PV-STMT22: COMBINED MULTI-SITE DATA

Line	Tissue[2]	Number of Sites[1] (Reps)	Mean[3]	Standard Error[4]	Range[5] µg/g tissue fresh wt.
RMBT22-82	Leaf	3 (18)	28.34	1.681	17.36 - 35.66
	Tuber	3 (12)	0.53	0.103	0.21 - 0.78
RB-Control	Leaf	3 (18)	0.07[6]	0.039	0.00 - 0.23
	Tuber	3 (12)	0.02[6]	0.015	0.00 - 0.07

[1]Combined data from tissue collected during 1995 field trials at Echo, OR; Ephrata, WA; and Pasco, WA.
[2]Leaf tissues were collected approximately ten weeks post-planting. Tubers were collected at harvest. The field trials consisted of eight replicates per line. Leaf expression was determined on five replicates from Echo, OR; six replicates from Pasco, WA; and seven replicates from Ephrata, WA. Tuber expression was determined on four replicates from each site.
[3]Overall mean for each line is the simple mean of the site means.
[4]Standard error of the overall mean was estimated as the standard deviation of the site means divided by the square root of the number of sites. These standard errors reflect the total precision of the mean and cannot be used to compare lines.
[5]"Range" denotes the highest and lowest individual assay sample for each line across sites.
[6]Value is within the background noise level of the assay.

4. Conclusions

Transgenic Russet Burbank potato plant line RBMT22-82 contains three introduced genes: the *Cry3A* gene which encodes for the CPB-active protein from *B.t.t.*, the *PLRVrep* gene which imparts resistance to potato leafroll virus and the *CP4 EPSPS* gene which served as a selectable marker during the potato transformation process. The *cry3A* and *CP4 EPSPS* genes and the respective proteins expressed by these genes are identical to the genes introduced in previously registered plant pesticides. These genes and gene products have been granted tolerance exemption by the EPA for usage as a pesticide or pesticide inert ingredient (EPA 1997, EPA 1998). The *PLRVrep* gene present is identical to the gene present in naturally occurring isolates of PLRV which are prevalent in commercially available potatoes.

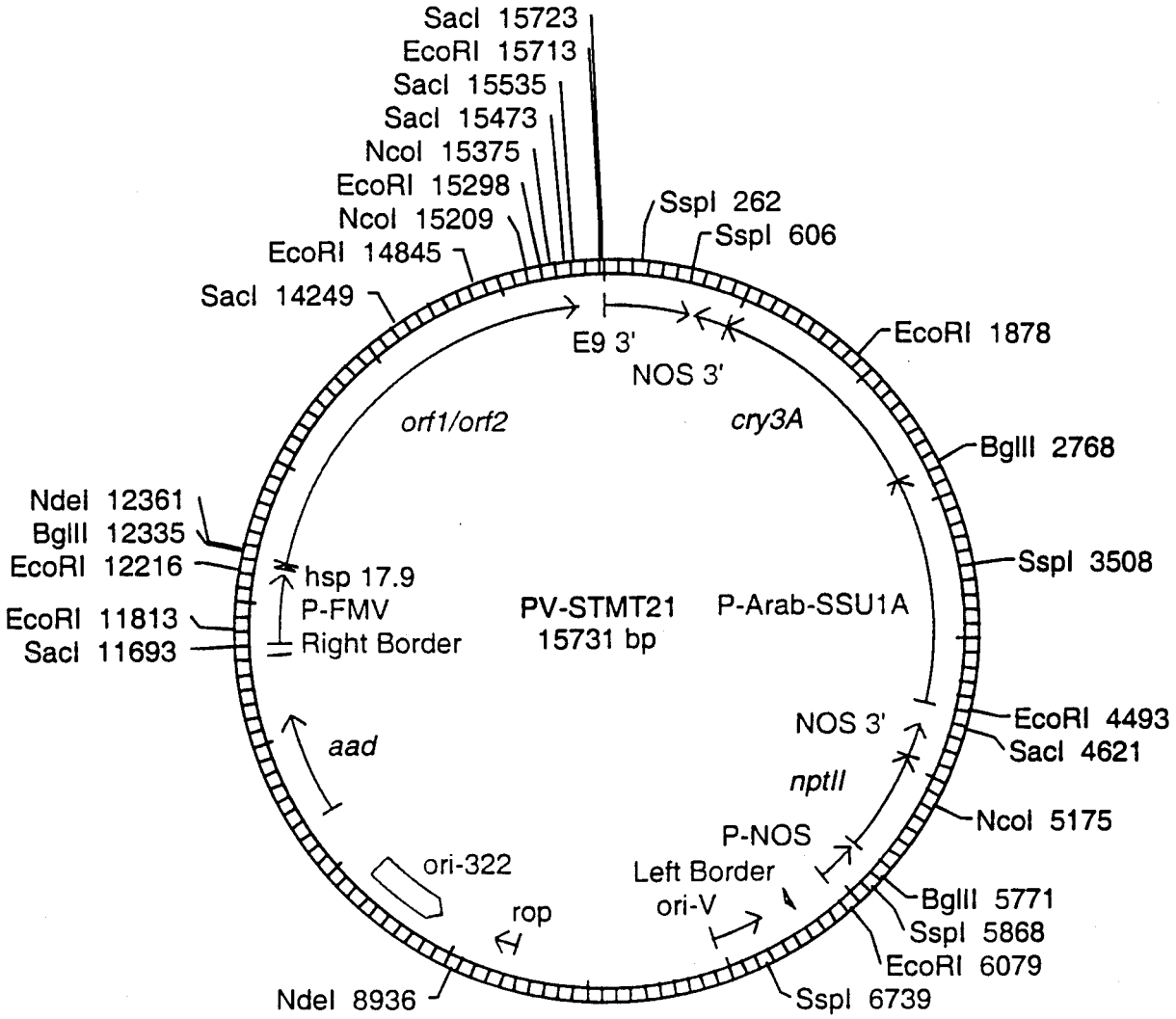

Figure V.1. Plasmid map of PV-STMT21.
This plasmid was include to aid the reviewer in interpretation of southerns as it was used as the spiked control plasmid in Figures V.5, V.6, V.8 and V.13. It was not used in the transformation of RBMT22-82, but was used to transform the previously approved lines of NewLeaf Plus potatoes. The plasmid map of PV-STMT22, which was used to transform RBMT22-82, is provided as Figure IV.1.

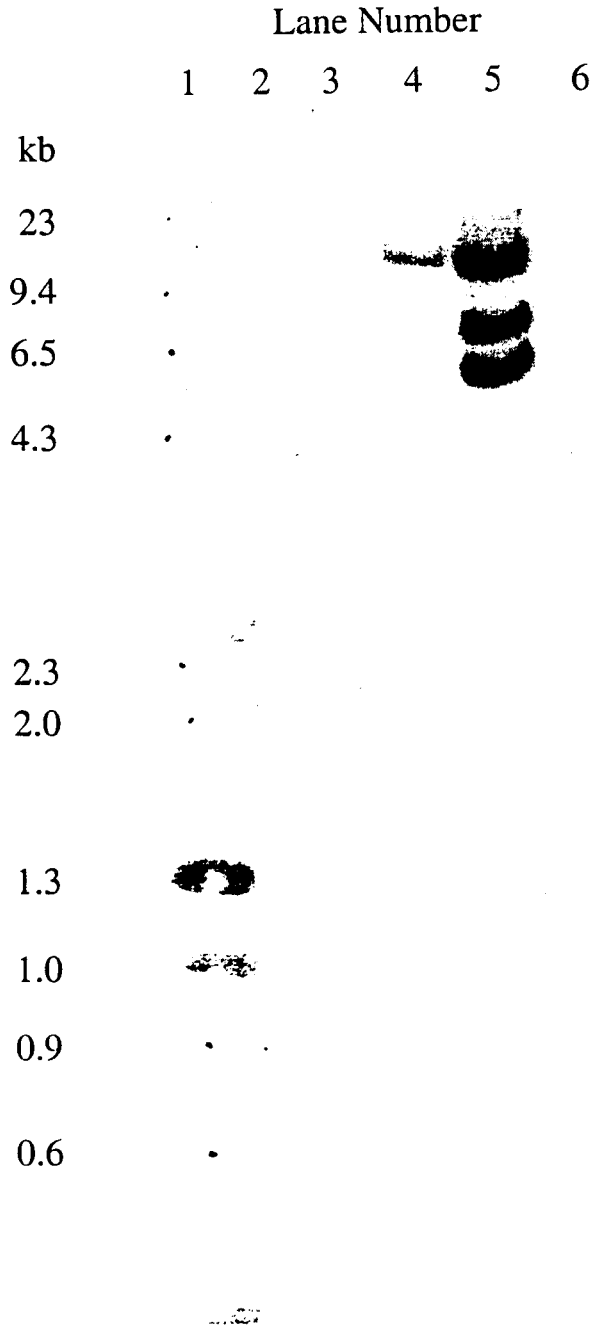

Figure V. 2. Digestion of genomic and plasmid DNA with *Nco*I; *orf1/orf2* probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with the endonuclease *Nco*I. The fragments were separated by gel electrophoresis and then transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *orf1/orf2* DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) blank, (3) genomic DNA isolated from non-transformed cv. Russet Burbank, (4) plasmid PV-STMT22 spiked into genomic DNA isolated from cv. Russet Burbank, (5) DNA isolated from NewLeaf Plus Russet Burbank Line No. RBMT22-82 and (6) blank.

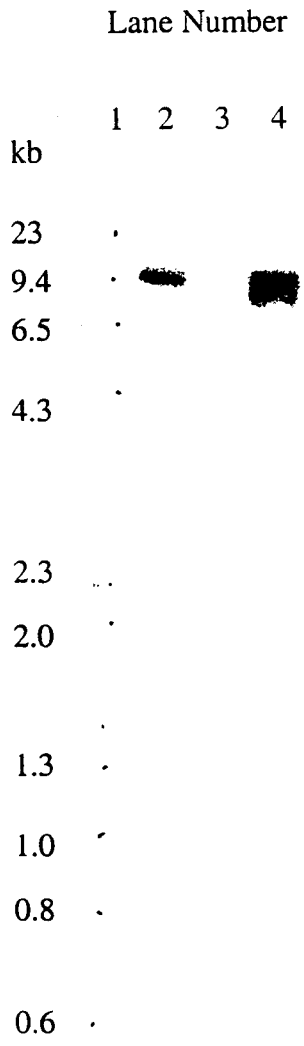

Figure V.3. Digestion of genomic and plasmid DNA with *Ssp*I; *orf1/orf2* probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Ssp*I endonuclease. The fragments were separated by gel electrophoresis and then transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *orf1/orf2* DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT22 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank, (3) genomic DNA isolated from non-transformed cv. Russet Burbank and (4) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82.

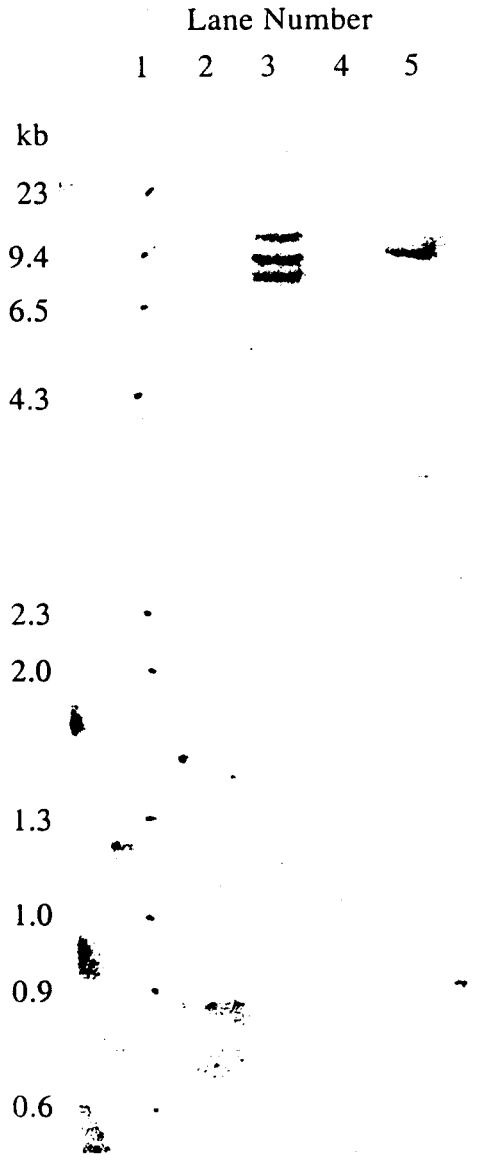

Figure V.4. Digestion of genomic and plasmid DNA with *BglII*; *CP4* EPSPS probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Bgl*II endonuclease. The fragments were separated by gel electrophoresis and then transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *CP4* EPSPS DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) blank, (3) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82, (4) DNA isolated from cv. Russet Burbank and (5) plasmid PV-STMT22 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank.

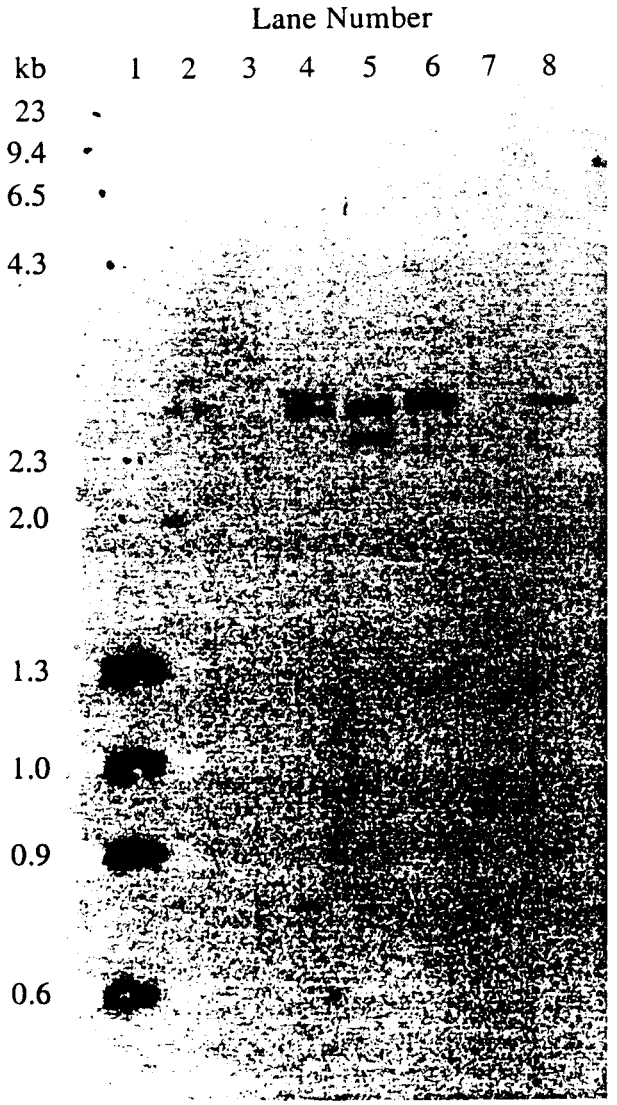

Figure V.5. Digestion of genomic and plasmid DNA with *Eco*RI; *orf1/orf2*$_{ft}$ probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT21 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Eco*RI endonuclease. The fragments were separated by gel electrophoresis and then transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *orf1/orf2*$_{ft}$ DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT21, not subject of this report, spiked into genomic DNA isolated from non-transformed cv. Russet Burbank. The plasmid, PV-STMT21, contains the *orf1/orf2* gene and produces a fragment of approximately 2.6kb when digested with *Eco*RI and probed with the *orf1/orf2* gene probe. The size of the band is the same as the predicted fragment from the plasmid, PV-STMT22, that is subject of this report. (3) genomic DNA isolated from cv. Russet Burbank, (4) genomic DNA isolated from NewLeaf Plus line not subject of this report, (5) genomic DNA isolated from NewLeaf Plus line not subject of this report, (6) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82, (7) blank and (8) plasmid PV-STMT21, not subject of this report, spiked into genomic DNA isolated from non-transformed cv. Russet Burbank.

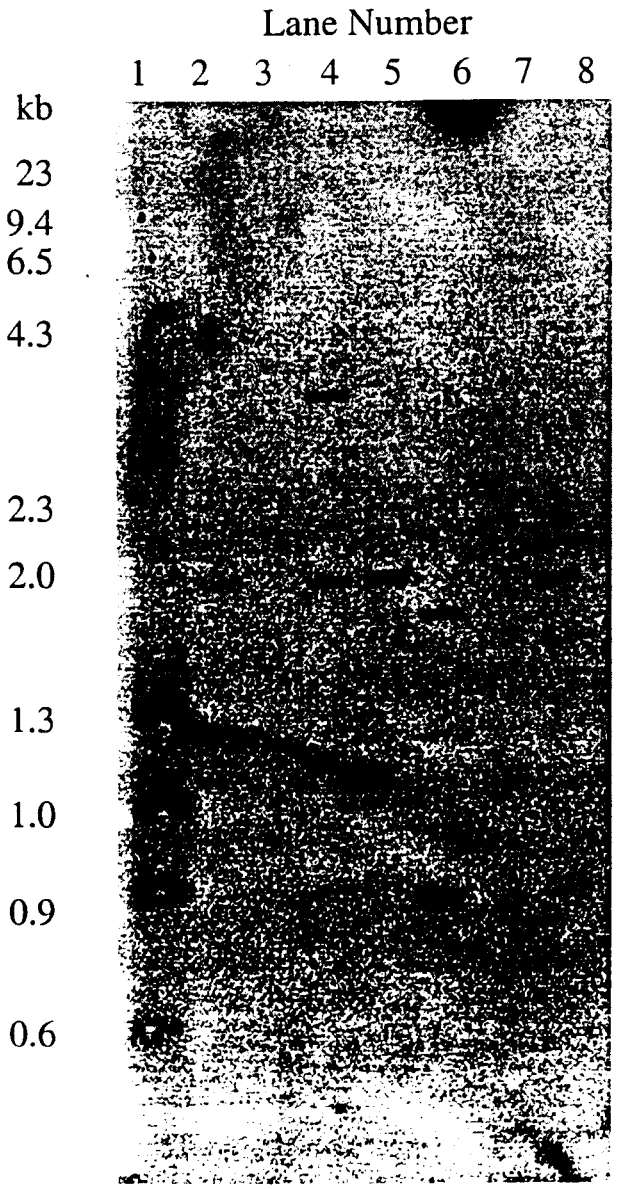

Figure V. 6. Digestion of genomic and plasmid DNA with *Eco*RI; E9 probe.
Lanes were loaded with 10 μg of genomic DNA and plasmid PV-STMT21 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Eco*RI endonuclease . The fragments were separated by gel electrophoresis and then transferred to a nylon membrane. The membranes were probed using ^{32}P labeled E9 DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT21, not subject of this report, spiked into genomic DNA isolated from non-transformed cv. Russet Burbank. The plasmid, PV-STMT21, contains the E9 terminator and produces a fragment of approximately 1.9kb when digested with *Eco*RI and probed with the E9 terminator. (3) genomic DNA isolated from cv. Russet Burbank, (4) genomic DNA isolated from NewLeaf Plus line not subject of this report, (5) genomic DNA isolated from NewLeaf Plus line not subject of this report, (6) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82, (7) blank and (8) plasmid PV-STMT21, not subject of this report, spiked into genomic DNA isolated from non-transformed cv. Russet Burbank.

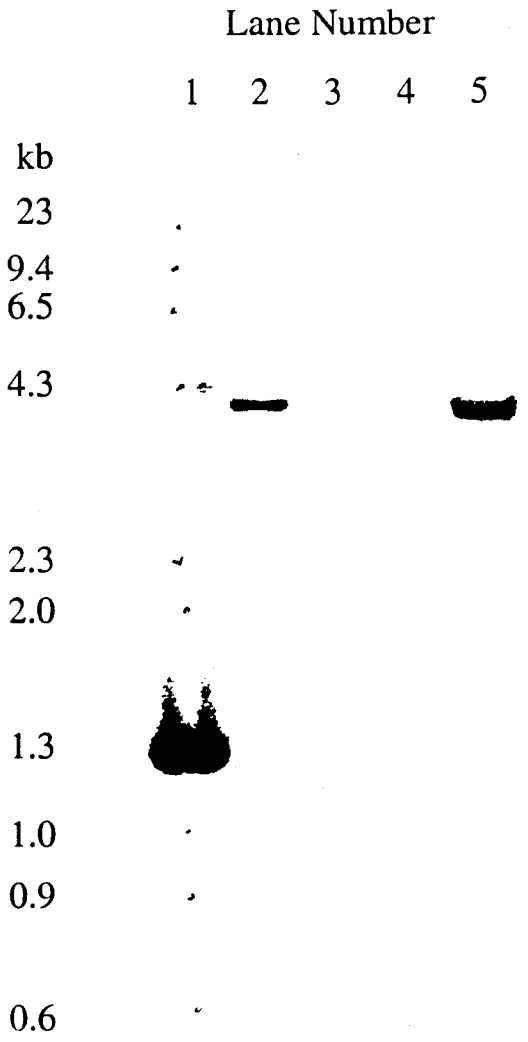

Figure V.7. Digestion of genomic and plasmid DNA with *Sac*I; *cry3A* probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Sac*I endonuclease. The fragments were separated by gel electrophoresis and then transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *cry3A* DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT22 spiked into genomic DNA isolated from cv. Russet Burbank, (3) blank, (4) DNA isolated from cv. Russet Burbank and (5) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82.

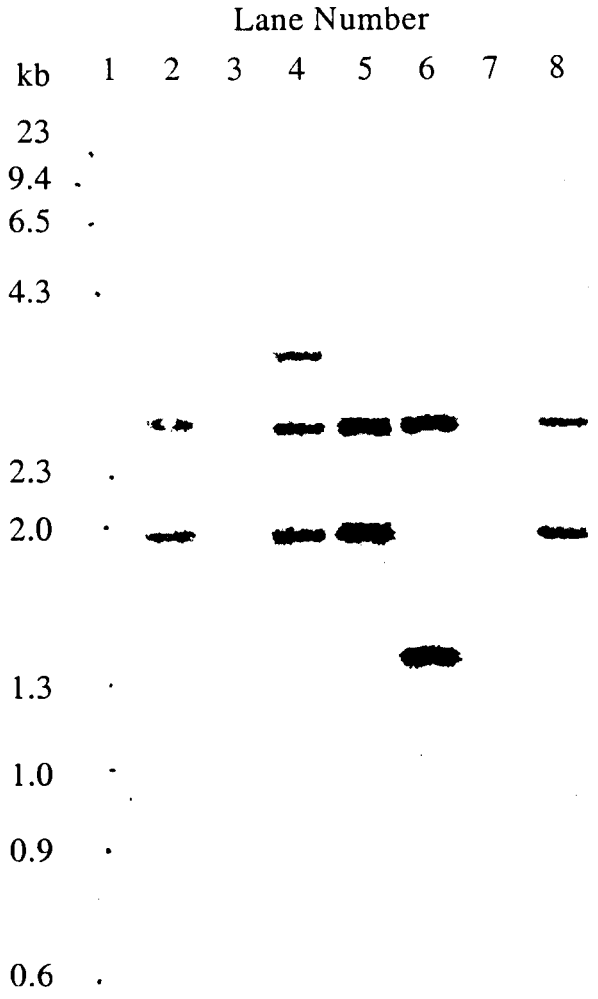

Figure V.8. Digestion of genomic and plasmid DNA with *Eco*RI; *cry3A* probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT21 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Eco*RI endonuclease. The fragments were separated by gel electrophoresis and then transferred to a nylon membrane. The membranes were probed using ^{32}P labeled *cry3A* DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT21, spiked into genomic DNA isolated from non-transformed cv. Russet Burbank. The plasmid, PV-STMT21, contains the *cry3A* and produces two fragments of approximately 1.9kb and 2.6kb when digested with *Eco*RI and probed with the *cry3A gene*. (3) genomic DNA isolated from cv. Russet Burbank, (4) genomic DNA isolated from NewLeaf Plus line not the subject of this report, (5) genomic DNA isolated from NewLeaf Plus line not subject of this report, (6) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82, (7) blank and (8) plasmid PV-STMT21, spiked into genomic DNA isolated from non-transformed cv. Russet Burbank.

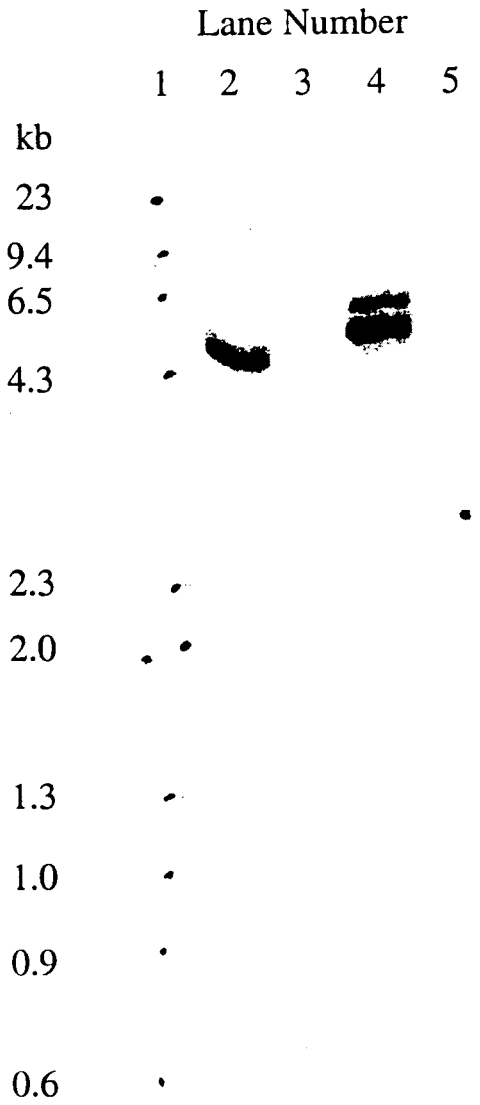

Figure V. 9. Digestion of genomic and plasmid DNA with *Ssp*I; *CP4 EPSPS* probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Ssp*I endonuclease. The fragments were separated by gel electrophoresis and transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *CP4* EPSPS DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT22 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank, (3) genomic DNA isolated from non-transformed cv. Russet Burbank, (4) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-082 and (5) blank.

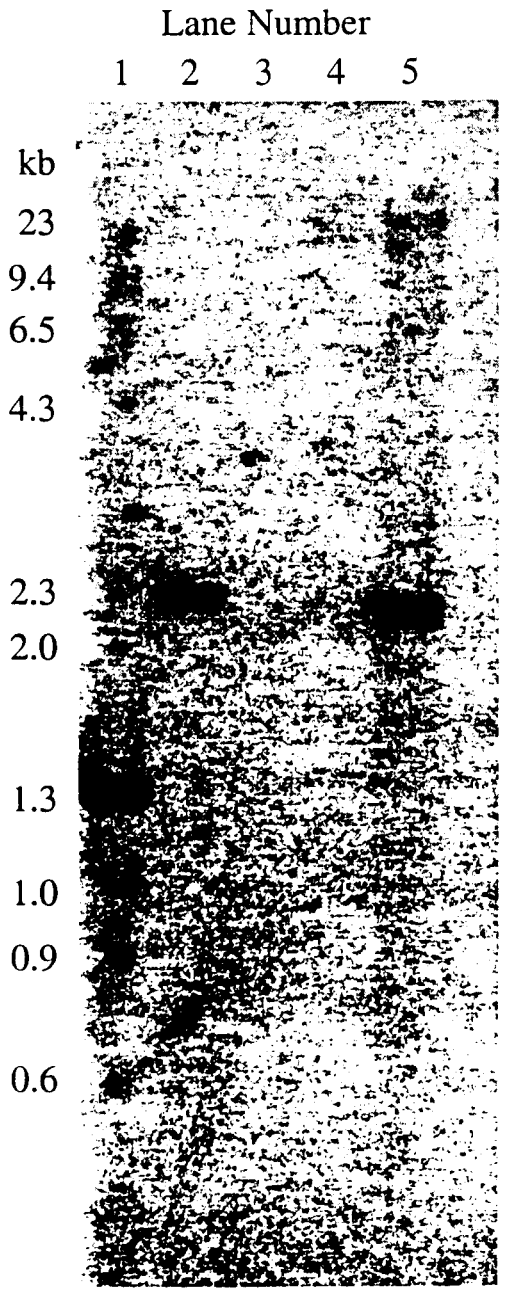

Figure V.10. Digestion of genomic and plasmid DNA with *Sac*I; *CP4 EPSPS* probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Sac*I endonuclease. The fragments were separated by gel electrophoresis and transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *CP4* EPSPS DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT22 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank, (3) blank, (4) genomic DNA isolated from cv. Russet Burbank and (5) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82.

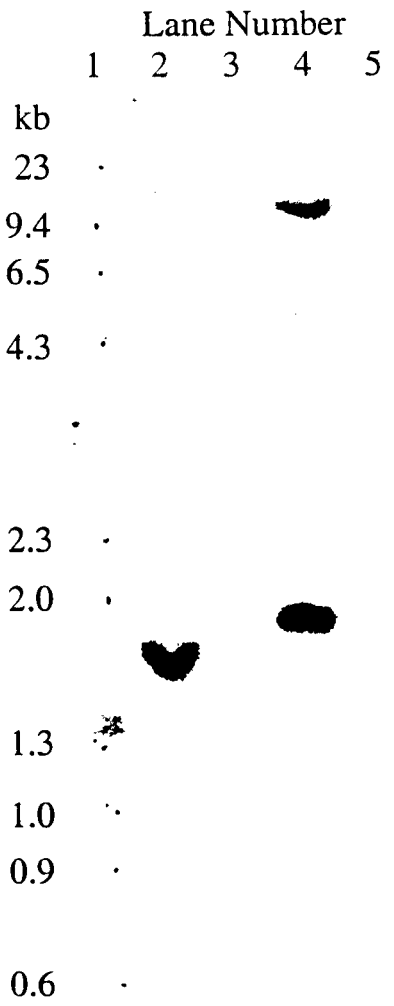

Figure V. 11. Digestion of genomic and plasmid DNA with *Eco*RI; *CP4 EPSPS* probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Eco*RI endonuclease. The fragments were separated by gel electrophoresis and transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *CP4* EPSPS DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT22 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank, (3) genomic DNA isolated from cv. Russet Burbank, (4) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82 and (5) blank.

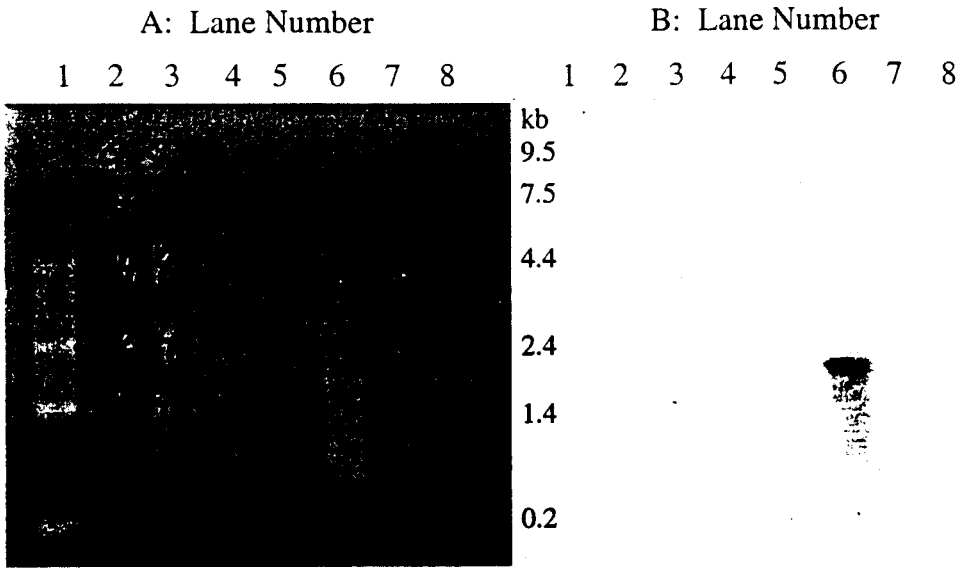

Figure V.12. Northern blot analysis of *CTP2-CP4 EPSPS* mRNA produced by NewLeaf Plus and NewLeaf Y potato varieties.

Northern blot analysis of NewLeaf Plus and NewLeaf Y genetically modified potato varieties, probed with random primer labeled *CP4 EPSPS* probe. The mRNA produced from the *CTP2-CP4 EPSPS* gene was characterized by northern blot analysis. For this analysis, leaf samples were obtained from genetically modified NewLeaf Plus potato Line No. RBMT22-082 from plants grown in the greenhouse at Monsanto's Chesterfield Village facility (Chesterfield, MO). Leaves were harvested from healthy plants. Samples were stored frozen at Monsanto at -80°C until analysis. For extraction of RNA, approximately 0.5 g of powdered leaf tissue was suspended in 10 mL of TRIZOL® Reagent (Gibco BRL, Life Technologies, Gaithersburg, MD) and total RNA was isolated from the tissue using the standard TRIZOL® protocol in accordance with the manufacturer's instructions. Poly-A mRNA was isolated from total RNA using magnetic agarose-poly-T beads in accordance with the manufacturer's instructions (Gibco BRL, Life Technologies, Gaithersburg, MD). For northern blot analysis, approximately 1 µg of mRNA was separated on an 1.2% agarose/formaldehyde gel and transferred to Hybond N (Amersham, Arlington Heights, IL) according to the method described by Sambrook *et al.* (1989). Membranes were probed with a PCR-generated fragment of the *CP4 EPSPS* gene. PCR fragments were labeled with α^{32}P using an α^{32}P-dCTP random primer kit in accordance with the manufacturers instructions (Gibco BRL, Life Technologies, Gaithersburg, MD). After hybridization, the membranes were washed in several changes of SSC buffer. The final wash was done in 0.1 X SSC + 0.1% SDS at 65°C and exposed to film for 120 hours. All exposures were done at -80°C.

Lanes contain mRNA (1 µg) isolated from genetically modified NewLeaf Plus and NewLeaf Y and non-modified potato varieties. Panel A: Poly-A mRNA electrophoresed on agarose gel; Panel B: Corresponding northern blot. Lanes were loaded with: (1) molecular weight markers, (2) blank, (3) genetically modified NewLeaf Y cv. Shepody line not subject of this report, (4) genetically modified NewLeaf Y cv. Shepody line not subject of this report, (5) genetically modified NewLeaf Y cv. Russet Burbank line not subject of this report, (6) genetically modified NewLeaf Plus Line No. RBMT22-82, (7) genetically modified NewLeaf Plus Line not subject of this report, and (8) genetically modified NewLeaf Plus Line not subject of this report. On the basis of the band pattern observed when the blot was hybridized with the *CP4 EPSPS* probe, it can be concluded that New Leaf Plus Line No. RBMT22-82 produces a full length CP4 EPSPS mRNA of the expected size (1.8 kb).

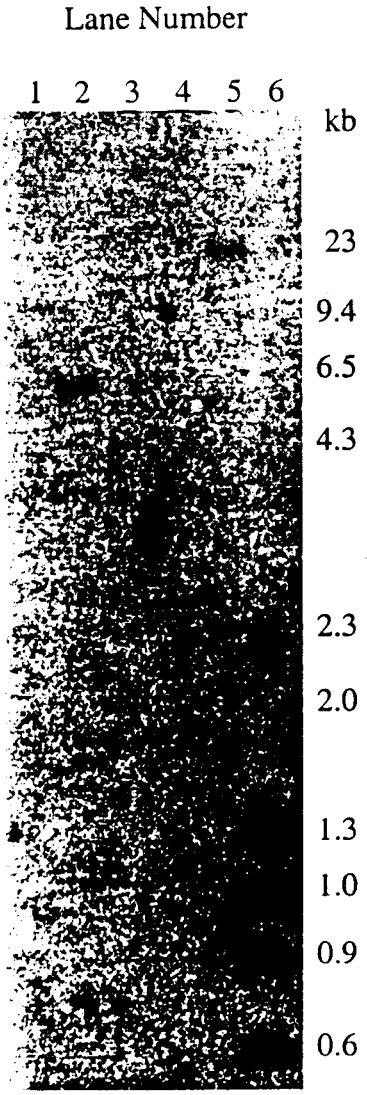

Figure V.13. Digestion of genomic and plasmid DNA with *EcoRI*; *aad* + *ori*322 + *ori*V probe. Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT21 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Eco*RI endonuclease. The fragments were separated by gel electrophoresis and transferred to a nylon membrane. The membrane was probed using combined ^{32}P labeled *ori*V, *ori*322, and *aad* DNA. Lanes were loaded with: (1) genomic DNA isolated from cv. Russet Burbank, (2) plasmid PV-STMT21 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank blank. The plasmid, PV-STMT21, contains the *aad*, *ori*-322 and *ori*-V genes and produces a fragment of approximately 6.4kb when digested with *Eco*RI and probed with the *aad*, *ori*322 and *ori*V genes. (3) genetically modified NewLeaf Plus Line not subject of this report, (4) genetically modified NewLeaf Plus Line not subject of this report, (5) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82 and (6) DNA molecular weight markers.

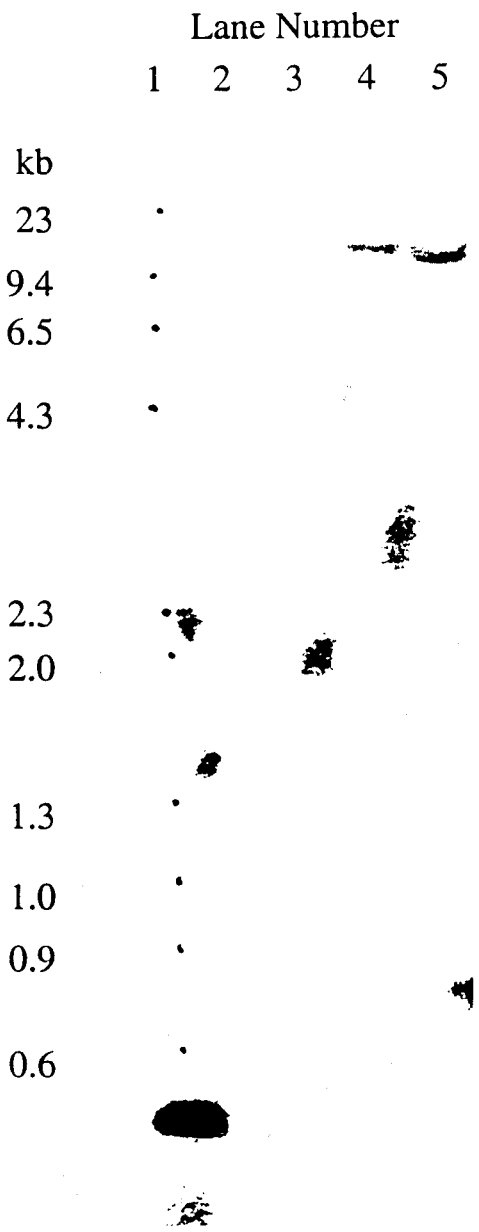

Figure V.14. Digestion of genomic and plasmid DNA with *Nco*I; *aad* + *ori*322 + *ori*V probe. Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Nco*I endonuclease. The fragments were separated by gel electrophoresis and transferred to a nylon membrane. The membrane was probed using combined ^{32}P labeled *ori*V, *ori*322, and *aad* DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) blank, (3) genomic DNA isolated from cv. Russet Burbank, (4) plasmid PV-STMT22 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank and (5) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82.

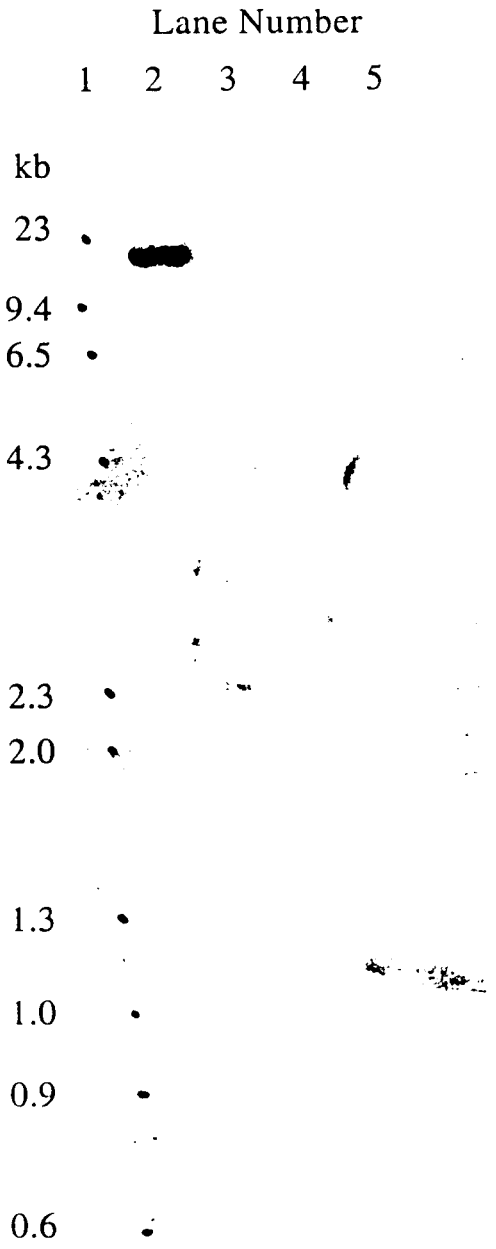

Figure V.15. Digestion of genomic and plasmid DNA with *Nde*I; *ori*V probe.
Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Nde*I endonuclease. The fragments were separated by gel electrophoresis and transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *ori*-V DNA. Lanes were loaded with: (1) DNA molecular weight markers, (2) plasmid PV-STMT22 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank, (3) genomic DNA isolated from cv. Russet Burbank, (4) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82 and (5) blank.

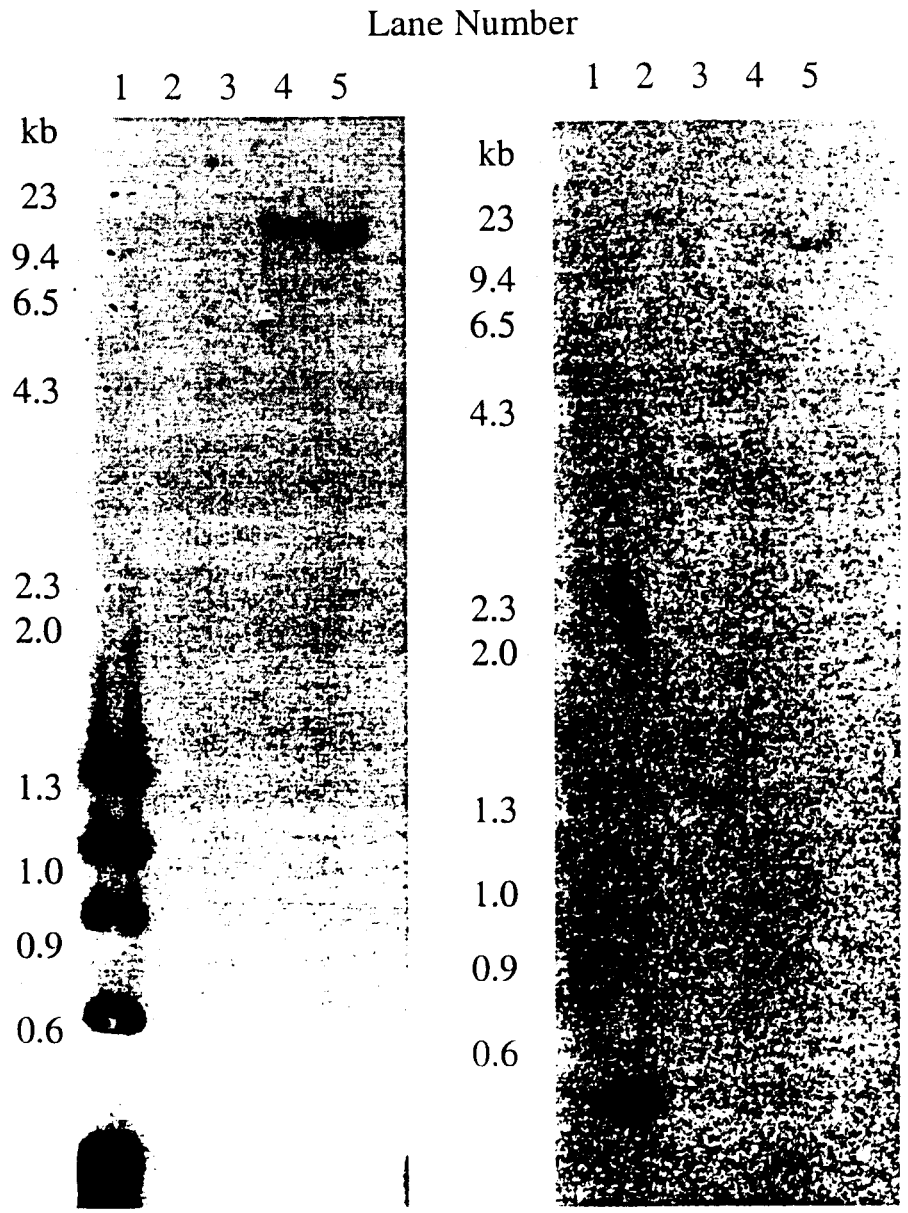

Figure V.16. Digestion of genomic and plasmid DNA with *Nco*I; Panel A - *aad* probe: Panel B - *ori*322 probe.

Lanes were loaded with 10 µg of genomic DNA and plasmid PV-STMT22 (30 pg) as a control where indicated. The DNA was subjected to digestion with *Nco*I endonuclease. The fragments were separated by gel electrophoresis and transferred to a nylon membrane. The membrane was probed using ^{32}P labeled *aad* DNA (Panel A), or *ori*322 DNA (Panel B). Lanes were loaded with: (1) DNA molecular weight markers, (2) blank, (3) genomic DNA isolated from cv. Russet Burbank, (4) plasmid PV-STMT22 spiked into genomic DNA isolated from non-transformed cv. Russet Burbank and (5) genomic DNA isolated from NewLeaf Plus Line No. RBMT22-82.

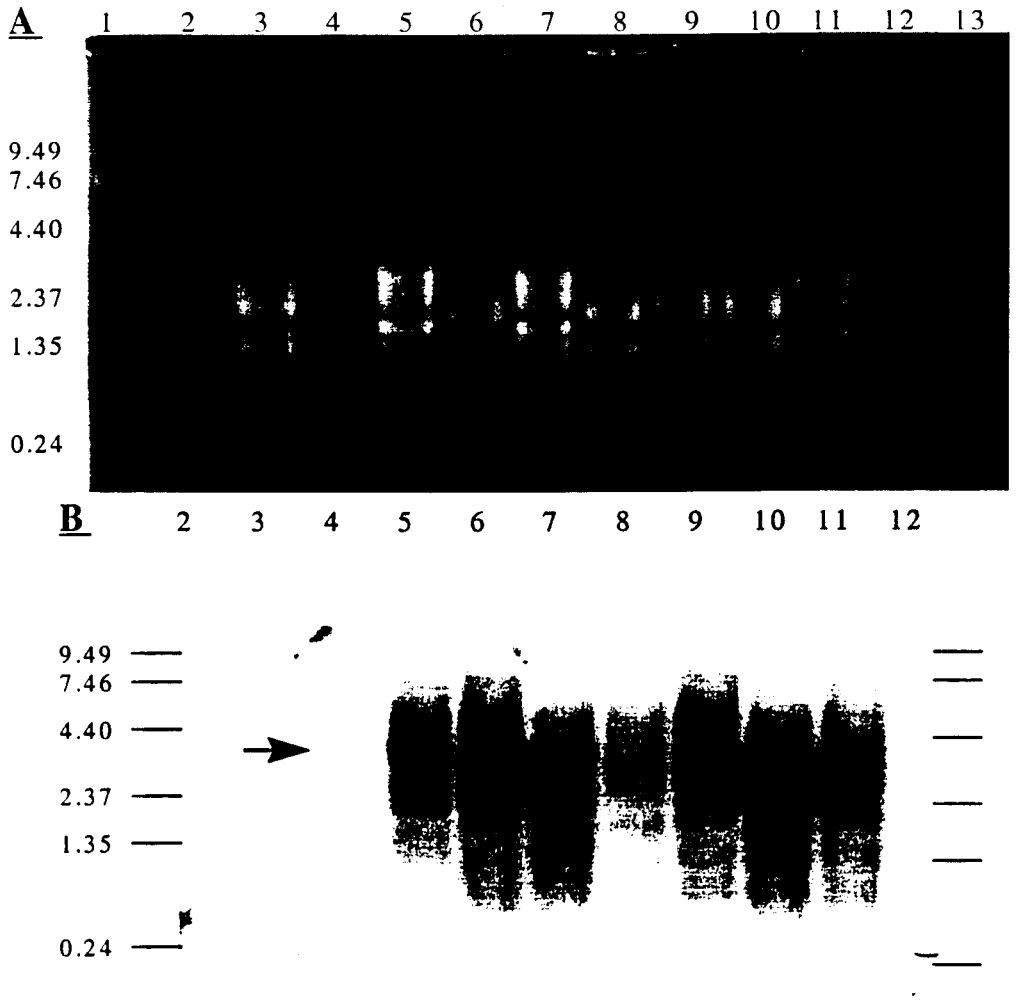

Figure V.17. Northern analysis of *PLRVrep* transgene mRNA.
For line RBMT22-82 and the Russet Burbank control, expression of the *PLRVrep* gene was analyzed using Northern hybridization for characterization. Lanes: (1) RNA molecular weight markers from BRL; (2) empty; (3) Russet Burbank control line; (4) empty; (5) **line RBMT22-82**; (6) line not subject of the petition; (7) line RBMT21-129; (8) line not subject of the petition; (9) line not subject of the petition; (10) line RBMT21-350; (11) line not subject of the petition; (12) blank; (13) RNA molecular weight markers. **Panel A**. Photograph of total plant RNA separated on an formaldehyde/agarose gel; 10 μg per well for markers, 20 μg per well for samples. **Panel B**. Total plant RNA from Panel A probed for PLRVrep mRNA. The arrow indicates the band of expected size for the full-length PLRVrep mRNA, appx. 3.8-3.9 kb.

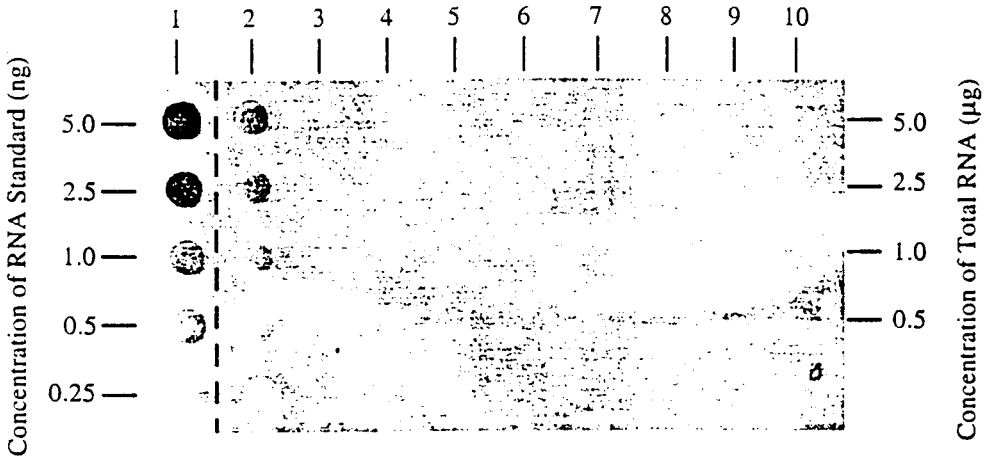

Figure V.18. Leaf PLRVrep RNA Dot Hybridization Analysis.
Lane 1 contains sense *PLRVrep* standard spiked into total RNA from control non-infected Russet Burbank plants. Lane 2 contains RNA from control Russet Burbank plants infected with PLRV. Lane 3 contains RNA from control uninfected Russet Burbank plants. Lane 7 contains RNA from transgenic line RBMT22-82. Lanes 4 and 6 contain RNA from transgenic line RBMT21-129 and RBMT21-350, respectively. Other lanes contain lines that are not the subject of the petition (lanes 5, 8-10). Total RNA from the transgenic lines was extracted from leaf tissues of field grown plants. Total RNA of infected and non-infected Russet Burbank control plants was obtained from leaf tissues of growth chamber grown plants. Standards consists of a dilution series of sense *PLRVrep* RNA spiked into total RNA extracted from leaf tissue of uninfected Russet Burbank control plants. Total RNA extracts were spotted onto charged nylon membrane, probed with fluorescein-labeled anti-sense *PLRVrep* RNA and detected with alkaline phosphatase conjugated antibody using BCIP as the substrate. Experimental details are described in Section V.A.2.

Figure V.19. Tuber PLRVrep RNA Dot Hybridization Analysis.
Lane 1 contains sense *PLRVrep* standard spiked into total RNA from control non-infected Russet Burbank plants. Lane 2 contains RNA from control Russet Burbank plants infected with PLRV. Lane 3 contains RNA from control uninfected Russet Burbank plants. Lane 7 contains RNA from transgenic line RBMT 22-82. Lanes 4 and 6 contain RNA from transgenic line RBMT21-129 and RBMT21-350, respectively. Other lanes contain lines that are not the subject of the petition (lanes 5, 8-10). RNA of the transgenic lines was extracted from tuber tissues of field grown plants. Total RNA of infected and non-infected Russet Burbank control plants was obtained from tuber tissues of growth chamber grown plants. Standards consists of a dilution series of sense *PLRVrep* RNA spiked into total RNA extracted from tuber tissue of uninfected Russet Burbank control plants. Total RNA extracts were spotted onto charged nylon membrane, probed with fluorescein-labeled anti-sense *PLRVrep* RNA and detected with alkaline phosphatase conjugated antibody using BCIP as the substrate. Experimental details are described in Section V.A.2.

B. Agronomic Performance

Potato line RBMT22-82 was evaluated in the field from 1994 - 1998 under USDA permits 93-362-01r, 94-217-02R, 94-342-01r, 96-277-01r, 97-017-03r; and notifications 98-06801n, 98-06808n, 98-06809n, 98-06810n, 98-12108n, and 98-13209n. The lines selected for commercialization, including RBMT22-82, are substantially equivalent agronomically, including weediness, to unmodified Russet Burbank potatoes, when analyzed for vigor, emergence, stem count, flowering, yield, tuber size and tubers per plant. In addition, the transgenic lines, including RBMT22-82 and those previously approved, are highly resistant to the Colorado potato beetle and the potato leafroll virus (see USDA Final Reports, submitted previously; and Certification of NewLeaf Plus Russet Burbank Potatoes in the U.S. and Canadian Seed Certification Programs in the submitted petition). During field trial evaluation of the transgenic potato lines, line RBMT22-82 was evaluated for the pests listed in the petition submission. The results of observations for these pests paralleled the results for the approved lines. Throughout the growing season, the insect pest and diseases noted varied depending on the location. Some level of mosaic disease, early blight, early dying, and canker were observed in all trial locations throughout the United States. Leafhoppers were seen but mostly in the Midwestern states (MN and WI). However, except for the intended PLRV and CPB resistance traits, no differences in susceptibility to insect pests or diseases between the transgenic lines previously approved, line RBMT22-82 and control lines were noted.

1. Control of CPB

NewLeaf Plus potatoes provide complete protection again the Colorado potato beetle without the input of synthetic insecticides. In field studies reported by Perlak et al. (1993), potatoes expressing 0.002% (appx. 0.32 ppm based on total protein level in foliage of 1.6% of fresh weight) protein as Cry3A caused 100% mortality of neonate CPB larvae. At levels of expression above 0.005% or appx. 0.8 ppm, feeding damage by adult beetles was negligible. In leaf tissue, NewLeaf Plus potato lines express between 7.7 and 40 ppm of Cry3A (see petition) and line RBMT22-82 expresses 14.97-26.61 (Table V.2.). This level is considerably beyond the level required to provide complete protection against neonate and adult CPB. As expected, Monsanto has seen no damage from CPB in any NewLeaf potato fields during the four years of commercial production. In field trials of NewLeaf Plus potatoes, line RBMT22-82 performed equally as well as previously approved lines RBMT21-129 and RBMT21-350. Monsanto has not observed any damage by CPB in any NewLeaf Plus field trials (see submitted reports for notifications and Appendix 7 of petition 97-204-01p).

2. Control of PLRV

The NewLeaf Plus potato line RBMT22-82 is highly resistant to PLRV. This transgenic line was field tested in 1994 and 1995, along with other NewLeaf Plus potato lines under consideration for commercialization at that time, two of which were approved under the submitted petition. In one set of experiments, plants were intentionally inoculated to challenge them under high virus pressure. In another experiment, the plants were challenged under natural conditions by allowing the virus to spread from adjacent potato fields.

PLRV RESISTANCE TO INTENTIONAL CHALLENGE. In the artificial challenge experiment, plants were field tested in 1994 and 1995 at the University of Idaho and Washington State University in cooperation with Dr. Thomas Mowry and Dr. Pete Thomas, respectively, for resistance to PLRV (Table V.4). Two (1994) or four (1995) replicates of ten plants of each transgenic line and the Russet Burbank parental line were planted in a randomized complete block design. Because of the number of lines tested, plants were seeded in three different plantings. Each planting had an independent Russet Burbank control. Three to four weeks after transplanting into the field, the plants were inoculated with PLRV isolate LR7 by transferring to the apex of each potato plant a small piece of PLRV-infected *Physalis floridana* leaf, which contained approximately ten aphids. After 7 days on the plants, the aphids were killed by treatment with an aphicide. Visual symptoms of PLRV infection (leaf rolling, yellowing, and stunting) were recorded six to eight weeks post-inoculation. In 1994, the plant lines were also

evaluated independently by planting a subset of all of the tubers harvested from virus challenged plants. Upon germination, the plants were assayed for PLRV by Agdia, Inc., using ELISA.

TABLE V.4. RESISTANCE OF NEWLEAF PLUS POTATO LINES TO INFECTION BY PLRV FOLLOWING INTENTIONAL CHALLENGE.

	Percent of plants with PLRV symptoms or Positive ELISA						
		1994				1995	
		Parma, ID		Prosser, WA		Parma, ID	Prosser, WA
Line	Planting Subset	Sym.	ELISA	Sym.	ELISA	Sym.	Sym.
RBMT21-129	1	0	0	0	NA	0	8
RBMT 21-152	3	5	0	0	20	3	0
RBMT 21-350	1	5	0	0	0	0	3
RBMT 22-82[1]	**2**	**5**	**11**	**0**	**0**	**0**	**3**
RBMT 22-186	2	0	0	0	7	0	0
RBMT 22-238	2	20	NA[2]	10	16	0	5
RBMT 22-262	3	0	NA	15	NA	0	3
RB 1	1	42	25	55	22	99	63
RB 2	2	87	30	34	44	NA	NA
RB 3	3	53	19	53	29	NA	NA

[1] Line that is the subject of this petition.
[2] Data not available.

PLRV RESISTANCE TO NATURAL CHALLENGE. In 1995, tubers from NewLeaf Plus line RBMT22-82, along with other lines including the two approved lines, and a Russet Burbank parental control line were planted in a randomized complete block design with eight replicates. Each plot consisted of 1 row containing 25 plants. This trial was planted adjacent to a commercial Russet Burbank potato field in Echo, OR which was moderately infected with PLRV. At harvest, 25 tuber samples were collected from each replicate of each transgenic line and control line. The tubers were then planted in Florida and the plants were assayed by seed certification specialists for symptoms of PLRV infection. This assay is used routinely by state and federal agencies to determine the percentage of PLRV infected plants in seedlots entered into the seed certification program. For the Russet Burbank control plants, 9.7+/- 1.9% showed symptoms of PLRV infections. Line RBMT22-82 did not show PLRV symptoms.

In 1996, tubers of NewLeaf line RBMT22-82, along with other lines including the two approved lines, and a Russet Burbank parental control line were planted in a randomized complete block design with seven replicates. Each plot consisted of 4 rows containing 20 plants. This trial was planted in a commercial potato growing area near Parma, ID. At harvest, 10 tubers per replicate were collected from each transgenic line and the control line. The tubers were then planted in Florida and the plants were assayed by seed certification specialists for visual symptoms typical of PLRV infection. For RBMT22-82, the assays found no evidence of PLRV infection. In the control line, 60% of the plants showed symptoms typical of PLRV infection. In addition, when a subsample (50-100 tubers per line) of stored tubers from this trial were assayed visually for net necrosis in January 1997, the non-transgenic controls had 11.7% of tubers showing net necrosis while RBMT22-82 tubers had none.

In conclusion, RBMT22-82 consistently showed a high level of resistance, but not immunity to PLRV. As shown in Table V.4, when the plants are exposed to very high levels of virus by artificial infestation, a percentage of the plants can become infected and exhibit symptoms similar to those of infected non-transgenic control plants. These plants show symptoms typical of systemic PLRV infection: leaf rolling, yellowing and stunting. They also tested positive for PLRV using ELISA. In winter grow-out tests from tubers collected from field trials conducted in 1996, RBMT22-82 did not exhibit evidence of PLRV infection. Therefore, as evident from the reduced incidence of PLRV infection and net necrosis, RBMT22-82 is highly resistant to PLRV.

C. Compositional Analysis

Monsanto consulted with FDA on the compositional analysis of potato tubers produced from this transformed line. FDA agreed with our assessment. Our data demonstrates that the potato tubers are substantially equivalent to non-engineered Russet Burbank potatoes with regard to nutritional composition (total solids, sugars, glycoalkaloid, and vitamin C) and proximate composition (soluble protein, moisture, total fat, ash, crude fiber, carbohydrates, and calories).

Glycoalkaloid determination was carried out by a procedure based on methods described by Bergers (1980) which measures total amount of solanines and chaconines. A single analysis was performed per sample. Glycoalkaloid level is reported as total milligrams of solanines and chaconines per 100 g fresh tuber weight (Table V.5.).

TABLE V.5. GLYCOALKALOID CONTENT OF TUBERS FROM NEWLEAF PLUS AND RUSSET BURBANK CONTROL LINES

Combined data from tubers grown in 1996 at three locations in Canada (Spruce Grove, Alberta; Winkler, Manitoba; and New Denmark, New Brunswick). For each line, the data includes analyses of four replicates per site.

Line	Total Glycoalkaloids, mg/100g FW
RBMT22-82	9.0
RB Control	8.3
Literature Reported Ranges	3 to 39[1,2]

[1]Pavek *et al.*, 1980-1992
[2]Sinden and Webb, 1972

VI. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION

NewLeaf Plus potatoes are resistant to the Colorado Potato Beetle and the Potato Leaf Roll Virus. The ecological consequences of both traits were discussed in the submitted petition (97-204-01p). Line RBMT22-82 is the same in its resistance to these pests as the lines previously approved under the conditions outlined in the submitted petition.

A. Overall Safety and Impact on Non-target Organisms

Data on genes and gene expression (Section V) show that the inserted genes in line RBMT22-82 function as expected and thus this line, since it is similar to the approved lines, is unlikely to 1) exhibit plant pathogenic properties, 2) become a weed, 3) increase the weediness potential for any other cultivated or wild species with which they can interbreed, 4) cause damage to raw or processed agricultural commodities, or 5) harm threatened or endangered species or other organisms, such as bees, that are beneficial to agriculture, or have an adverse impact on the ability to control non-target insect pests.

As an update to information on regulatory reviews provided in the submitted petition 97-204-01p, the PLRV replicase protein has been evaluated by EPA and has been granted an Exemption from the Requirement of a Tolerance in all crops and raw agricultural commodities (EPA 1997). The EPA has registered as a plant pesticide, the PLRV resistance gene and genetic material for its expression (EPA 1998). Monsanto consulted with FDA on the nutritional qualities of the potatoes from line RBMT22-82 and FDA agreed with Monsanto's conclusions (FDA

1998). Data show that the potatoes of the approved lines and RBMT22-82 are substantially equivalent to non-engineered Russet Burbank potatoes.

VII. ADVERSE CONSEQUENCES OF INTRODUCTION AND CONCLUSIONS

No adverse consequences are expected from the introduction of line RBMT22-82 based on its conformance to the product identity standards specified herein.

In summary, NewLeaf Plus potato line RBMT22-82 and the approved lines demonstrated no significant differences over non modified Russet Burbank potatoes apart from the intended change. There were no morphological differences between RBMT22-82 and the two approved lines (RBMT21-129 and RBMT21-350) and no differences relating to survival or propagation. The benefits of introduction of commercial lines of NewLeaf Plus potatoes are provided in the submitted petition.

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99-173-01p
Addendum 2

MONSANTO
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MONSANTO COMPANY
700 CHESTERFIELD PARKWAY NORTH
ST. LOUIS, MISSOURI 63198
PHONE (314) 694-1000
http://www.monsanto.com

September 17, 1999

Coordination, and Technical Assistance,
Biotechnology, and Scientific Services,
Plant, Protection, and Quarantine,
Animal and Plant Health Inspection Service,
U.S. Department of Agriculture
4700 River Road
Riverdale, MD 20737

Attention: Dr. James White

Subject: Application for Extension of a Determination of Nonregulated Status - NewLeaf ®Plus Potatoes: Petition 99-173-01p (PLRV + CPB resistant potatoes)

Attached are three copies of Addendum 2 for the above referenced petition. Included in Addendum 2 are the following:

1. Additional information to be added to the information on page 5 of the petition which is a description of the event.
2. Summaries of most recent data which demonstrate that these potato plants are resistant to the pest CPB and to net necrosis (page 39 and page 40 of the petition).
3. Descriptions of the preparation of the probes described on page 11 of the petition.

Please phone me or email me if you need additional information to complete your review.

Signature

Elizabeth D. Owens, Ph.D.
Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway North
BB1K
St. Louis, MO 63198
Phone: 314-737-5721
FAX: 314-737-7085
Email: Elizabeth.D.Owens@monsanto.com

Attachments : 3 copies of Addendum 2 to petition 99-173-01p

9/20/99

Petition 99-173-01p (PLRV + CPB resistant potatoes)

Addendum 2

Date:

September 17, 1999

Submitted by:

Monsanto Company

Document No.: 99-246-U (Addendum 2)

Submitter:
Elizabeth D. Owens, Ph.D.
Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway North
BB1K
St. Louis, MO 63198
Phone: 314-737-5721
FAX: 314-737-7085
Email: Elizabeth.D.Owens@monsanto.com

9/20/99

Included in Addendum 2 to Petition 99-173-01p are the following:

Tab:	Information
1.	Additional information to be added to the information on page 5 of the petition which is a description of the event.
2.	Summaries of most recent data which demonstrate that these potato plants are resistant to the pest Colorado Potato Beetle and to net necrosis (page 39 and page 40 of the petition).
3.	Descriptions of the preparation of the probes described on page 11 of the petition.

Tab 1. **Additional information to be added to the information on page 5 of the petition which is a description of the event.**

The following paragraph is to be added to page 5 to complete the description of the event (revised pages 5-7 are also included herein for substitution within the original petition if needed):

- The right border in plasmid PV-STMT22 is from the *Pvu*I to *Bcl*I restriction fragment from the pTiT37 plasmid containing the nopaline-type T-DNA right border region (Depicker *et al.*, 1982) The left border in plasmid PV-STMT22 is from the restriction fragment of the octopine Ti plasmid, pTi15955, containing the 24 bp T-DNA left border used to terminate the transfer of the T-DNA from *Agrobacterium tumefaciens* to the plant genome (Barker *et al.*, 1983). For transfer of the DNA by *Agrobacterium* transformation, the plasmid DNA is nicked between the nucleotides 3 and 4 of the borders; therefore, it is expected that each transformed line contains some of the border sequences (Albright *et al.*, 1987; Wang *et al.*, 1987).

The following are additional references cited:

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I. RATIONALE FOR THE SUBMISSION OF A REQUEST FOR EXTENSION FOR NEWLEAF® PLUS POTATOES

There are no changes in the rationale from the previously approved petition number 97-204-01p, used to support deregulation of two other lines (USDA 1998). The specific genetic elements of the new line are summarized below and discussed in the appropriate sections of this application. The only difference between NewLeaf Plus line RBMT22-82 and the previously approved lines is that the plasmid used to transform this line contains the chimeric gene for selection of transformed plant cells on media containing a herbicide, glyphosate.

Information on the potato plant line derived from one specific independent transformation event is presented in this petition. The parental potato line RB1, of the Russet Burbank variety, was transformed with PV-STMT22 to insert the DNA between the left and right border of the *Agrobacterium tumefaciens* Ti plasmid (Klee and Rogers, 1989).

This plant line (Line No. RBMT22-82) contains the following genetic elements:

- The right border in plasmid PV-STMT22 is from the *PvuI* to *BclI* restriction fragment from the pTiT37 plasmid containing the nopaline-type T-DNA right border region (Depicker *et al.*, 1982) The left border in plasmid PV-STMT22 is from the restriction fragment of the octopine Ti plasmid, pTi15955, containing the 24 bp T-DNA left border used to terminate the transfer of the T-DNA from *Agrobacterium tumefaciens* to the plant genome (Barker *et al.*, 1983). For transfer of the DNA by *Agrobacterium* transformation, the plasmid DNA is nicked between the nucleotides 3 and 4 of the borders; therefore, it is expected that each transformed line contains some of the border sequences (Albright *et al.*,1987; Wang *et al.*, 1987).
- The chimeric gene for selection of transformed plant cells on glyphosate (FMV/*CP4 EPSPS*/E9 3') which consists of the 35S promoter region of the Figwort mosaic virus (FMV) (Richins *et al.*,1987), the *CP4 EPSPS* gene which encodes the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (Barry *et al.*, 1992) and the nontranslated 3' region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) referred to as E9 3' (Coruzzi *et al.*, 1984). . Results from Southern blot analysis indicate that two copies of T-DNA contain an intact FMV/*CTP2-CP4 EPSPS* coding region, and that insertion of the third copy of T-DNA resulted in a less than full length copy of the FMV/*CTP2-CP4 EPSPS* coding region in which the FMV promoter and *CTP2* coding region are intact, and that a predicted truncation occurs somewhere downstream in the *CP4 EPSPS* coding region
- The chimeric gene responsible for the control of CPB (AraSSU1A/*cry3A*/nos 3') which consists of the *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase small subunit ats1A promoter (Almeida *et al.*, 1989; Wong *et al.*, 1992), the *cry3A* gene which encodes the Cry3A protein (McPherson *et al.*, 1988; Perlak *et al.*, 1993) and the nontranslated 3' region of the nopaline synthase gene referred to as NOS 3' (Depicker *et al.* 1982; Bevan *et al.*, 1983).
- The chimeric gene responsible for control of PLRV (FMV/*PLRVrep*/E9 3') which consists of the 35S promoter region of FMV (Richins *et al.*,1987), the full-length ORF1 and ORF2 (hereafter referred to collectively as *PLRVrep)* from a naturally occurring PLRV isolate (Miller *et al.*, 1995; Murphy *et al.*, 1995; Van der Wilk *et al.*, 1989), the PLRV intergenic region and the nontranslated 3' region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) referred to as E9 3' (Coruzzi *et al.*, 1984).
- Backbone sequence inserted in this line includes the *aad* gene (bacterial selectable marker), and the bacterial origin of replication, *ori*-322. The inserted *aad* gene is the coding region for TN7 adenylyltransferase conferring spectinomycin or streptomycin resistance in *E. coli* (Fling *et al.*, 1985). The *ori*-322 segment (rop) is a segment of pBR322 which provides the origin of replication for maintenance of the PV-STMT22 plasmid in *E.coli* and *bom* site for conjugational transfer into *Agrobacterium* (Bolivar *et al.*, 1977; Sutcliffe, 1978). Because the *aad* gene is under the control of a bacterial promoter, no AAD protein was expected to be produced in the line. This expectation was confirmed from results using an AAD specific ELISA.

Field experiments with this transgenic potato line were conducted beginning in 1994 under permits/notifications from the USDA (93-362-01r, 94-217-02R, 94-342-01r, 96-277-01r, 97-017-03r, 98-06801n, 98-06808n, 98-6809n, 98-06810n, 98-12108n, and 98-13209n). The final reports for these permits/notifications are on file with USDA. This petition provides information to demonstrate that the modified potato plants do not present a plant pest risk and are not otherwise deleterious to human health or the environment.

II. THE POTATO FAMILY

OECD compiled a report on the biology of the potato (OECD 1997). This is a Consensus Document on the Biology of *Solanum tuberosum* subsp. *tuberosum* in the OECD Series on the Harmonization of Regulatory Oversight in Biotechnology. The document, prepared by the Netherlands as the lead country in collaboration with the United Kingdom, addresses the biology of the crop plant *Solanum tuberosum* subsp. *tuberosum* and contains information for use during the regulatory assessment of potato products. This supersedes the information provided in the petition submission. The cultivar, Russet Burbank, is the same as in the petition submission.

III. TRANSFORMATION SYSTEM

The line RBMT22-82 was transformed with a different plasmid than the previously approved lines. The transgenic Russet Burbank potato line was developed by transforming one Russet Burbank parental line (RB1) with the PV-STMT22 vector using the *Agrobacterium tumefaciens* transformation system.

The transformation vector PV-STMT22 was created and used in transformation of potato using the methods as described in the submitted petition with the plant selection media used being one containing glyphosate instead of kanamycin.

IV. THE DONOR GENES AND REGULATORY SEQUENCES

The line RBMT22-82 was transformed with a different plasmid than the previously approved lines. Genetic elements contained in the vector PV-STMT22 (Figure IV.1) are summarized in Table IV.1. For clarity, please note that when reference is made to "replicase" in the context of the transgene or its product(s), this refers to PLRV ORF1 and ORF2 which are thought to encode a fusion protein having both helicase and a RNA-dependent RNA polymerase activity, respectively.

A. Construction of the Plant Vector

The plant transformation vector used in the generation of transformed Russet Burbank potatoes was PV-STMT22. Plant vector PV-STMT22 employs the *CP4 EPSPS* gene which allows selection of transformed plants in a medium containing glyphosate.

PV-STMT22 is a double border binary transformation vector, which is described in the submitted petition. The genes contained in the vector are described below.

Three chimeric genes, with signals for plant expression, were introduced between the right and left border regions of the plant transformation vector. For vector PV-STMT22, the chimeric genes and their components are as follows. The chimeric gene for selection of transformed plant cells on glyphosate (FMV/*CP4 EPSPS*/E9 3') which consists of the 35S promoter region of the Figwort mosaic virus (Richins *et al.*, 1987), the *CP4 EPSPS* gene which encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (Barry *et al.*, 1992) and the nontranslated 3' region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) referred to as E9 3' (Coruzzi *et al.*, 1984). The chimeric gene responsible for the control of CPB (AraSSU1A/*cry3A*/NOS 3') which consists of the

A. thaliana ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit ats1A promoter (Almeida *et al.*, 1989; Wong *et al.*, 1992), the *cry3A* gene which encodes the Cry3A protein (McPherson *et al.*, 1988; Perlak *et al.*, 1993), and the nontranslated region of the 3' region of the nopaline synthase gene referred to as NOS 3' (Depicker *et al.*, 1982; Bevan *et al.*, 1983). The chimeric gene responsible for control of PLRV (FMV/*PLRVrep*/E9 3') which consists of the 35S promoter region of the Figwort mosaic virus (Richins *et al.*, 1987), the full length *PLRVrep* gene from a naturally occurring PLRV isolate (Miller *et al.*, 1995; Murphy *et al.*, 1995; van der Wilk *et al.*, 1989) and the nontranslated 3' region of the pea small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) referred to as E9 3' (Coruzzi *et al.*, 1984).

B. Description of the Inserted Genes

1. *Cry3A* gene and its encoded Cry3A *protein*

This gene is the same as described in the petition submission.

2. *PLRVrep* gene

This gene is the same as described in the petition submission.

3. *CP4 EPSPS* gene and its encoded CP4 EPSPS protein

The *CP4 EPSPS* gene, present in plant vector PV-STMT22, was isolated from *Agrobacterium* sp. strain CP4 (Barry *et al.*, 1992). The *CP4 EPSPS* gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation (Howe *et al.*, 1992). The *CP4 EPSPS* gene encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in all plants, bacteria and fungi (Levin and Sprinson, 1964). The aromatic amino acid pathway is not present in mammalian metabolic pathways (Cole, 1985). EPSPS enzyme is inhibited by the herbicide glyphosate (N-phosphonomethylglycine). Plant cells treated with glyphosate are unable to synthesize the aromatic amino acids essential for protein synthesis which result in death of the plant cells (Barry *et al.*, 1992). In contrast, CP4 EPSPS enzyme is not inhibited by glyphosate. Thus, insertion of the *CP4 EPSPS* gene in a plant cell results in plant cells which are tolerant to glyphosate. The purpose of inserting the *CP4 EPSPS* gene into potato cells with the *cry3A* and *PLRVrep* genes is to have an effective method of selecting cells that contain the insect and viral resistant genes using glyphosate as the selective agent. Consequently, cells selected for plant regeneration contain the *CP4 EPSPS*, *cry3A* and *PLRVrep* genes.

4. *AAD* and *ORI-322* genes

Backbone sequence in this T-DNA insertion includes the *aad* gene (bacterial selectable marker), and the bacterial origin of replication, *ori*-322. The inserted *aad* gene is the coding region for TN7 adenylyltransferase conferring spectinomycin or streptomycin resistance in *E. coli* (Fling *et al.*, 1985). The *ori*-322 segment (rop) is a segment of pBR322 which provides the origin of replication for maintenance of the PV-STMT22 plasmid in *E.coli* and *bom* site for conjugational transfer into *Agrobacterium* (Bolivar *et al.*, 1977; Sutcliffe, 1978). Because the *aad* gene is under the control of a bacterial promoter, no AAD protein was expected to be produced in the line. This expectation was confirmed from results using an AAD specific ELISA.

Tab 2. Summaries of most recent data which demonstrate that these potato plants are resistant to the pest CPB and to net necrosis (page 39 and page 40 of the petition).

CPB: In the petition, a statement is made on page 39 that "NewLeaf Plus potatoes provide complete protection against the Colorado potato beetle without the input of synthetic insecticides". Included in this tab are summaries of research conducted in 1998 and 1999 that support this statement within the petition. The summaries are as follows:

1. Cry3A Expression Levels of NewLeaf® Plus Potato Foliage and Colorado Potato Beetle Feeding Through the 1998 Growing Season
2. Comparison Study of Colorado Potato Beetle Control Methods Under Commercial Conditions in Wisconsin During the 1999 Growing Season

Net Necrosis: In the petition, a statement is made on page 40 that "Therefore, as evident from the reduced incidence of PLRV and net necrosis, RBMT22-82 is highly resistant to PLRV". Included in this tab is the summary of research conducted in 1997 and 1998 that support this statement relative to the symptoms known as "net necrosis". The summary is as follows:

1. Summary of 1997 and 1998 Research on the Development of Net Necrosis in NewLeaf ® Plus Line RBMT22-82

Cry3A Expression Levels in NewLeaf® Plus Potato Foliage and Colorado Potato Beetle Feeding Through the 1998 Growing Season

NewLeaf potato plants were genetically modified by plant transformation techniques to contain the Cry3A gene from *Bacillus thuringiensis* subsp. *tenebrionis* (Perlak, *et al.*, 1993). Data derived from product characterization studies, agronomic line selection trials and biological assays show that NewLeaf Plus Line No. RBMT22-082 provides season-long control of the Colorado Potato beetle.

In 1998, an over the season Cry3A protein expression study was completed on NewLeaf Plus which showed that levels were consistently above that necessary for complete control of feeding (Bookout and Dodson 1998). For determining Cry3A protein levels in leaves, leaf tissue was obtained from field trials conducted in 1998 in New Maryland, NB Canada, and Saint Croix de Lotbiniere, QB, Canada. Samples were collected at 6, 10 and 14 weeks post planting and immediately frozen on dry ice. Samples were shipped on dry ice to Monsanto Co. (Chesterfield, MO) and stored at approx. -80°C until tissue processing. Cry3A protein levels in leaf tissue were determined using an Enzyme Linked Immunosorbent Assay (ELISA) method (Dean, 1993; Grace, 1996).Leaf samples were taken from field grown CPB/PLRV resistant Russet Burbank lines RBMT22-82, RBMT21-129, and RBMT21-350. .

Results from this study showed that NewLeaf Plus potato plants produced an efficacious level of the Cry3A protein over the course of the growing season. The mean expression levels of Cry3A protein in NewLeaf Plus lines were 13.90, 14.56, and 12.83 μg/g tissue fresh weight at 6, 10, and 14 weeks post-planting, respectively. The means for line RBMT22-82 were higher than the overall means for all NewLeaf Plus lines (Table 1). When calculated on a percent leaf protein basis, the Cry3A protein levels in line RBMT22-82 ranged from 0.07 to 0.20% of total leaf protein in this study (potato total leaf protein level is 1.6% of tissue fresh weight), well above the 0.002% reported in Perlak *et al.*, 1993 as necessary to control CPB feeding.

A parallel efficacy study conducted in 1988 demonstrated that CPB did not damage plant foliage. Field data from an efficacy trial conducted for Canadian variety registration further support that line RBMT22-82 is protected from CPB feeding. In 1998, line RBMT22-82 was compared to non-transformed Russet Burbank (control) for CPB damage over the season at the New Maryland, New Brunswick site, the field site from which leaf samples were taken for determining season long expression of Cry3a protein. In the trial, there were four replicates of 18 foot rows for each treatment which were

not sprayed with insecticides, but otherwise husbanded according to commercial practices for the area. At four times during the growing season, all treatment plots were visually rated for CPB feeding damage based on a scale of zero (no defoliation) to 7 (75 - 90% of plants with at least 1 stem with >50% defoliation) (Boiteau, 1994). Beetle populations overall were low, but all four replicates of the control sustained a low level of injury (1-2). There was no visible feeding damage to line RBMT22-82 (0) (Boiteau, 1998).

Perlak *et al.* (1993) demonstrated that a low level of plant expressed Cry3A protein is sufficient to control feeding by all stages of the Colorado Potato Beetle (CPB). Levels above 0.002% of total protein in leaf tissue were sufficient to essentially stop CPB feeding. As a result of this observation, all genetically modified potato lines developed by NatureMark undergo ELISA analyses prior to field testing to assure that the level of the Cry3A protein is above a pre-determined efficacious cut-off level. Analysis of the Cry3A concentration in leaf tissue by ELISA and field observations of feeding damage by CPB in these current studies continue to support the strong correlation between the level of the Cry3A protein produced by the genetically modified plant and the biological activity (insecticidal efficacy) of NewLeaf potato plants. On the basis of these current and previously conducted studies, it is concluded that NewLeaf Plus Line No. RBMT22-082 provides season long control of CPB through high dose expression of biologically active Cry3A protein.

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Table 1. Evaluation of Cry3A protein expression in Newleaf® varieties at three times post planting.

		µg/g Tissue Fresh Weight		
Sample ID	**Site**	**6 weeks**	**10weeks**	**14weeks**
Newleaf Plus (CPB/PLRV)				
RBMT21-129	NM[1]	8.84	7.19	6.41
RBMT21-350	NM	14.62	9.63	4.51
RBMT22-82	**NM**	**18.22**	**22.97**	**13.21**
RBMT21-129	QB[2]	NA[3]	7.25	7.48
RBMT21-350	QB	NA	29.58	19.98
RBMT22-82	**QB**	**NA**	**10.73**	**25.36**

[1]**NM** = New Maryland, New Brunswick Canada field site.
[2]**QB** = Saint Croix de Lotbiniere, Quebec, Canada field site.
[3]Six week samples from this site could not be obtained.

Comparison Study of Colorado Potato Beetle Control Methods Under Commercial Conditions in Wisconsin During the 1999 Growing Season

Introduction

Colorado potato beetle (CPB), is an annual pest that requires control every year in most potato production regions. In the past, growers have relied on both foliar and systemic insecticides for control of this and other pests. Imidacloprid insecticide has been widely adopted in recent years due to its efficacy against CPB and aphids. However, researchers continue to seek alternatives to this compound so that growers have multiple options for pest control, thereby reducing selection pressure and the corresponding risk of resistance development.

In 1999, a farm-scale experiment was initiated at a 1200 acre potato and vegetable farm in Coloma, Wisconsin to evaluate the efficacy of various pest control tactics for control of CPB and other insect pests. Reported below are the CPB efficacy data using NewLeaf® Plus potatoes or other registered insecticides.

Methods

Russet Burbank potatoes were planted in a 65 acre field at Coloma Farms, Coloma, WI using standard production practices. Each insect control treatment consisted of 48 rows X 2000 feet with treatments replicated four times and arranged in a modified Randomized Complete Block design (Figure 1). Colorado potato beetle adults, egg masses, small larvae, and large larvae were counted once per week on five plants at four locations within each plot.

Five "conventional" or "low risk" management regimes using various combinations of insect control measures were evaluated. Management regimes were selected to control the following key insect pests: Colorado potato beetle (CPB), potato leafhopper (PLH) and various aphid species (aphids) (Table 1).

Table 1. Management regimes selected to control the key insect pests

	Insecticides* Applied Against Specific Pests		
Management Regime	**Colorado Potato Beetle**	**Potato Leafhopper**	**Aphids**
1. Conventional Systemic	imidacloprid 13 oz.	dimethoate	methamidophos
2. Low Risk Systemic	imidacloprid 10 oz.	permethrin (low rate)	pymetrozine**
3. Conventional Foliar	esfenvalerate endosulfan	dimethoate	methamidophos
4. Low Risk Foliar	spinosad	permethrin (low rate)	pymetrozine
5. Low Risk Transgenic	Line RBMT22-82	permethrin (low rate)	pymetrozine

*Imidacloprid (Admire®); methamidophos (Monitor®); permethrin (Ambush®); pymetrozine (Fulfill®); esfenvalerate (Asana®+PBO); endosulfan (Thiodan®); dimethoate (Dimethoate®); spinosad (SpinTor®); Line RBMT22-82 (NewLeaf ®Plus Russet Burbank).

** Pymetrozine was submitted to EPA for pesticide registration on May 3, 1999 by Novartis.

Plots were scouted for CPB, potato leafhoppers, and green peach and potato aphids beginning in early June. Insecticides targeting key insects were applied on the basis of established thresholds. All applications were made using a commercial 48-row boom sprayer.

Results

Colorado potato beetle adults, eggs, and larvae were found in all insecticide treatments except NewLeaf Plus (Tables 2-5). No CPB adults, larvae or eggs were found in any of the NewLeaf Plus plots on any date. No CPB defoliation was observed in any plot at any time during the season, indicating that all treatment regimes provided commercially acceptable control despite significant adult and larval populations on some dates in all but NewLeaf Plus plots. Only Bt-expressing NewLeaf Plus potatoes completely prevented CPB infestation and survival throughout the entire season.

Table 2. Colorado potato beetle adults per 5 plants, Coloma, WI, 1999

Treatment	8-Jun	16-Jun	22-Jun	30-Jun	7-Jul	14-Jul	23-Jul	27-Jul	4-Aug	11-Aug	18-Aug
1. Admire 13 oz	0	0	0	0	0.1	0	0.1	***1.9***	***1.2***	***1.0***	0
2. Admire 10 oz	0	0	0	0	0	0	0.1	0.5	0.3	***1.0***	0.2
3. Asana/PBO	0	0	0	0.1	0.1	0.4	***0.6***	***2.1***	***4.6***	***3.4***	***1.2***
4. Spintor	0	0.1	***0.3***	0.1	0.3	***2.4***	***1.9***	***1.6***	***2.3***	***4.2***	***0.9***
5. NewLeaf Plus	0	0	0	0	0	0	0	0	0	0	0
Prob>F	ns	ns	0.0008	ns	ns	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Table 3. Colorado potato beetle egg masses per 5 plants, Coloma, WI, 1999

Treatment	8-Jun	16-Jun	22-Jun	30-Jun	7-Jul	14-Jul	23-Jul	27-Jul	4-Aug	11-Aug	18-Aug
1. Admire 13 oz	0	0	0	0	0	0	0	***4.1***	0.1	1.7	0
2. Admire 10 oz	0	0	0	0	0	0	0	0	1.8	***3.7***	0
3. Asana/PBO	0	0	0	0	0	0	0.8	***6.6***	***5.7***	***13.3***	***0.3***
4. Spintor	0	0.1	0.1	0	0	0.1	1.9	***7.1***	1.8	***9.7***	0.1
5. NewLeaf Plus	0	0	0	0	0	0	0	0	0	0	0
Prob>F	ns	ns	ns	ns	ns	ns	ns	0.0001	0.0002	0.0001	0.05

Table 4. Colorado potato beetle small larvae per 5 plants, Coloma, WI, 1999

Treatment	8-Jun	16-Jun	22-Jun	30-Jun	7-Jul	14-Jul	23-Jul	27-Jul	4-Aug	11-Aug	18-Aug
1. Admire 13 oz	0	0	0	0	0	0	0	***3.0***	0.4	0.9	0.4
2. Admire 10 oz	0	0	0	0	0	0	0	0	0.2	***2.9***	0
3. Asana/PBO	0	1.4	0	***2.1***	2.3	0	0	***6.9***	***1.3***	***10.9***	***2.7***
4. Spintor	0	***2.1***	0.1	***3.9***	1.6	0.1	***1.0***	***12.7***	0.3	0.4	0.1
5. NewLeaf Plus	0	0	0	0	0	0	0	0	0	0	0
Prob>F	ns	0.01	ns	0.0001	ns	ns	0.0004	0.0001	0.02	0.0001	0.0001

Table 5. Colorado potato beetle large larvae per 5 plants, Coloma, WI, 1999

Treatment	8-Jun	16-Jun	22-Jun	30-Jun	7-Jul	14-Jul	23-Jul	27-Jul	4-Aug	11-Aug	18-Aug
1. Admire 13 oz	0	0	0	0	0.1	0.1	0	0.4	0.1	0	0.1
2. Admire 10 oz	0	0	0.1	0	0	0.3	0	0	0.4	0.1	0.1
3. Asana/PBO	0	***1.6***	0.3	***1.6***	1.3	0.1	0.1	***1.6***	0.4	***0.2***	0.3
4. Spintor	0	***1.0***	0.3	***2.9***	***3.3***	***5.8***	***0.7***	0.3	0.2	0	0.2
5. NewLeaf Plus	0	0	0	0	0	0	0	0	0	0	0
Prob>F	ns	0.02	ns	0.0001	0.001	0.0002	0.01	0.01	0.07	0.05	ns

Significantly different from NewLeaf Plus by orthogonal contrast

Summary of 1997 and 1998 Research on the Development of Net Necrosis in NewLeaf® Plus Line RBMT22-82

Net necrosis is thought to develop if Potato Leaf Roll Virus (PLRV) infection occurs at the time of most rapid tuber expansion (bulking) (W. Regional Res. Pub. 1992). If plants are infected early or heavily, as in the PLRV resistance screening tests, susceptible plants are severely stunted and tubers from such plants are small and show little or no net necrosis. Consequently, resistance to net necrosis was evaluated by replicated trials in which test clones were allowed to become naturally infected with PLRV.

Natural infection trials were established at three different test sites in Idaho and Oregon in April, 1997. Each trial consisted of six replications of standard Russet Burbank and the transgenic clone RBMT22-082 arranged in a randomized complete block design. Each replicate was two rows wide by 15 feet long with rows spaced 36" apart and seed tubers planted every 12" in each row. Standard cultural and pest management were carried out at each trial site except that no aphicide was applied to the trial area, allowing natural infestation with aphids which transmit PLRV. All tubers were harvested in September, 1997, stored at 45°F and 95% relative humidity at NatureMark's Boise, ID warehouse until February, 1998, at which time 100 tubers from each replicate were cut open and visually examined for net necrosis.

In addition, commercial-scale demonstration trials were established at seven different sites in Washington, Oregon, and Idaho in 1998 where Russet Burbank was grown on one part of a commercial field which received standard aphicide treatment and RBMT22-082 was grown in an adjacent portion of the field which received no aphicide treatment. At harvest, a 400 tuber sample of Russet Burbank and RBMT22-082 was collected at random from each field, stored until February under conditions as described above, and then cut open and visually examined for net necrosis. Results from the 1997 and 1998 trials are shown in Table 1.

In the replicated natural exposure trials (no aphid control used) the incidence of net necrosis in RBMT22-082 was significantly less than for standard Russet Burbank by a large margin at all locations. In commercial-scale trials, the average level of net necrosis in RBMT22-082 was still approximately 8 times lower than that of Russet Burbank that had received a standard insecticide program to control aphids (Table 1). These results indicate that RBMT22-082 has a high degree of resistance to net necrosis caused by infection with the PLRV.

Reference: Western Regional Research Publication 011: Integrated Pest Management for Potatoes in the Western United States. 1992. Page 89.

Table 1. Resistance of RBMT22-082 to net necrosis.

Clone	Incidence of net necrosis(%) in trial:[a]			
	1	2	3	4[b]
R. Burbank	16.9	8.8	3	2.4
RBMT22-082	0.2	0.2	0.3	0.3
p-value[c] =	0.0007	0.004	<.0001	0.02

[a] 1997 natural exposure trials at: 1) Kimberly, ID, 2) Parma, ID and 3) Hermiston, OR. Mean of six replicates per site.

[b] Means of 7 commercial trials in 1998.

[c] Means are significantly different if $p<.05$.

3

TAB 3: Descriptions of the preparation of the probes described on page 11 of the petition.

The following information is to be added to complete the description of the preparation of the probes described on page 11 (an additional page, 11a, is included herein for inclusion within the original petition if needed):

DNA probes for Southern Blot Analysis. The probes used to estimate the number of loci and assessment of gene integrity in NewLeaf Plus line No. RBMT22-82 were synthesized by the polymerase chain reaction (PCR). Protocols for the amplification of these probes were essentially as described by Sambrook *et al.* (1989). The plasmid DNA template, either PV-STMT21 (Figure V.1) or PV-STMT22 (Figure IV.1), containing the genes of interest was used as the target for gene specific primers that anneal at the ends of the respective genetic elements. The PCR products were gel purified using the Qiagen gel extraction kit (Qiagen, QIAquick Gel Extraction Kit, Chatsworth, CA). A second PCR reaction was performed using the gel extracted product as the template and the same gene specific primers that anneal at the ends of the respective elements. The purpose of the second PCR reaction was to remove residual plasmid DNA within the initial PCR sample and enhance probe specificity. The secondary PCR reactions were gel purified as above. A Hoeffer DyNA Quant 200 fluorometer was used to quantitate the probes and they were radiolabelled using the RadPrime DNA labeling Kit (GibcoBRL, Gaithersburgh, MD).

Element	Position of primers on PV-STMT22	Length of probe product
orf1/orf2	13882-13907 16991-16964	3.4Kb
CP4 EPSPS	5581-5604 6916-6943	1.4Kb
orf1/orf2$_{ft}$	13894-13918 15504-15482	1.6Kb
E9	631-651 4-24	0.63Kb
*cry*3A	2556-2583 4326-4349	1.8Kb
*ori*V	8181-8200 8560-8580	1.3Kb
*ori*322	10704-10726 11323-11347	1.8Kb
aad	11880-11898 12633-12662	0.8Kb

The following additional reference is cited:

Sambrook, J., Fritsch, E. F., and Maniatis, T.1989. "Molecular Cloning." Cold Spring Harbor Laboratory Press, 2nd Edition.

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aad	11880-11898 12633-12662	0.8Kb

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99-173-01p
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addendum 2
information

MONSANTO COMPANY
700 CHESTERFIELD PARKWAY NORTH
ST. LOUIS, MISSOURI 63198
PHONE (314) 694-1000
http://www.monsanto.com

September 30, 1999

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COPY

Coordination, and Technical Assistance,
Biotechnology, and Scientific Services,
Plant, Protection, and Quarantine,
Animal and Plant Health Inspection Service,
U.S. Department of Agriculture
4700 River Road
Riverdale, MD 20737

Attention: Dr. James White

Subject: Application for Extension of a Determination of Nonregulated Status - NewLeaf ®Plus Potatoes: Petition 99-173-01p (PLRV + CPB resistant potatoes) - Addendum 2

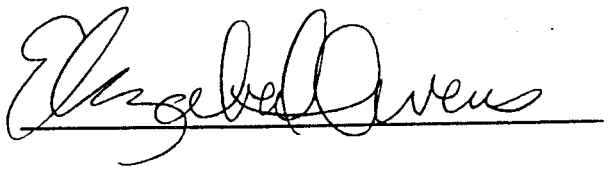
In response to your questions of September 29th, we are providing information as noted below:

1. In Perlak, *et al.*, the 0.002% Bt calculates to 0.32 µg/g tissue fresh weight.
2. In Table 1 of "Cry3A Expression Levels of NewLeaf® Plus Potato Foliage and Colorado Potato Beetle Feeding Through the 1998 Growing Season", the leaf tissues tested were sampled as follows:
   For Cry3A analysis, the youngest fully expanded, compound leaf was collected from six plants per plot. The leafs were placed inside of a plastic sampling bag and immediately frozen on dry ice. Leaf tissue was shipped on dry ice to Monsanto Life Sciences Research Center (Chesterfield, MO) and stored frozen at -80°C until used for this study.
3. A copy of "Perlak, *et al.*" was faxed to Dr. Heron on 9/29.
4. In Tables 2-5 of "Comparison Study of Colorado Potato Beetle Control Methods Under Commercial Conditions in Wisconsin During the 1999 Growing Season", the sampling method was as follows: Five random plants were visually inspected for adults, larvae and egg masses in four locations of each of the four plots for a total of 80 plants sampled for each treatment weekly. Data is shown as an average per five plant sample. I also note that I did not include Figure 1 showing the plot design so I have attached it to this letter.
5. Monsanto reports cited are included as a confidential attachment to this letter. We have also included a non confidential summary of each report. The reports are as follows:
   - Bookout and Dodson, 1998
   - Dean 1993
   - Grace 1996
6. The lengths of the probes for oriV and ori322 are 0.4kb and 0.64kb respectively.
7. In the report "Cry3A Expression Levels of NewLeaf® Plus Potato Foliage and Colorado Potato Beetle Feeding Through the 1998 Growing Season", we cited a study conducted in 1998 (incorrectly listed as completed in 1988) and have attached a copy here of portions of a report by Boiteau, including the relevant data on line RBMT22-82 and the control.

10/4/99

8. In Table 1 of "Summary of 1997 and 1998 Research on the Development of Net Necrosis in NewLeaf ® Plus Line RBMT22-82", there is a typo which should be corrected to read RBMT22-82.

Please phone me or email me if you need additional information to complete your review.

Signature 

Elizabeth D. Owens, Ph.D.
Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway North
BB1K
St. Louis, MO 63198
Phone: 314-737-5721
FAX: 314-737-7085
Email: Elizabeth.D.Owens@monsanto.com

Attachment : Additional information: Figure 1; non confidential summaries of reports; non confidential research report; confidential copies of reports;.

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CONFIDENTIAL BUSINESS INFORMATION JUSTIFICATION

The information claimed as confidential within this submission consists of Monsanto research reports. The reports contain information on internal procedures and unpublished data on products.

LEGAL BACKGROUND

The Freedom of Information Act ("FOIA"), 5 U.S.C. § 552, specifically exempts from release "trade secrets and commercial or financial information obtained from a person and privileged or confidential" ("Exemption 4"). 5 U.S.C. § 552(b)(4). Exemption 4 applies where the disclosure of information would be likely to cause substantial harm to the competitive position of the owner, or where, in the case of voluntarily submitted information, the submitter would be less likely in the future to share data with the agency voluntarily. National Parks & Conservation Association v. Morton, 498 F.2d 765, 770 (D.C.Cir. 1974); Gulf & Western Industries, Inc. v. U.S., 615 F.2d 527, 530 (D.C.Cir. 1979).

A party seeking to demonstrate "substantial competitive harm" need not show actual competitive harm, but must only demonstrate the presence of competition and the likelihood of substantial competitive injury. Id. at 530; National Parks & Conservation Association v. Kleppe, 547 F.2d 673, 679 (D.C.Cir. 1976); Miami Herald Pub. Co. v. U.S. Small Business Administration, 670 F.2d 610, 614 (5th Cir. Unit B 1982).

For the purposes of FOIA, courts have defined the term "trade secret" to mean a "secret, commercially valuable plan, formula, process, or device that is used for the making, preparing, compounding, or processing of trade commodities and that can be said to be the end product of either innovation or substantial effort. Public Citizen Health Research Group v. FDA, 704 F.2d 1280, 1288 (D.C.Cir. 1983); Anderson v. Dept. of Health & Human Services, 907 F.2d 936, 943-44 (lOth Cir. 1990).

Information on gene description and commercial development falls squarely within this definition, and is the type of information accorded trade secret protection by the courts under Exemption 4 of the Freedom of Information Act. It is well established that information on the formulation and chemistry of a product should be treated as confidential for FOIA purposes. See, e.g., Anderson v. Dept. of Health & Human Services, 907 F.2d 936 (lOth Cir. 1990). This is exactly the type of information provided by each and every subcategory listed above in the gene description category. Where, as in the case of the Monsanto products subject to this FOIA request, the development time and costs of the product have been substantial and the information can only be obtained by competitors at considerable cost, disclosure is prohibited. Greenberg v. Food and Drug Administration, 803 F.2d at [213, 1216-1218 (D.C. Cir. 1986); Worthington Compressors, Inc. v. Costie, 622 F.2d 45, 51-52 (D.C.Cir. 1981). The existence of confidentiality agreements binding employees not to reveal the information is another factor considered by the courts. Greenberg v. FDA, 803 F.2d at 1216-1218.

The courts have also been very clear in finding commercial development information covered by Exemption 4 where the release of such information could allow competitors to procure a clear understanding of a company's business practices and allow a competitor to cause harm to a company's competitive standing. See, e.g., Braintree Electric Light Dept. v. Dept. of Energy, 494 F.Supp. 287, 289-291 (D.D.C. 1980). Information on distribution channels, market strategies, pricing structures, and patterns of competition fall squarely within the Exemption because such information enables a competitor to gain an accurate picture of a company's marketing activities and the competitive structure of the market. Timken v. U.S. Customs Service, 531 F.Supp. 194, 200 (D.D.C. 1981). Typically, information concerning marketing strategies, and the names of independent contractors participating in a company's studies have been accorded confidential treatment. See, e.g., Teich v. Food & Drug Administration, 751 F. Supp. 243, 253 (D.D.C. 1990).

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Petition 99-173-01p (PLRV + CPB resistant potatoes)

Addendum 2

Additional Information

Date:

September 30, 1999

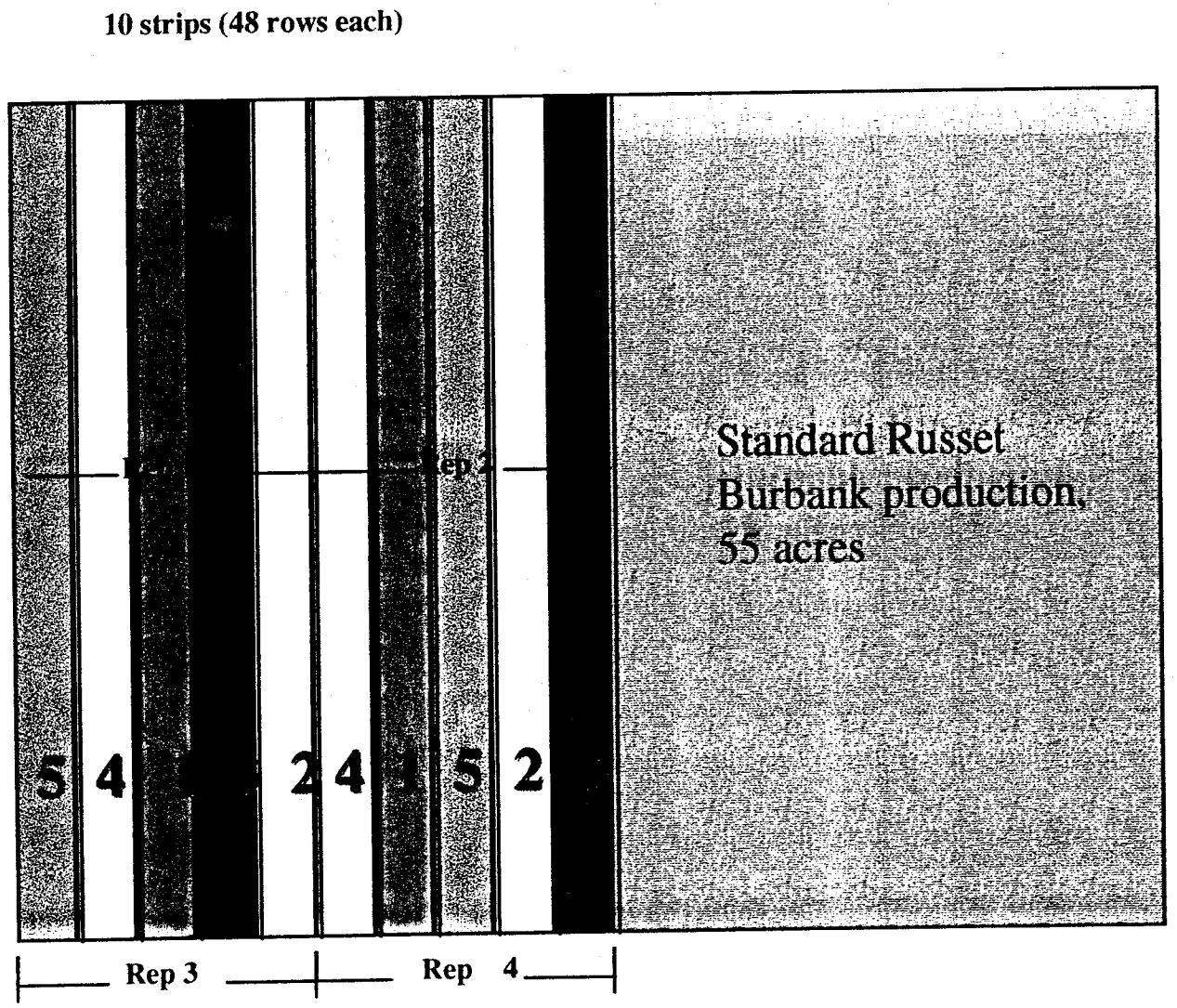
Submitted by:

Monsanto Company

Document No.: 99-246-U (Addendum 2)

Submitter:
Elizabeth D. Owens, Ph.D.
Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway North
BB1K
St. Louis, MO 63198
Phone: 314-737-5721
FAX: 314-737-7085
Email: Elizabeth.D.Owens@monsanto.com

Figure 1. Experimental design of CPB efficacy trial, Coloma Farms, 1999



Treatments:

1. Conventional Systemic: Admire 13 oz, dimethoate, Monitor
2. Low Risk Systemic: Admire 10 oz., low rate permethrin, Fulfill
3. Conventional Foliar: Asana+PBO, Thiodan, dimethoate, Monitor
4. Low Risk Foliar: SpinTor, low rate permethrin, Fulfill
5. Low risk transgenic: NewLeaf Plus, low rate permethrin, Fulfill

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Petition 99-173-01p (PLRV + CPB resistant potatoes)

Addendum 2

Additional Information

Date:

September 30, 1999

Non Confidential Report Summaries and Report

CBI DELETED

Report No: MSL -155931

Date: 1998

Title: Overseason Expression Levels of Cry3A Protein in Tissues Derived from Shepody, HiLite, and Russet Burbank Potato Plants Resistant to CPB/PVY or CPB/PLRV

Authors: J. T. Bookout and H. C. Dodson

Summary:

The purpose of this study was to determine expression levels, throughout the growing season, of Cry3A protein in tissue from genetically modified potato plants. The tested varieties have been genetically improved to confer resistance to Colorado potato beetle (CPB) and Potato Virus Y (PVY) or CPB and Potato Leaf Roll Virus (PLRV). In order to evaluate Cry3A protein expression levels, leaf samples were obtained from three potato varieties (seven distinct potato lines) at 6, 10, and 14 weeks of growth from New Maryland, New Brunswick, Canada, and at 10 and 14 weeks of growth from Saint Croix de Lotbiniere, Quebec, Canada field sites.

Results for NewLeaf® Plus potatoes: Season-long expression levels of the Cry3A protein were determined in Newleaf Plus (RBMT22-82, RBMT21-129, RBMT21-350) potato lines. Overall, Cry3A protein expression levels peak at 6 to 10 weeks and decline over the course of the growing season. The mean expression levels of Cry3A protein in Newleaf Plus lines were 13.90, 14.56, and 12.83 µg/g tissue fresh weight at 6, 10, and 14 weeks post-planting, respectively.

Results from this study showed that Cry3A protein expression levels peak at six to ten weeks and decline over the course of the growing season. These current results are consistent with Cry3A protein expression levels observed in previously approved Newleaf® Atlantic lines which contain the same gene - promoter construct.

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Report No: MSL-12341

Date: 1993

Title: Development and validation of an enzyme-linked immunosorbent assay (ELISA) for detection and quantitation of *Bacillus thuringiensis tenebrionis* (*B.t.t.*) insecticidal protein in Colorado Potato Beetle Resistant Potatoes

Author: Duff A. Dean

Summary:

The report describes the results of the development and validation studies of an ELISA for detection of *Btt* insecticidal protein in genetically modified plants. The *Btt* insecticidal protein gene is derived from the microbe, *Bacillus thuringiensis tenebrionis*. When expressed in genetically modified potato plants, this protein confers resistance to coleopteran insect infestation, most notably, the Colorado potato beetle. The *Btt* ELISA has been optimized to quantitate the *Btt* protein in leaf, tuber, old whole plant, and young whole plant extracts. It employs a polyclonal antibody adsorbed to a 96-well microtiter plate well to capture the *Btt* protein from the extract solution. The same polyclonal antibody, linked with horseradish peroxidase, is used to detect the captured protein. The development of color in the assay is directly related to the amount of *Btt* protein present in each sample. The quantity of *Btt* protein in a sample can be determined by extrapolation from the standard curve.

Several areas of validation were investigated, including accuracy, precision, range of analysis, stability, and ruggedness. Within the parameters outlined in the report, a reliable quantitation of *Btt* protein in the potato tissues can be obtained for the range of 31 to 2000 picograms of *Btt* protein loaded per microtiter plate well.

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Report No: BtP-PRO-032-04

Date: 1996

Title: Quantitation of *Btt* in extracts of potato tissues. Monsanto Standard Operating Procedure.

Author: A. M. Grace

Summary:

This SOP describes the tissue extraction and quantitation of Cry3A protein in potato tissues for the Biotechnology Regulatory Science Group.

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DIVIDER PAGE
BETWEEN
REPORTS

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Global Agri Services Inc.
376 New Maryland Highway
New Maryland, New Brunswick
E3C 1E5
Tel: (506) 458-8765
Fax: (506) 454-0567
E-Mail: globalag@nbnet.nb.ca

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NATUREMARK
NORTH TILLEY, NEW BRUNSWICK

OBJECTIVE

The objective of this experiment was two folds: to evaluate the efficacy of the *(B.t.t.)* protein expressed in transgenic lines in controlling the Colorado potato beetle and to prepare a botanical description for the Plant Breeders' Rights.

MATERIALS & METHODS

Varieties:

There were 28 lines and varieties in the trial. The varieties Atlantic, Superior, Snowden, Atlantic, Shepody, Hilite Russet, Russet Norkotah, Dark Red Norland, FL1533 and Russet Burbank were used as standard varieties.

The seed used in the trial was produced in Island Falls, Maine. For the exception of FL1533 and NL1533 that was produced in Rhinelander, Wisconsin.

Design:

A randomized complete block with 28 varieties and four replicates. All entries were planted in single-row plots. Each entry had one plot per replicate. A plot was 18' long. Spacing between adjacent plots was 36". Spacing between adjacent hills within plot was 12". The design and methodology were developed in consultation with Dr. Jeff Stewart from Agriculture Canada Charlottetown, P.E.I.

Planting:

The trial was planted on May 26, 1998.

Fertility:

1500 lbs/A 10-10-20 (McCain Fertilizer formulation)

Spacing:

All = 12"

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Crop Husbandry:

No insecticide was applied to control the Colorado potato beetle or any other pest. All other husbandry activities were done according to commercial practices.

Data Collection for Efficacy:

The efficacy was evaluated by measuring the degree of defoliation using the following ratings compared to the standards.

0 = no defoliation
1 = 2-60% of plants with leaflets slightly damage
2 = 2% of plants with at least 1 compound leaf with more than 50 % defoliation
3 = 2-9% of plants at least 1 stem with more than 50% defoliation
4 = 1-24% of plants at least 1 stem with more than 50% defoliation
5 = 25-49% of plants at least 1 stem with more than 50% defoliation
6 = 50-74% of plants at least 1 stem with more than 50% defoliation
7 = 75-90% of plants at least 1 stem with more than 50% defoliation

Source: Boiteau, G. 1994. Visual index for the estimation of defoliation in the potato crop. pp 6-7. In: Entomology Group Research Summary 3, Fredericton Research Centre, Agriculture and Agri-Food Canada.

GENERAL OBSERVATIONS

The environmental conditions at planting of the trial were very dry. The month of July brought some nice weather with good rainfall and August was dry and hot. The flea beetle pressure early in the season caused some stress to the plants.

The transgenic lines had no defoliation or very limited defoliation. The lines HLBTVY15-46 and DRBT00-3 had very limited defoliation with less then 2-60% of plants with leaflets slightly damage. However all the standard in the trial had at one time or another some defoliation with 2% of the plants with at least one compound leaf with more than 50% defoliation. Some of the variability of defoliation on the standard varieties in reference to the replication was possibly caused by the distance between the standard and the transgenic lines. In area that you had two standard varieties side by side you had a tendency of having more defoliation.

It seems that under a single row plot that it is very difficult to properly increase a population of Colorado potato beetle and sustain that population long enough to have severe damage to the plants. Thus, the distance between the standard variety and the transgenic lines must be increase. This could be accomplished with a three to four row plot.

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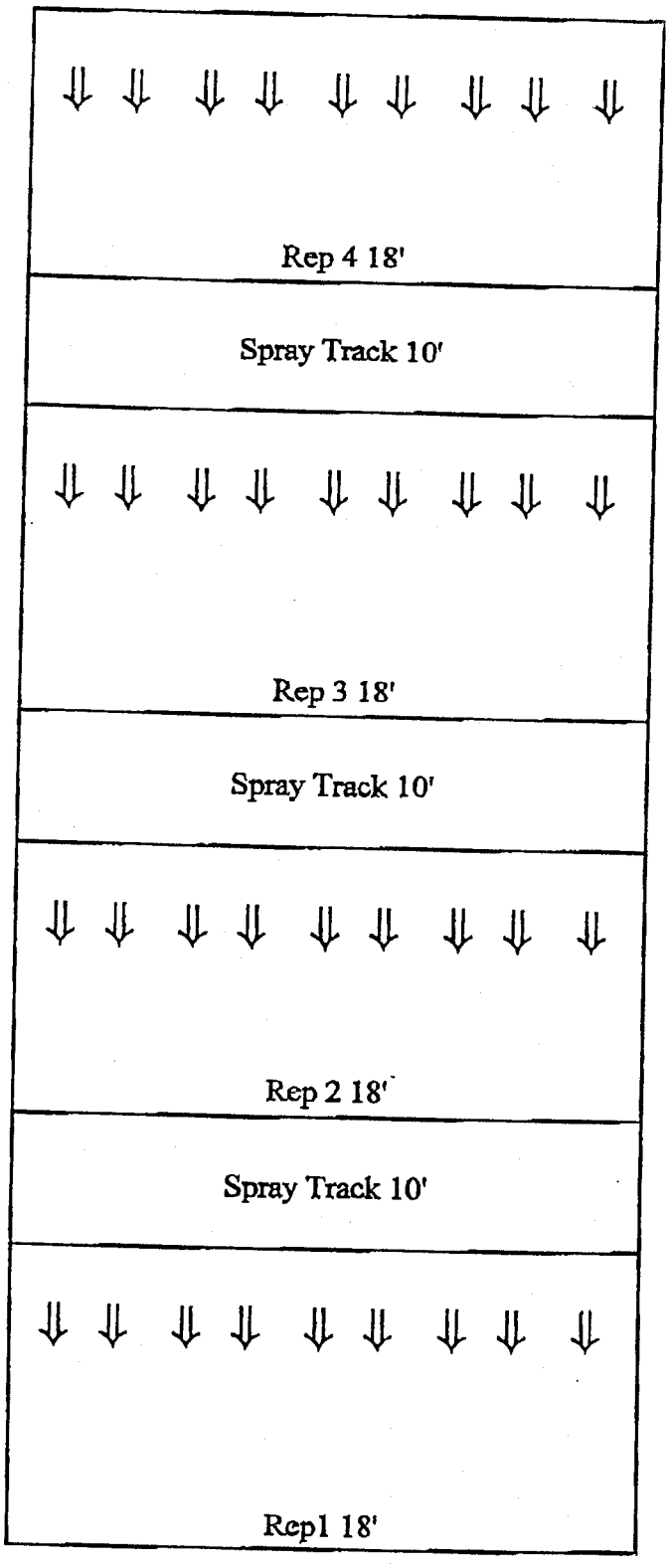

Global Agri Services Inc.

1998 EFFICACY TRIAL

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	Variety	July 21	July 29	August 6	August 18
REP1	RBBT02-6B2	0	0	0	0
REP2	RBBT02-6B2	0	0	0	0
REP3	RBBT02-6B2	0	0	0	0
REP4	RBBT02-6B2	0	0	0	0
REP1	RBBTLR21-129	0	0	0	0
REP2	RBBTLR21-129	0	0	0	0
REP3	RBBTLR21-129	0	0	0	0
REP4	RBBTLR21-129	0	0	0	0
REP1	RBBTLR21-350	0	0	0	0
REP2	RBBTLR21-350	0	0	0	0
REP3	RBBTLR21-350	0	0	0	0
REP4	RBBTLR21-350	0	0	0	0
REP1	RBBTLR22-82	0	0	0	0
REP2	RBBTLR22-82	0	0	0	0
REP3	RBBTLR22-82	0	0	0	0
REP4	RBBTLR22-82	0	0	0	0
REP1	RBBTVY15-101X	0	0	0	0
REP2	RBBTVY15-101X	0	0	0	0
REP3	RBBTVY15-101X	0	0	0	0
REP4	RBBTVY15-101X	0	0	0	0
REP1	RBCTLR00-12	1	2	2	2
REP2	RBCTLR00-12	0	1	1	1
REP3	RBCTLR00-12	1	1	1	1
REP4	RBCTLR00-12	1	1	1	1
REP1	RNBTVY15-245	0	0	0	0
REP2	RNBTVY15-245	0	0	0	0
REP3	RNBTVY15-245	0	0	0	0
REP4	RNBTVY15-245	0	0	0	0
REP1	RNBTVY15-350	0	0	0	0
REP2	RNBTVY15-350	0	0	0	0
REP3	RNBTVY15-350	0	0	0	0
REP4	RNBTVY15-350	0	0	0	0
REP1	RNBTVY15-360	0	0	0	0
REP2	RNBTVY15-360	0	0	0	0
REP3	RNBTVY15-360	0	0	0	0
REP4	RNBTVY15-360	0	np	np	np
REP1	RNCTLR00-5	1	1	1	1
REP2	RNCTLR00-5	0	1	0	1
REP3	RNCTLR00-5	2	2	2	2
REP4	RNCTLR00-5	1	np	np	np
REP1	SDBTVY15-24X	0	0	0	0
REP2	SDBTVY15-24X	0	0	0	0
REP3	SDBTVY15-24X	0	0	0	0
REP4	SDBTVY15-24X	0	0	0	0

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Petition 99-173-01p (PLRV + CPB resistant potatoes)

Addendum 2

Additional Information

Date:

September 30, 1999

CONFIDENTIAL REPORTS

The pages 13 - 94 are claimed
as Confidential Business Information by the
Monsanto Company
and are CBI- deleted from this copy.

99-173-01p
Addendum 3

Petition 99-173-01p
(PLRV & CPB resistant potatoes)
Addendum 3

October 22, 1999

99-173-01p
Addendum 3

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MONSANTO COMPANY
700 CHESTERFIELD PARKWAY NORTH
ST. LOUIS, MISSOURI 63198
PHONE (314) 694-1000
http://www.monsanto.com

October 22, 1999

Coordination, and Technical Assistance,
Biotechnology, and Scientific Services,
Plant, Protection, and Quarantine,
Animal and Plant Health Inspection Service,
U.S. Department of Agriculture
4700 River Road
Riverdale, MD 20737

Attention: Dr. James White

Subject: Application for Extension of a Determination of Nonregulated Status - NewLeaf ®Plus Potatoes: Petition 99-173-01p (PLRV + CPB resistant potatoes) - Addendum 3

In response to your need for further information, Monsanto is providing as Addendum 3, the four studies, submitted to the EPA in support of the registration of the CryIIIA protein, listed below. These studies are not confidential business information, but are considered to be property of the Monsanto Company for purposes of compensation for use in registration of pesticide products under FIFRA.

1. Effect of the *Bacillus thuringiensis* insecticidal proteins CryIA(b), CryIA©, CryIIA, and CryIIIA on *Folsomia candida* and *Xenylla grisea* (Insecta: Collembola). (Pages 1-22)
2. Evaluation of the Dietary Effect(s) of Purified *B.t.t.* Protein on Honey Bee Larvae (Pages 23-91)
3. *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) Protein: An acute toxicity study with the earthworm in artificial soil substrate (Pages 92-145)
4. Aerobic Soil Degradation of Colorado Potato Beetle Active Protein from *Bacillus thuringiensis* subsp. *tenebrionis* (Pages 146-186)

Since there is no confidential business information in this submission, I have included just the three copies. Please phone me or email me if you need additional information to complete your review.

Signature Elizabeth D Owens

Elizabeth D. Owens, Ph.D.
Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway North
BB1K
St. Louis, MO 63198
Phone: 314-737-5721
FAX: 314-737-7085
Email: Elizabeth.D.Owens@monsanto.com
Attachment : Addendum 3.

11/3/99

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