97-204-01p
Revised

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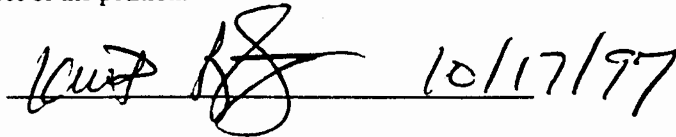
Subject: PETITION FOR DETERMINATION OF REGULATORY STATUS FOR NEWLEAF® PLUS POTATOES.

Enclosed is a copy of a petition for determination on the regulatory status of *Solanum tuberosum* cultivar Russet Burbank, that has been modified to be resistant to the Colorado potato beetle and the potato leafroll virus, and which is currently deemed a regulated article. Based on the data and information contained in the enclosed petition, 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 petition 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 petition.

Signature 

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PETITION FOR DETERMINATION OF REGULATORY STATUS FOR NEWLEAF® PLUS POTATOES

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MONSANTO ID 97-224U
USDA NUMBER 97-204-01P

SUBMITTED 17 October, 1997

Keith Reding, Ph.D.
Monsanto Company

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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
nptII	Gene encoding neomycin phosphotransferase II
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
RBMT21, etc.	Plant lines
sp.	Species
T-DNA	Transfer DNA
TGMV	Tomato Golden Mosaic Virus
TRV	Tobacco Rattle Virus
USDA	United States Department of Agriculture

I. RATIONALE FOR THE DEVELOPMENT OF NEWLEAF® PLUS POTATOES

Potato is the world's fifth most abundant crop with 265 million metric tons produced world-wide in 1994 (National Potato Council, 1996). Monsanto Company has developed NewLeaf Plus potatoes which are highly resistant to infection by potato leafroll virus (PLRV) and to the Colorado Potato Beetle (CPB), two of the most damaging potato pests in North America (Perlak *et al.*, 1993; Kaniewski *et al.*, 1994). Fourteen potato lines expressing the *cry3A* gene for CPB resistance have already been grant a Determination of Nonregulated Status by the USDA, Animal and Plant Health Inspection Service (USDA Determinations for 95-338-01p and 95-290-01p). NewLeaf Plus potato lines were developed using a new transformation event to have the additional trait of PLRV resistance.

The Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) is the most damaging pest of the 2.3 billion dollar U.S. potato crop (Krieg *et al.*, 1983; Casagrande, 1987; National Potato Council, 1992). To date, no traditionally bred cultivars have been produced through traditional breeding which are resistant to the CPB. For traditional potato varieties, approximately one-third of the 2.8 million pounds of chemical insecticides annually applied to potatoes are targeted for its control (USDA, 1993). CPB damage is particularly severe in the eastern and north central potato production areas and is becoming an increasing problem in the northwest. Both larval and adult stages feed on potato foliage and, if not controlled, can undergo population growth rates exceeding 40 fold per generation (two and potentially three generations per year are possible in many areas) and a potential overwintering survival rate of more than 60% (Groden and Casagrande, 1986; Harcourt, 1971). If poorly managed, the CPB is capable of completely defoliating potato plants, resulting in yield reductions of as much as 85%, which is sufficient to prevent potato production in some areas (Roush, 1993; Hare, 1980; Ferro *et al.*, 1983; Shields and Wyman, 1984). Loss of revenue due to the CPB in Michigan alone was estimated at more than 15 million dollars in a state where total potato production in 1991 was valued at 70 million dollars (Potato Growers of Michigan, Inc. and the Michigan Potato Industry Commission, 1992; Olkowski *et al.*, 1992).

Current control of CPB relies heavily upon the use of chemical insecticides that are variably effective due to environmental factors or insect sensitivity. These insecticides are also expensive with costs that can exceed $200 per acre per season (Ferro and Boiteau, 1992). Additional management options for CPB include, crop rotation, vacuum suction (Boiteau *et al.*, 1992), propane flaming (Moyer, 1992; Moyer *et al.*, 1991), polyethylene-lined trenches (Roush, 1993) and trap plots (Roush, 1993; Roush and Tingey, 1992). These options are not often practical, effective, economical nor easily implemented throughout the season (Roush, 1993).

A major viral disease of potato, leafroll disease, is caused by the potato leafroll virus (PLRV). PLRV is member of the luteovirus group which consist of positive-sense RNA viruses. It is the only member of the luteovirus group of viruses known to infect potatoes (Thomas, 1996). PLRV infects potatoes in most geographical locations where potatoes are grown, resulting in reduction in total yields of some cultivars by as much as 75% (Broadbent et. al., 1957). In the U.S., a major problem with PLRV infection is net necrosis, i.e. death of the vascular tissues in the tuber resulting in discoloration (Banttari *et al.*, 1993). Potatoes with net necrosis are normally rejected by potato processors so care must be taken to control PLRV infection. Loss of marketable yield from net necrosis can exceed 25% in susceptible cultivars such as Russet Burbank in years of rapid spread of PLRV (Manzer *et al.*, 1982). Although some Polish cultivars of *S. tuberosum* do contain genes for natural resistance, these potatoes are not utilized because of poor agronomic properties (Kaniewski, 1997).

PLRV can be introduced into a potato field either by planting infected seed tubers or by an aphid vector that brings the pathogen to the field from an outside source infection, such as other potato fields (Barker, 1992). The spread of PLRV within the potato field is dependent on aphid infestation, as PLRV is not spread mechanically (Banttari *et al.*, 1993). Potato growing areas which have low aphid populations generally have a reduce incidence of PLRV infection (Barker, 1992).

To control PLRV infection in potatoes, growers depend on seed certification programs (Slack, 1993) and the use of insecticides to control the aphid vectors. For seed to be certified, generally less than 1% of the seed tubers are allowed to be infected with PLRV. Therefore, seed producers rely on growing the seed potatoes in remote

locations, where aphid populations are expected to be low, and the use of insecticides to control the spread of the virus within the field. Farmers also must use insecticides to control aphids which may spread PLRV from infected plants within the field (originating from infected seed tubers or volunteers from the previous growing season).

In 1995, growers applied insecticides to 88% of the potato acreage in the U.S. amounting to 2,553,000 total pounds. Of these insecticides, 1,927,000 lb. were applied to control damage from the Colorado potato beetle and potato leafroll virus. Commercial planting of NewLeaf Plus potatoes could drastically reduce the need for these insecticides in potato farming by providing insecticide-free control of the Colorado potato beetle and potato leafroll virus. An article entitled "Biologic and Economic Assessment of Genetically Modified CPB- and PLRV-Resistant Potatoes" was prepared by A. Schreiber and J.F. Guenthner to address in detail the benefits of NewLeaf Plus potatoes (Appendix 1).

Information on potato plant lines derived from seven independent transformation events are presented in this petition.

Three plant lines (Line Nos. RBMT21-129, RBMT21-152 and RBMT21-350) were transformed with plasmid vector PV-STMT21. The lines containing the following genetic elements:

- The chimeric gene for selection of transformed plant cells on kanamycin (NOS/*nptII*/NOS 3') which consists of the promoter region of the nopaline synthase gene from Ti plasmid of *A. tumefaciens* (Fraley *et al.*, 1983), the neomycin phosphotransferase type II (*nptII*) gene (Beck *et al.*, 1982) 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 the control of CPB (AraSSU1A/*cry3A*/E9 3') which consists of the *Arabidopsis 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 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 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 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).

Four plant lines (Line Nos. RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262) were transformed with plasmid vector PV-STMT22. These lines containing 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 (Richins *et al.*, 1987), the *CP4 EPSPS* gene which encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase enzyme (Barry *et al.*, 1992) and the 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*/E9 3') which consists of the *Arabidopsis 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 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 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 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).

Field experiments with the transgenic potato lines were conducted during 1994 through 1996 under permits from the USDA (93-362-01r, 94-217-02R, 94-342-01r, 96-277-01r, and 97-017-03r). The final reports for these permits

are supplied in Appendix 7 of this petition. 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

The following section was written by Dr. Steve Love, University of Idaho, Aberdeen.

The Potential for Gene Escape from Cultivated Transgenic Potatoes Within the U.S.

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A. Summary

Potato genetic engineering has caused concern that exotic genes will escape into wild relatives of potato and develop the potential for ecological disruption. In some situations this could happen. Over nine hundred species of *Solanum* have been identified, most near the centers of origin in Central and South America and many cross freely with the cultivated potato (*S. tuberosum*). However, within the borders of the U.S., only two species of tuberizing *Solanum*, *S. fendleri* and *S. jamesii*, have been confirmed to exist. Neither species hybridizes with *S. tuberosum* due to differences in ploidy level, differences in endosperm balance number (EBN) or a combination of the two. Both species are found in high elevation, arid climates and are seldom geographically adjacent to potato production areas. Several species of *Solanum* are considered weeds in cultivated fields, including several species of nightshade. None of these species are closely related and none will hybridize with potatoes. The lack of compatible wild species and the clonal propagation system used in potatoes leads to the conclusion that within the borders of the U.S. no opportunity exists for the escape of introduced genes from cultivated types to wild relatives of potato.

B. History and Geography of Potato Production and Use

The potato (*Solanum tuberosum*) is native to the western hemisphere and occurs in abundance from the tropical highlands of Mexico, southward throughout western South America. Around 1570, South American cultivated potatoes were introduced into Europe. Descendants of these early European potatoes were permanently introduced into the U.S. in 1719 by Irish immigrants when they established a colony in New Hampshire (Stevenson, 1951). As Europeans settled in North America, potato production spread throughout the geographical area currently controlled by the U.S.

Potatoes are currently produced to some extent in all fifty states and the U.S. ranks fourth in world production (National Potato Council, 1992). The major commercial production areas are located in the northernmost states of the continental United States and include Maine, New York, Michigan, Wisconsin, Idaho, Oregon and Washington. Exceptions are substantial potato acreages in California and Florida, and minor, but significant acreage in Alabama, Arizona, Louisiana, Nevada, New Mexico, North Carolina, Texas and Virginia (National Potato Council, 1992).

Per capita consumption of potatoes in the United States is ca. 130 pounds per year or more than one 150 g potato each per day (USDA, 1991). In 1990, 85% of the crop was used for human consumption (either processed or as tablestock), 6% was planted as seed, and less than 1% was used for animal feed. The feed use is limited by region, by season and is confined to a few integrated potato grower/processors or individual farmers. Shrinkage, loss, and home use represented the remaining 8% (National Potato Council, 1992).

C. Modern Potato Production and Potato Life Cycle

Cultivated potatoes are a clonally propagated crop, grown as an annual, with tubers from the previous year's crop serving as propagules. In the U.S., potato acreage is rotated with other crops on a cycle of two to five years. In most potato growing regions of the U.S., winters are severe enough to freeze and destroy tubers left after the harvest season, eliminating the possibility of escapes. However, in areas with heavy snow cover or mild winters, clonally generated volunteer potatoes are common and may persist for several years. The number of volunteers is reduced, but not eliminated by cultivation and herbicide usage in subsequent crops. Small grains are a common rotation crop and herbicides used in small grains are effective for reducing the number of volunteers. The rate of decline for volunteers has not been well documented but is highly dependent on the severity of the environment. Volunteers from true seed following berry production by fertile varieties will germinate for up to eleven years following seed production with a 40-50% reduction in emergence each year (Lawson, 1983). However, in the long term, potatoes are not competitive with other cultivated crop species and are even less competitive in noncultivated areas. There has been no documented case of cultivated potatoes (*S. tuberosum*) becoming a persistent weed outside of cultivated areas.

In wild species, the predominant method of propagation is also clonal (Hawkes, 1978). Sexual reproduction occurs readily, but is not obligatory and only occasionally results in viable hybrids populations. Nearly all potato species are at least partial outcrossers and require insects, in particular bumblebees, for pollination. Insects rarely visit flowers of cultivated species because they lack nectaries (Pavek, pers. comm.). This results in very limited pollen dissemination. In the only definitive study completed to date, Tynan *et al.* (1990) found that dispersal of pollen from transgenic plants did not occur outside a range of five meters.

D. Taxonomy of Genus *Solanum*

Potatoes belong to the family *Solanaceae* and the genus *Solanum*. This family comprises 2000 species and includes tomatoes, peppers, eggplant, tobacco, petunia and several forms of the weed commonly called nightshade (Benson, 1959). The genus *Solanum* contains more than 900 species (Correll, 1962; Hawkes, 1990). All potatoes cultivated in the U.S. belong to a single species, *Solanum tuberosum*. Native cultivated potatoes in South America are taxonomically divided among several species including *S. ajanhuiri, S. curtilobum, S. goniocalyx, S. x chaucha, S. phureja, S. tuberosum, S. stenotomum*, and *S. juzepczukii* (Bavyko, 1978). Most can be hybridized with *S. tuberosum*. Native cultivated types are found in Peru, Columbia, Ecuador, Bolivia, and Argentina with *S. tuberosum* subsp. *tuberosum* limited to Chile (Hanneman and Bamberg, 1986).

Only two close relatives of potato, *S. fendleri* and *S. jamesii*, occur naturally within the borders of the U.S. (Hawkes, 1990). They are considered close relatives because both are tuber bearing *Solanum* (section petota) with at least some possibility of producing hybrids with *S. tuberosum*. *S. fendleri* belongs to the series longipedicellata, is tetraploid, and has been found in Arizona, Colorado, New Mexico and Texas. It resides in dry forests at altitudes of 5,000 to 10,000 feet. *S. jamesii* belongs to the series pinnatisecta, is a diploid, and has been found in Arizona, Colorado, New Mexico, Texas and Utah. It resides in environments similar to those where *S. fendleri* is found.

Several other *Solanum* species are either native or introduced weeds in the U.S. including bitter nightshade (*S. dulcamara*), silverleaf nightshade (*S. elaeagnifolium*), black nightshade (*S. nigrum*), hairy nightshade (*S. sarrachoides*), cutleaf nightshade (*S. triflorum*), buffalobur (*S. rostratum*), and turkeyberry (*S. torvum*) (Whitson *et al.*, 1991). All of these are non-tuber bearing and will not hybridize with tuberizing *Solanum* species.

E. Genetics of Potato

The genetic structure, and crossability of potato species are important considerations in understanding the flow of genes from cultivated to wild species. A brief description follows.

1. Genetic structure

A basic chromosome number of 12 was established by Smith (1927) for the genus *Solanum*. Polyploidy is common in both wild and cultivated potatoes. Most species are diploid (73%), or tetraploid (15%), but triploids (4%), pentaploids (2%) and hexaploids (6%) have also been documented (Hawkes, 1990). Russet Burbank is an tetraploid.

The production of numerically unreduced gametes is common in many diploid cultivated and wild species (Camadro and Peloquin, 1980; Yerk and Peloquin, 1990). The result is a production of tetraploid progeny from diploid x tetraploid, tetraploid x diploid, or diploid x diploid crosses with a resultant transfer of genes from the diploid into the tetraploid population. Triploid potatoes are occasionally partially female fertile, producing a limited number of both n and 2n eggs. Triploids may also be crossed as pollen parents with cultivated tetraploids (Brown, 1988; Brown and Adiwilaga, 1990). These may act as triploid bridges, serving to allow gene flow in both directions (Jackson *et al.*, 1978). In nature, this is probably a rare event. Crosses of either tetraploids or 2n egg producing diploids with hexaploid species are usually easily made.

2. Crossability

Three major factors influence the crossability of species. The ploidy level, the endosperm balance number (EBN), and cross incompatibility. The ploidy level, as has been discussed above, restricts the frequency of interspecies hybrids and the direction of gene flow, but by and large, does not prevent such events.

EBN is a term given to the ratio of maternal to paternal genomes in the endosperm of a species. Crosses of species with unequal EBN's result in a nonviable endosperm, causing the embryo to abort. The result is a very effective hybridization barrier between many *Solanum* species. Most South American diploid species and nearly all tetraploid species, including *S. fendleri*, have an EBN of 2. *Solanum tuberosum*, a tetraploid, is an exception with an EBN of 4. Most Mexican diploids have an EBN of 1, including *S. jamesii* (Hanneman and Bamberg, 1986). The production of 2n gametes in 2 EBN diploids effectively doubles the EBN, allowing hybridization with *S. tuberosum* to occur. EBN is an important guideline for determination of crossability, however, many exceptions have been noted.

Most diploid species are self-incompatible due to the presence of S-alleles (Howard, 1970). Many closely related species are also cross incompatible because they share identical S-alleles. For reasons not completely understood, cultivated tetraploids and tetraploids derived from self-incompatible diploids show a weakened effect of the S-alleles and are usually self-compatible.

Hawkes (1990) cites evidence from a number of studies that hybrids between wild and cultivated, or between two wild species outside of the United States occur frequently in nature. However, the adaptability of the hybrids is poor and they rarely survive more than one or two seasons. Crosses of *S. tuberosum* with intrageneric species outside the section petota, such as with many types of nightshade, have been attempted, but no fertile progeny have been recovered (Dale *et al.*, 1992 and Rick, 1979).

3. Hybridization of potato with wild relatives

Within the borders of the 50 United States, no opportunity exists for gene flow from cultivated potatoes to wild species. None of the solanaceous weedy species growing in and around potato fields will hybridize with cultivated potatoes. *S. jamesii* and *S. fendleri* are the only closely related species that are endemic to the U.S. Both are very difficult to hybridize with *S. tuberosum* due to incompatible EBN's. The only documented hybrids have been created under carefully controlled conditions in a laboratory situation (Adiwilaga and Brown, 1991; Novy and Hanneman, 1991). *S. fendleri* is the most likely of the two to produce hybrids with *S. tuberosum* because the development of a 2n gamete will produce a compatible EBN. However, no 2n gametes have been reported *for S.*

fendleri. Any resulting progeny would be hexaploid with an EBN of 4 and would not be compatible parents for further hybridization with *S. fendleri*.

In addition to genetic incompatibility, the possibility of outcrossing is diminished due to geographical separation. Both *S. fendleri* and *S. jamesii* are found in high elevation, dry forest environments, isolated from all potato production areas. In the event an unlikely hybridization event does occur, the progeny probably will not be adapted to either environment and will not survive.

4. Hybridization of potato with other cultivated varieties

Other than the common occurrence of sterility, there is no genetic mechanism to prevent the hybridization of two cultivated varieties within the U.S. However, due to production methods, it is unlikely that gene transfer will occur in this manner. Pollen transfer occurs infrequently and over short distances. Tynan and his coworkers (1990) demonstrated no pollen dispersal in a field interplanted with genetically engineered and control potatoes beyond 4 - 5 meters and Dale *et al.* (1992) in a similar study, reported no pollen transfer beyond 10 meters. Hybrid seed that does occur is not used for further propagation and will remain in the field. If this seed germinates, long term propagation and survival of the resulting seedlings is not expected due to standard cultivation practices, and in fact has never been documented. In the event of self-pollination within a fertile variety containing the transgenes, germination of the resulting seed will present no more concern than clonal volunteers (J. Pavek, pers. comm.).

5. Escape of transgenic plant materials

Escape of plant materials will take the form of lost tubers. Other plant parts are not suitable for propagation. Once in commerce, tubers can and will be lost during all phases of the growing and marketing operations. The major recipient locations of lost tubers will be fields where the crop is grown, roadsides, and areas around buildings where the potatoes are stored and shipped. Given the non-competitive nature of potatoes in these locations, escape will be inconsistent and temporary. No unusual steps need be taken to control escape through vegetative plant parts.

6. Ecological impact of gene escape

If the transgenes escape into the environment in a persistent manner it is most likely to do so in Central or South American where appropriate wild species are present. Even there, gene movement into a diploid wild species is unlikely due to the infrequent flow of genes from tetraploids into diploids via triploid bridges, an event never documented in nature. Hybrids are more likely with tetraploid and hexaploid species, but in a native situation will likely be noncompetitive (Love, 1994).

F. Properties of the Non-transformed Cultivar Russet Burbank

Russet Burbank is the dominant potato cultivar produced in the U.S. and is estimated by the USDA to represent 50.9% of total Fall potato production of 1.14 million planted acres. This potato variety is grown primarily in the northern tier of the United States in the following states: Colorado, Idaho, Maine, Michigan, Minnesota, North Dakota, Oregon, Washington, and Wisconsin (National Potato Council, 1993).

Multiple end uses make Russet Burbank unique among the major cultivars with good consumer quality for boiling, and excellent for baking and french fry processing. Russet Burbank is classified as a table and processing variety. Principal markets include the fresh market and processing trades for the manufacture of french fries. It is the standard of french fry quality on the North American continent. A smaller percentage of Russet Burbank production, mostly that which does not meet the quality standards for the fresh market and processing, is utilized for dehydration and cattle feed.

- **Parentage**: Parentage goes back to the variety Burbank. Luther Burbank was the breeder of Burbank variety which was released in 1874 (from seed ball from cv. Early Rose). Lon D. Sweet selected the russeted mutation (Barkley and Schrage, 1993).

- **Description**: Russet Burbank tubers are long with numerous well distributed shallow eyes. It has a russeted and heavily netted skin with white flesh. Russet Burbank plants are medium size and spreading with few white flowers.

- **Characteristics**: Russet Burbank is a male sterile, tetraploid potato variety. It is a high yielding, high specific gravity potato with a very late maturity. It is resistant to common scab and *Fusarium* storage rot, and is highly resistant to blackleg. It is highly susceptible to potato virus Y and potato leaf roll virus (which causes tuber net necrosis). It produces a heavy set of tubers and is normally spaced at 12 to 14 inches when planted for seed and 14 to 16 inches when planted for table and processing. Russet Burbank production requires a regular moisture supply to avoid second growth (Barkley and Schrage, 1993).

III. DESCRIPTION OF TRANSFORMATION SYSTEM

The seven transgenic Russet Burbank potato lines were developed by transforming one Russet Burbank parental line (RB1) with either the PV-STMT21 or PV-STMT22 using the *Agrobacterium tumefaciens* transformation system.

The *A. tumefaciens* transformation method used in the generation of the seven transformed Russet Burbank lines has been reviewed by Klee and Rogers (1989). The transformation vectors PV-STMT21 and PV-STMT22 contain well-characterized DNA segments required for selection and replication of the plasmid vector in bacteria and transfer of the transgenes into plant cells. The vectors were assembled in *E. coli* MV1190, a derivative of the common laboratory *E. coli* K-12 strain (Bachmann, 1987) and mated into the *Agrobacterium* strain ABI. The ABI strain contains the disarmed pTi58 plasmid pMP90RK which does not carry the T-DNA phytohormone genes (Koncz and Schell, 1986). Therefore, the *Agrobacterium* is unable to cause crown gall disease and is no longer considered a threat as a plant pest (Huttner *et al.*, 1992). The pMP90RK plasmid was engineered to provide the *trfA* gene functions required for autonomous replication of the plasmid vector after conjugation into the ABI strain. The *Agrobacterium* strain ABI containing the plant expression vector PV-STMT21 or PV-STMT22 was added to potato stem sections (Newell *et al.*, 1991) in tissue culture dishes. The transgene expression cassettes, which include the *cry3A*, *PLRVrep* and *nptII* genes or the *cry3A*, *PLRVrep* and *CP4 EPSPS* genes, were transferred into the genome of individual potato cells thereby allowing selection in either kanamycin or glyphosate, respectively. After a few days, the remaining viable *Agrobacterium* cells were killed using carbenicillin and cefotaxime. Prior to planting any new line, the plants were grown on medium without antibiotics for two weeks, then a sample of tissue from each plant was placed in LB broth and shaken a 25°C. The plant was rejected if bacteria is found in either of these assays. Subsequently, the potato tissues were treated to stimulate regeneration of transgenic cells into shoots. Ultimately platelets were grown in soil and assayed for CPB and PLRV resistance.

IV. THE DONOR GENES AND REGULATORY SEQUENCES

Genetic elements contained in the vectors PV-STMT21 (Fig. IV.1) and PV-STMT22 (Fig. IV.2) 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 Plant Vectors

Two plant transformation vectors were used in the generation of Transformed Russet Burbank potatoes, PV-STMT21 and PV-STMT22. These vectors differ only in the selectable maker gene. Plant vector PV-STMT21

employs the *nptII* gene which allows selection of transformed plants in a kanamycin containing medium. Plant vector PV-STMT22 employs the *CP4 EPSPS* gene which allows selection of transformed plants in a medium containing glyphosate.

PV-STMT21 and PV-STMT22, are double border binary transformation vectors. They contain well-characterized DNA segments required for selection and replication of the plasmid in bacteria as well as right and left borders for delineating the region of DNA (T-DNA) to be transferred into the plant genomic DNA. The host for all DNA cloning and vector construction was the *E. coli* MV1190, a derivative of the common laboratory *E. coli* K-12 strain (Bachmann, 1987). These vectors are composed of the following genetic elements. The first segment is the 0.45 Kb fragment from the octopine Ti plasmid, pTi15955 (a *Cla*I to *Dra*I restriction fragment), which contains the T-DNA left border region (Barker *et al.*, 1983). This is joined to the 1.3 Kb origin of replication (*oriV*) region derived from the broad-host range RK2 plasmid (Stalker *et al.*, 1981). The next segment (*ori-322/rop*) is a 1.8 Kb segment of pBR322 plasmid which provides the origin of replication for maintenance in *E. coli* and the *bom* site for the transfer by conjugation into the *A. tumefaciens* cells (Bolivar *et al.*, 1977; Sutcliffe, 1978). This is fused to the 0.93 Kb fragment isolated from transposon Tn7 which encodes the 0.79 Kb *aad* gene that allows for bacterial selection on spectinomycin or streptomycin (Fling *et al.*, 1985) which is fused to a 0.36 Kb *Pvu*I to *Bcl*I restriction fragment from the pTiT37 plasmid containing the nopaline-type T-DNA right border region (Depicker *et al.*, 1982).

Three chimeric genes, with signals for plant expression, were introduced between the right and left border regions of the plant transformation vectors. For vector PV-STMT21, the chimeric genes and their components are as follows. The chimeric gene for selection of transformed plant cells on kanamycin (NOS/*nptII*/NOS 3') which consists of the promoter region of the nopaline synthase gene from Ti plasmid of *A. tumefaciens* (Fraley *et al.*, 1983), the neomycin phosphotransferase type II (*nptII*) gene (Beck *et al.*, 1982) 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 the control of CPB (AraSSU1A/*cry3A*/NOS 3') which consists of the *Arabidopsis 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). And, 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).

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 enzyme (Barry *et al.*, 1992) and the 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). And, 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*

The *cry3A* gene, present in vectors PV-STMT21 and PV-STMT22 and introduced in the transformed Russet Burbank, was isolated from the DNA of *B.t.t.*, strain BI 256-82 (Krieg *et al.*, 1983). This gene encodes the protein which is responsible for the control of CPB. A full length clone and the complete nucleotide sequence has been reported by McPherson *et al.* (1988) and Perlak *et al.* (1993). The *cry3A* gene encodes a protein of 644 amino acids with a molecular weight of 73 kD which is produced by the bacterium during sporulation. This protein has insecticidal properties with selective activity against a narrow spectrum of Coleoptera (Sims, 1993; MacIntosh *et al.*, 1990). Upon ingestion by susceptible species, feeding is inhibited with disruption of the gut epithelium, which results in the eventual death of the insect (Slaney *et al.*, 1992). In addition to the 73 kD full-length protein, the *B.t.t.* bacterium also produces a smaller form of this protein called *B.t.t.* band 3. The *B.t.t.* band 3 protein has a molecular weight of 68 kD (597 amino acids) which results from an internal translational initiation event within the same gene starting at amino acid 48 (McPherson *et al.*, 1988; Perlak *et al.* 1993). This protein has been shown to possess the same insecticidal potency and selectivity to CPB larvae as the full-length protein (McPherson *et al.*, 1988). The gene encoding the *B.t.t.* band 3 protein, modified with potato preferred codons for increased plant expression, was introduced in the Transformed Russet Burbank potato plants. The plant-preferred codon modifications changed 399 out of 1791 nucleotides within the gene which codes for the *B.t.t.* band 3 protein, without altering any of the encoded amino acids. This modified gene encodes the identical amino acid sequence of band 3 protein as produced by the *B.t.t.* bacterium (Perlak *et al.*, 1993).

2. *PLRVrep* gene

The *PLRVrep* gene, introduced for PLRV resistance, was isolated from a cDNA library made from RNA extracted from purified virions of PLRV isolate LR-7 (Table IV.1), a naturally occurring isolate obtained from USDA-ARS in Prosser, Washington, USA (Thomas, 1986). Using the nomenclature of Miller et al., 1995), the *PLRVrep* gene comprises the two overlapping ORF1 and ORF2 sequence of the PLRV genome which together encode the full length 110 kD PLRV replicase protein (Miller *et al.*, 1995; Murphy *et al.*, 1995; van der Wilk *et al.*, 1989). It is believed that ORF1/ORF2 of the PLRV genome functions as a helicase and RNA-dependent RNA polymerase, respectively, in plants infected with PLRV (van der Wilk *et al.*, 1989). Both the putative helicase and polymerase enzymes are produced from a single mRNA. Translation of the replicase (ORF2) portion of the protein is believed to occur by a -1 translational frameshift of the ribosome on the viral RNA (Prufer *et al.*, 1992; Kujawa *et al.*, 1993). The context of this gene as a transgene for *in vivo* frameshifting was best demonstrated by Pufer et al. (1992). They demonstrated that the expression of a chimeric PLRV ORF1/ORF2 frameshift region fused to a β-glucuronidase (GUS) open reading frame would create a GUS product at ~1% efficiency in potato protoplasts. Transcription of this fusion construct was driven by the CaMV 35S promoter similar to the FMV 35S promoter driving the PLRV ORF3 and ORF2 in transgenic lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262. The FMV 35S promoter is stronger than that of the CaMV 35S promoter used by Pufer *et al.* (1992). However, the efficiency of frameshifting is not expected to change as a result of possibly more mRNA being transcribed by the FMV promoter. The *in vitro* analysis of the frameshifting efficiency of PLRV ORF1/ORF2 frameshift region-GUS fusion gene in rabbit reticulocyte lysate was also observed by Pufer et al. (1992) to be about 1%. Similar *in vitro* experiments conducted in rabbit reticulocyte lysate and wheat germ extracts were also able to establish that the frameshifting occurred and was dependent on the slippery site and the presence of a pseudoknot structure in the mRNA (Kujawa *et al.* 1993). They observed a frameshift efficiency of ~30% with the unmodified PLRV frameshift region made as a fusion with the chloramphenicol acetyltransferase (CAT) gene. Because PLRV ORF1 and ORF2 in the transgenic plant lines are the full-length native sequence, translational frameshifting is expected to occur with the transgene as has been demonstrated in transient and *in vitro* systems by two independent research groups. The conclusions of these two research groups is that the RNA sequence and structure in the frameshift region is capable of producing a -1 frameshift. The frameshift results in the translation of a fusion protein which would continue until a translational stop codon occurs in frame. The expected products of

translation of the PLRV ORF1/ORF2 in the transgenic potato lines would be a ~70 kD protein and a ~110 kD protein. The efficiency of this frameshifting in transgenic plant lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 has not been determined, however, frameshifting between ~1% and possibly as high as 30% is possible.

TABLE IV.1. DESCRIPTION OF PLRV DONOR ORGANISM FOR *PLRVrep*.

Characteristic	Description	Reference(s)
Taxonomic Name	Potato leafroll luteovirus isolate LR7	Murphy et al, 1995; Thomas et al., 1995
Nucleic Acid Type	ssRNA	Murphy et al, 1995
Localization	Phloem Enhanced	van den Heuvel et al., 1995
Association with any satellite or helper viruses	None	OECD, 1996
Natural Host Range	Solanaceous plants, mostly potato	Murphy et al, 1995; Thomas et al., 1995
Means of Transmission	Aphid [primarily green peach aphid (*Myzus persicae* (Sulzer)]	Murphy et al, 1995; Thomas et al., 1995
Mode of Transmission	Persistent	Murphy et al, 1995; Thomas et al., 1995
Known Synergy	None	OECD, 1996
Origin of Virus	Infected potato plant in Washington State, USA	Thomas et al., 1995

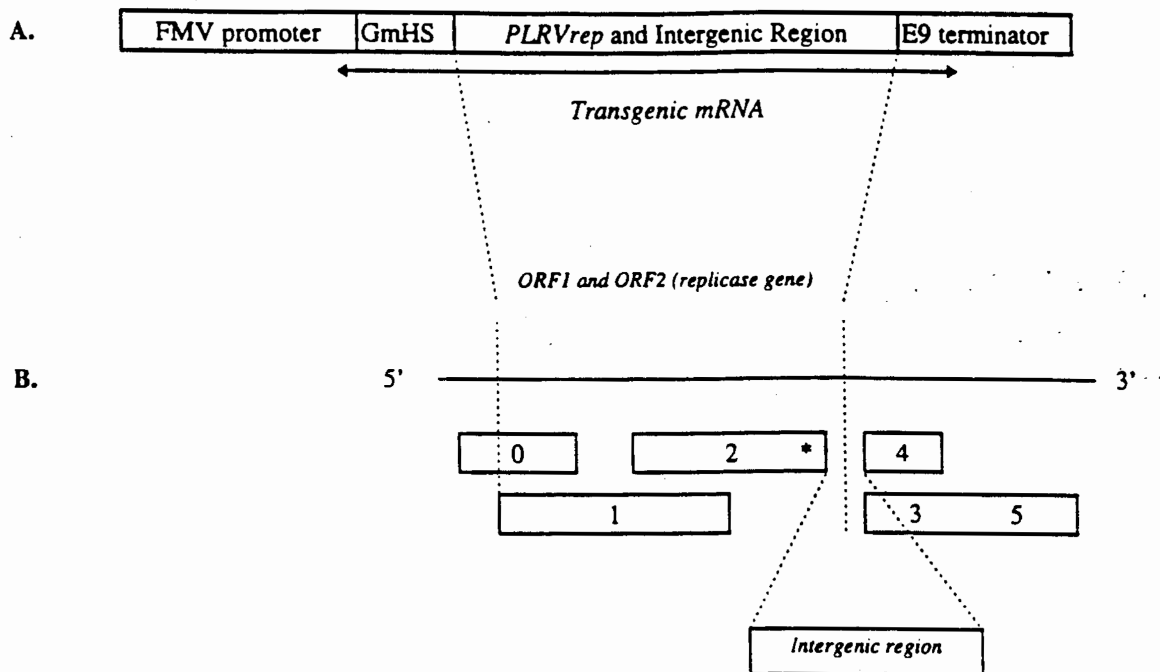

FIGURE IV.1. SCHEMATIC OF THE PLRV GENOME ORGANIZATION AND THE SECTION OF THE GENOME CLONED INTO PV-STMT21 AND PV-STMT22.
A. PLRV replicase expression cassette transformed into the potato genome (boxes) and the corresponding mRNA (line under boxes). See text for details.
B. PLRV RNA genomic organization and portion cloned in PV-STMT21 and PV-STMT22. Presumed function of Open Reading Frames (ORFs): 0, unknown; ORF , helicase; 2, polymerase; 3, coat protein; 4, putative "movement-like" protein; 5, vector transmission (Martin *et al.*, 1990).
* denotes subgenomic promoter (ACAAAAGA) located at the 3' end of ORF2 (Mayo and Ziegler-Graff, 1996).

3. *NptII* gene and its encoded NPTII protein

The *nptII* gene, present in plant vector PV-STMT21 was isolated from the prokaryotic transposon Tn5 present in *E. coli* (Beck *et al.*, 1982). The *nptII* gene encodes the NPTII protein which functions as a dominant selectable marker in the initial laboratory stages of plant cell selection following transformation (Horsch *et al.*, 1984; DeBlock *et al.*, 1984). The NPTII protein uses ATP to phosphorylate neomycin and the related kanamycin, thereby inactivating these aminoglycoside antibiotics and preventing them from killing the cells producing the NPTII protein. The sole purpose of inserting the *nptII* gene into potato cells with the *cry3A* and *PLRVrep* genes is to have an effective method of selecting cells that contain the insect and virus-resistant genes. In general, the frequency of cells that are transformed is often as low as 1 in 10,000 or 1 in 100,000 of the cells treated (Fraley *et al.*, 1984). Therefore to facilitate this process, a selectable marker gene *nptII* and selective agent kanamycin are used. Consequently, cells selected for plant regeneration contain the *nptII*, *cry3A* and *PLRVrep* genes.

4. *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). As is the case with the *nptII* gene, the *CP4 EPSPS* gene also 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. As was the case for the *nptII* gene, 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.

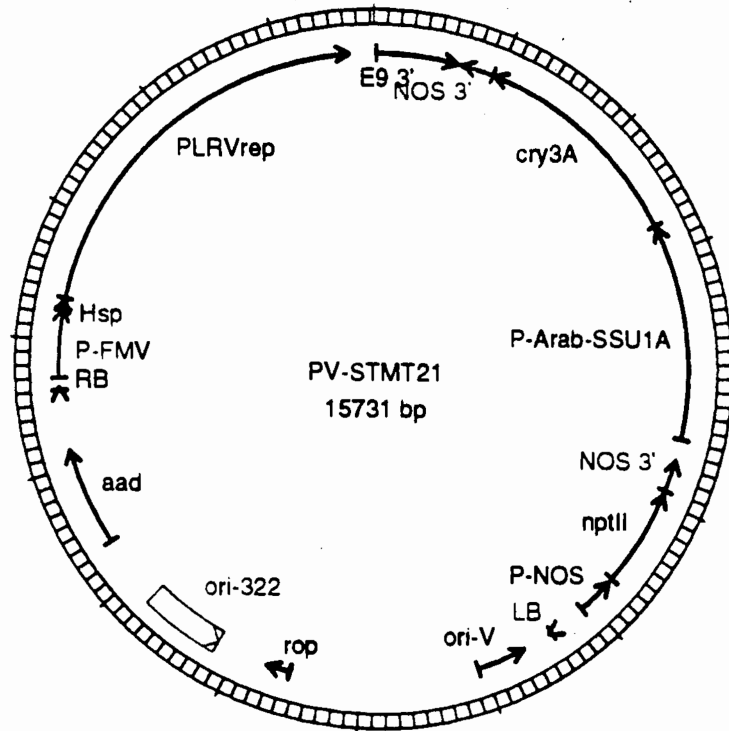

FIGURE IV.1. PLASMID MAP OF PV-STMT21.
See text in Section IV for details.

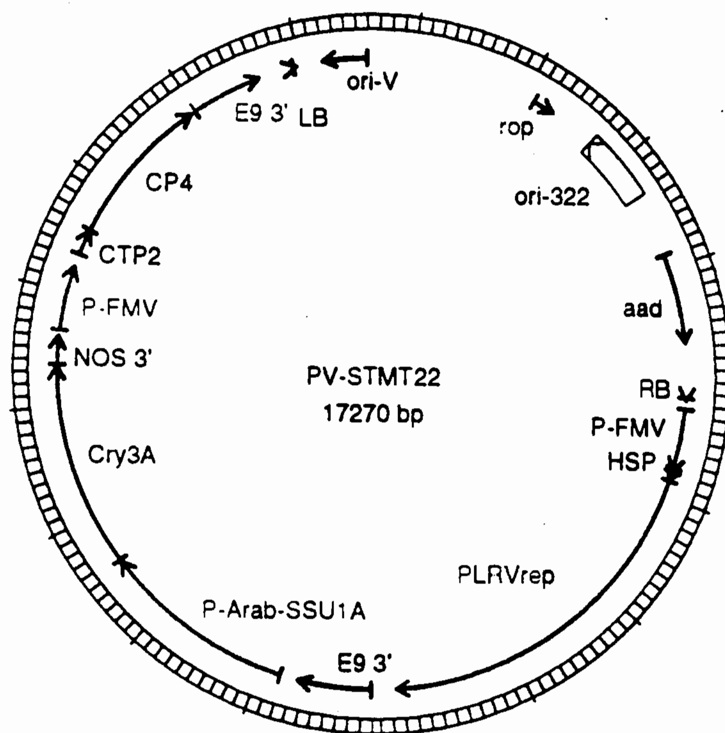

FIGURE IV.2. PLASMID MAP OF PV-STMT22.
See text in Section IV for details.

Table IV.1. Summary of DNA Components in PV-STMT21 and 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).
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 PRLV 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 (rbcS) 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[1]	0.3	The promoter region of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens* (Fraley *et al.*, 1983).
nptII[1]	0.79	The gene isolated from Tn5 which is present in *E. coli.* The gene encodes neomycin phosphotransferase II. Expression of this gene in plant cells confers kanamycin resistance and serves as a selectable marker for transformation (Fraley *et al.*, 1983).
CTP2		The 0.23 Kb chloroplast transit peptide leader sequence from the Arabidopsis thaliana EPSPS gene (CTP2) (Klee et al. 1987).
CP4[2]	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-STMT21 or PV-STMT22 plasmids in *E.coli* (*rop*) and *bom* site for conjugational transfer into *Agrobacterium* (Bolivar *et al.*, 1977; Sutcliffe, 1978).

[1] Present only in vector PV-STMT21. [2] Present only in vector PV-STMT22.

V. GENETIC ANALYSIS, AGRONOMIC PERFORMANCE AND COMPOSITIONAL ANALYSIS

A. Genetic Analysis

Product characterization studies were carried out on the transgenic Russet Burbank potato plant lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262. The studies included characterization of the DNA inserted, protein or RNA presence, and levels of proteins expressed in the recipient plants.

1. Characterization of inserted DNA

DNA was isolated from 0.5 - 1.5 g of frozen leaf tissue by grinding using a mortar and pestle. The DNA was precipitated with isopropanol and purified by ultracentrifugation in a cesium chloride gradient (Rochester and Lavrik, 1997). Polymerase chain reaction was used to characterize the DNA inserted into transformed potato lines. Each PCR reaction contained approximately 100 ng genomic DNA, 40 pmoles of each PCR primer, 45 µl of PCR Supermix (GIBCO BRL, Gaithersburg, MD, Catalog No. 10572-014) in a total reaction volume of 50 µl. In general, PCR conditions were as follows: 1 cycle of 96°C, 5 minutes; 35 cycles of 96°C, 45 seconds, "Anneal Temp" 30 seconds, 68°C, 4 minutes; 1 cycle of 68°C, 10 minutes. Following thermocycling samples were stored at 4°C until analysis by gel electrophoresis. At times, in order to optimize amplification, the "Anneal Temp" for different primer sets varied between 44°C and 64°C. The Anneal Temp" used for each primer set is shown in Table V.1. For the *oriV* and *aad* primer sets, no PCR product was expected following amplification. Therefore, separate reactions using annealing temperatures of 44°C, 48°C, 52°C, 56°C, 60°C, and 64°C were used. This data was generated to insure that specific annealing temperature parameters used in the study would not give negative results under conditions in which the specific genetic element was present in the genome.

TABLE V.1. PCR PRIMERS

Primer 1[a]	Size (n.t.)	Primer 2[1]	Size (n.t.)	Annealing Temp (°C)
P1	24	P2A	27	54
B1	27	B2	24	60
C1	23	C2	27	60
PB3	19	SS2	29	56
SS1	29	PB2	23	60
BC1	19	BC2	25	58
O1	19	02	21	44-64
S1	24	S2	29	44-64

[a]Primer position is shown in Figures V.1. And V.6.

Data on the DNA inserted into transformed potato lines are shown in Figures V.1 - V.11. Characterization of inserted DNA was carried out on genomic DNA isolated from young leaf tissue from each of the seven transgenic Russet Burbank lines. The isolated DNA was analyzed by polymerase chain reaction (PCR) reaction. The analysis defined the genetic elements which were transferred from either plasmid PV-STMT21 or PV-STMT22 to the genome of the potato lines and the linkage between the three genetic elements (*PLRVrep*, *cry3A*, and *nptII* or *CP4 EPSPS*). Additionally, the presence of PLRVrep mRNA, Cry3A and CP4 EPSPS proteins, and kanamycin

resistance demonstrates that the expression cassettes for each gene are intact and functional. PCR analysis was also used to establish the absence of genetic elements outside the left and right borders (i.e. *aad* and *oriV*) as defined by the plasmid vectors.

2. Characterization of Expressed Proteins

Cry3A, NPTII, 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. The NPTII protein characterization is based on the identity of the *nptII* gene which was introduced in the *E. coli* which produced the NPTII reference standard and in the transformed lines.

PLRV Replicase

RNA Analysis
For lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 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, appx. 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 (Ausubel et al., 1993). A random-labeled ^{32}P-dCTP probe was made from PRLV 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 appx. 3.8-3.9 kb, the expected size for the full-length replicase mRNA (Figure V.12.). No hybridization was seen with the RNA from the Russet Burbank control line. This datum 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.13 and V.14, respectively. *PLRVrep* RNA transcript was not detected by dot hybridization analysis in either leaf or tuber tissue of the seven transgenic lines in this assay. Based on the amount of *PLRVrep* RNA standard spotted (Lane 1, Figure V.12 and V.13), 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. Since the average amount of RNA isolated from leaf and tuber of the seven transgenic lines corresponded to 840 ng/mg leaf fresh weight and 190 ng/mg tuber fresh weight, the limits 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 seven transgenic lines.

Protein Analysis
To assay for the presence of the PLRV replicase (combined helicase and polymerase) protein, the PLRV 1.3 kb ORF1 gene was cloned into *E. coli* as a N-terminal histidine-thrombin-S-tag fusion protein to allow for affinity

purification and identification of the expressed ORF1 protein. Both the helicase from ORF1 and the putative RNA-dependent-RNA polymerase from ORF2 are made as a fusion protein resulting from a translational frameshift at a frequency of ~1 to as high as 30%; therefore, the molar amount of helicase translated is much higher compared to the polymerase. The helicase portion of the protein may be synthesized independently of the RNA-dependent RNA polymerase, but never vice-versa. The modified ORF1 protein was isolated using a nickel column and then purified using PAGE. Amino acid sequence analysis of trypic digests of the isolated protein confirmed that the recovered protein was produced from the ORF1 gene.

To produce anti-ORF1 protein antiserum, two rabbits each were injected (Vaitukaitus et al., 1982) with 300 µg of the protein. Additional injects were made monthly over a 7-month period. Serum (25 ml per rabbit) was collected 10-12 days post-injection and stored at -20°C. Antiserum titer was evaluated using western blot analysis. The serum showing the highest band intensity using the ORF1 protein as the standard was chosen for future analysis.

Protein was extracted from leaf samples using an aqueous extraction buffer [100 mM Tris, 1 Mm EDTA, 25 mM KCl, 20% glycerol, 5 mM ascorbic acid, pH 8.0, with protease inhibitors (Boehringer Mannheim, Indianapolis, IN, cat. No. 1697498)] at a 1:1 tissue to volume ratio. Extracts were centrifuged to pellet cellular debris and the supernatant fluid was diluted using SDS-polyacrylamide reducing sample buffer (0.0625 M Tris-Cl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, pH 6.8). The equivalent of approximately 10 mg (fresh weight) of tissue was subjected to electrophoresis. The samples were separated by electrophoresis using a 4-20% gradient SDS PAGE gel. The proteins were transferred from the SDS PAGE gel to a nitrocellulose membrane (transfer buffer: 25 mM Tris, 0.192 M glycine, 20 % methanol) for one hour. The membrane was blocked for one hour using 3% BSA in Tris saline-Tween (10 mM Tris, 150 mM NaCl, 0.05 % Tween-20, pH 8.0). The membrane was probed with rabbit anti-ORF1 protein antibody at a 1:1000 dilution and specific binding detected using alkaline-phosphatase labeled goat anti-rabbit antiserum and BCIP color development.

No immunoreactive bands of the expected molecular weight in tissue from transgenic or PLRV-infected Russet Burbank control lines were observed. However, the purified protein standard was detected. Repeating this experiment yielded similar results. Therefore, even though Northern analysis demonstrates that the full-length mRNA is present in the plant, no protein was detected. Difficulty in detection or the inability to detect replicase protein in transgenic or virus-infected plants is not uncommon. Similar results have been reported in the literature for other viral replicases (Carr et al., 1992; 1994; Sijen et al., 1995; and Tenllado et al., 1995).

3. Levels of Expressed Proteins

The level of *Cry3A*, NPTII and CP4 EPSPS proteins expressed in leaf and tuber tissues of the transformed Russet Burbank plant lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 were determined using enzyme-linked immunosorbent assays. The tissues were obtained from potato plants grown in field trials during the summer of 1995 at Ephrata, WA; Echo, OR and Pasco, WA. Leaf samples were collected at approximately ten weeks post-planting, tuber samples were collected at harvest. In these field trials, each line was replicated eight times. Four to seven plots were selected from each site for estimation of *Cry3A* and NPTII or CP4 EPSPS protein expression levels. The expression levels were estimated in potato tissue extracts using validated enzyme linked immunosorbent assays (ELISAs), which are highly specific sandwich assays developed and validated to estimate the concentration of the respective protein in extracts derived from potato tissues. The ELISAs employ highly specific antibodies which recognize only B.t.t. (Dean, 1993), NPTII (Grace and Rogan, 1995); or CP4-EPSPS proteins EPSPS (Grace and Lavrik, 1996) in extracts. The assay systems are capable of measuring these proteins in crude tissue extracts and are amenable to high sample throughput. These analytical methods are robust and the most appropriate procedures to use for estimation of the expression levels of either B.t.t., NPTII or CP4-EPSPS proteins in potato tissue extracts. The protein reference standard for each ELISA was produced and purified from E. coli. The three reference substances for this experiment were: (a) B.t.t. protein produced in E. coli Batch No. 5192101, Lot No. 5002056; (b) NPTII protein standard produced in E. coli, Lot No. 4821020, Batch No. 5524618; and (c) CP4-EPSPS produced in E. coli, Lot No. 5192245. The antibodies used for each ELISA were produced by immunization of animals using the appropriate purified antigen. Specific details describing antibodies which are used in each ELISA are described in the technical report for each assay.

The mean Cry3A protein expression level in leaf tissue of the seven lines collected across three sites was estimated to be in the range of 12.8 to 29.9 µg/g tissue fresh weight (Table V.2.). The mean Cry3A protein expression level in tuber was estimated to be in the range of 0.08 to 0.63 µg/g tissue fresh weight. The Cry3A protein expression levels correspond to a range of 0.08 to 0.2% of total foliage protein and 0.0004 to 0.003% of total tuber protein, using total protein levels of 1.6 and 2.0% for foliage and tuber fresh weight, respectively.

The mean CP4 EPSPS protein expression level in leaf tissue of four lines transformed with PV-STMT22 (RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262) collected across three sites was estimated to be in the range of 1.6 to 28.3 µg/g tissue fresh weight (Table V.3.). The mean tuber CP4 EPSPS protein expression level was estimated to be in the range of 0.04 to 0.72 µg/g tissue fresh weight. The CP4 EPSPS protein expression levels correspond to a range of 0.01 to 0.2% of total foliage protein and 0.0002 to 0.004% of total tuber protein, using total protein levels of 1.6 and 2.0% for foliage and tuber fresh weight, respectively.

The expression level of NPTII protein in leaf and tuber tissue of the three lines transformed with PV-STMT21 (RBMT21-129, RBMT21-152, and RBMT21-350) was below the detection limits of the assay (0.3 ng/g tissue fresh weight). However, recovery of the transformed lines from medium containing 200 µg/ml of kanamycin demonstrates that the NPTII expression cassette is intact and that functional protein is synthesized.

As discussed above in section V.A.2., the PLRV replicase has not been detected in any of the transgenic plant lines.

Overall, the Cry3A protein expression in the transgenic potato lines is comparable to the corresponding expression levels in tissue of previously registered NewLeaf® Atlantic plants which utilizes the same promoter (the *A. thaliana* ribulose-1,5-bisphosphate carboxylase small subunit *ats1A* promoter) to control the expression of the *cry3A* gene. The NPTII protein expression in leaves and tubers of lines RBMT21-129, RBMT21-152, and RBMT21-350 is much lower than previously registered NewLeaf® cvs. Russet Burbank, Atlantic and Superior plants. The difference in NPTII protein expression reflects the strength of promoters used in these plants. Previously registered lines utilize the Cauliflower mosaic virus 35S promoter to regulate the NPTII protein expression; whereas, the lines RBMT21-129, RBMT21-152, and RBMT21-350 utilize the nopaline synthase promoter (NOS).

Table V.2. CRY3A Protein Expression in Tissue of Russet Burbank Lines transformed with either PV-STMT21 or PV-STMT22: Combined Multi-site Data

Line	Tissue[2]	Number of Sites[1] (Reps)	Least Square Mean[5]	Standard Error[6]	Range[7] µg/g tissue fresh wt.
RBMT21-129	Leaf	3 (18)	12.81	1.099	9.12 -16.53
	Tuber	3 (12)	0.35	0.220	0.11 - 0.90
RBMT21-152	Leaf[3]	2 (13)	28.85	0.317	21.59 - 39.99
	Tuber[3]	2 (8)	0.63	0.102	0.42 - 0.80
RBMT21-350	Leaf	3 (18)	20.54	3.101	7.71 - 35.66
	Tuber[4]	3 (11)	0.28	0.124	0.12 - 0.61
RBMT22-82	Leaf	3 (18)	20.97	2.061	14.97 - 26.61
	Tuber	3 (12)	0.63	0.058	0.49 - 0.79
RBMT22-186	Leaf	3 (18)	19.42	1.192	14.40 26-61
	Tuber	3 (12)	0.63	0.058	0.49 - 0.79
RBMT22-238	Leaf[3]	2 (13)	19.42	1.192	14.40 - 26.61
	Tuber[3]	2 (8)	0.08	0.003	0.05 - 0.10
RBMT22-262	Leaf	3 (18)	29.91	3.397	18.56 - 45.26
	Tuber	3 (12)	0.53	0.218	0.00 - 0.85
RB-Control	Leaf	3 (18)	0.01[8]	0.007	0.00 - 0.07
	Tuber	3 (12)	0.03[8]	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]Plant lines RBMT21-152 and RBMT22-238 were planted at two sites only: Ephrata, WA and Pasco, WA.
[4]Tuber expression for line RBMT21-350 from Echo, OR was determined on three replicates.
[5]Overall mean for each line is the simple mean of the site means.
[6]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.
[7]"Range" denotes the highest and lowest individual assay sample for each line across sites.
[8]Value is within the background level of the assay.

TABLE V.3. CP4 EPSPS PROTEIN EXPRESSION IN TISSUE OF RUSSET BURBANK LINES TRANSFORMED WITH EITHER PV-STMT21 OR PV-STMT22: COMBINED MULTI-SITE DATA

Line	Tissue[2]	Number of Sites[1] (Reps)	Least Square Mean[4]	Standard Error[5]	Range[6]
RMBT22-82	Leaf	3 (18)	10.84	1.024	6.82 - 16.07
	Tuber	3 (12)	0.53	0.103	0.21 - 0.78
RBMT22-186	Leaf	3 (18)	10.84	1.024	6.82 - 16.07
	Tuber	3 (12)	0.57	0.027	0.42 - 0.80
RBMT22-238	Leaf[3]	2 (13)	1.60	0.205	0.99 - 2.32
	Tuber[3]	2 (8)	0.04	0.000[8]	0.02 -0.05
RBMT22-262	Leaf	3 (18)	11.29	0.861	6.73 - 15.55
	Tuber	3 (12)	0.72	0.056	0.55 - 1.39
RB-Control	Leaf	3 (18)	0.07[7]	0.039	0.00 - 0.23
	Tuber	3 (12)	0.02[7]	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]Plant line RBMT22-238 was planted at two sites only: Ephrata, WA and Pasco, WA.
[4]Overall mean for each line is the simple mean of the site means.
[5]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.
[6]"Range" denotes the highest and lowest individual assay sample for each line across sites.
[7]Value is within the background noise level of the assay.
[8]Site means identical to four decimal places, which resulted in estimate of standard error being very close to zero.

4. Conclusions

Transgenic Russet Burbank potato plant lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT21-129, RBMT21-152 and RBMT21-350 contain three introduced genes which encode for the CPB-active protein from *B.t.t.*, the *PLRVrep* gene which imparts resistance to potato leafroll virus and either the *nptII* or *CP4 EPSPS* genes which served as a selectable marker during the potato transformation process. The *cry3A*, *nptII* or *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. The *PLRVrep* gene present is identical to the gene present in naturally occurring isolates of PLRV which are prevalent in commercial available potatoes.

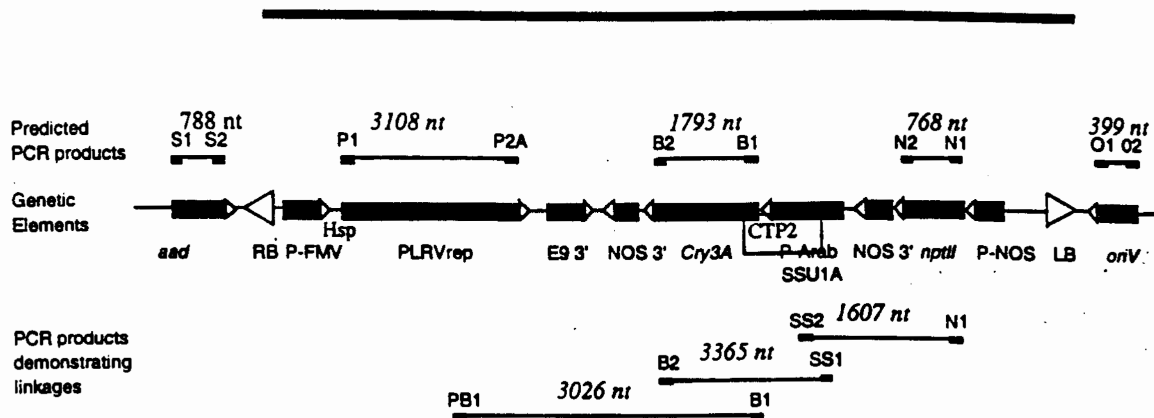

FIGURE V.1. SCHEMATIC REPRESENTATION OF PREDICTED PCR PRODUCTS FROM LINES TRANSFORMED WITH PV-STMT21.

Detailed map of the portion of the PV-STMT21 (Fig. IV.1) containing all the genetic elements (T-DNA) expected to be integrated as well as elements flanking the right border (*aad*) and left border (*oriV*). The expected T-DNA, delineated by the right and left borders, is indicated by the line above the map. Directly above the map is a representation of PCR products expected using primer sets designed to amplify specific elements. Below the map is a representation of PCR products from reactions designed to assess linkages between genetic elements. The italicized values are the expected size of the PCR product in nucleotides (nt). Hsp and CTP2 (Table IV.1) are leader sequences for the *PLRVrep* and *Cry3A* genes, respectively. Figure not to scale.

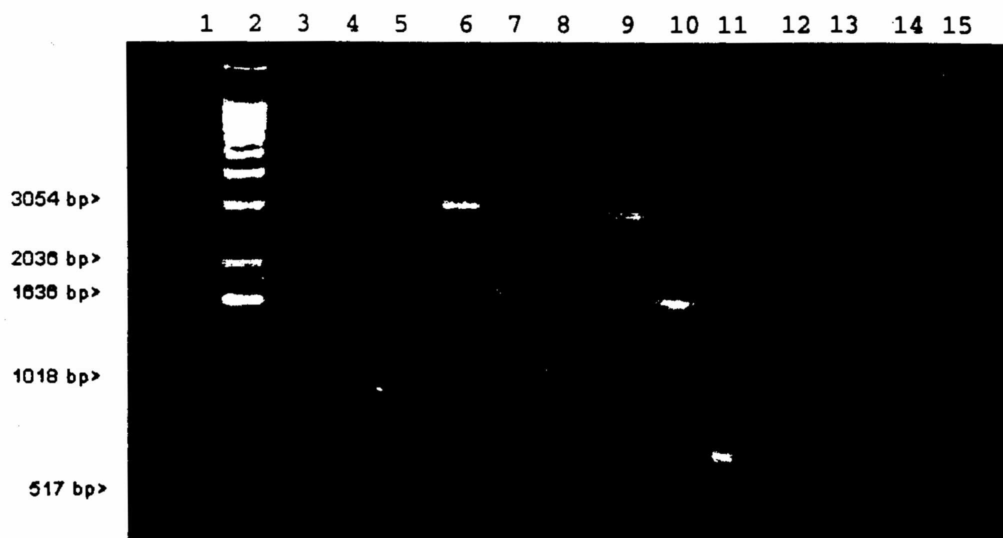

FIGURE V.2. PCR ANALYSIS OF INTEGRATED GENETIC ELEMENTS IN LINE RBMT21-350.
Photograph of agarose gel electrophoresis of PCR products following amplification of genomic DNAs using primers for specific genetic elements. Lane 2 contains molecular weight markers. PCR products from amplification of control genomic DNA from nontransformed tissue (Lanes 3-5), RBMT21-350 (Lanes 6-8), and control genomic DNA from nontransformed tissue plus PV-STMT21 plasmid (9-11). Lanes 3, 6, and 9 contain PCR products from amplification with primers directed toward the *PLRVrep* gene (Fig. V.1, P1-P2A). Lanes 4, 7, and 10 contains PCR products from amplification with primers directed toward the *cry3A* gene (Fig. V.1, B2-B1). Lanes 5, 8, and 11 contains PCR products from amplification with primers directed toward the *nptII* gene (Fig V.1, N2-N1). Lanes 1 and 12-15 are empty.

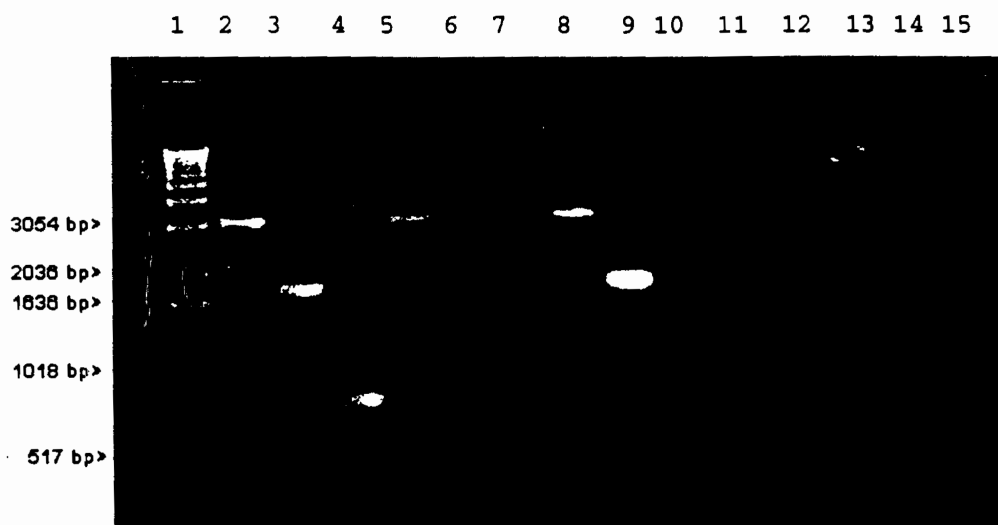

FIGURE V.3. PCR ANALYSIS OF INTEGRATED GENETIC ELEMENTS IN LINES RBMT21-129 AND RBMT21-152. Photograph of agarose gel electrophoresis of PCR products following amplification of genomic DNA using primers for specific genetic elements. Lane 1 contains molecular weight markers. PCR products from amplification of control genomic DNA from nontransformed tissue plus PV-STMT21 plasmid DNA (Lanes 2-4), RBMT21-129 genomic DNA (Lanes 5-7), RBMT21-152 genomic DNA (Lanes 8-10). Lanes 2, 5, and 8 contain PCR products from amplification with primers directed toward the *PLRVrep* gene (Fig. V.1, P1-P2A). Lanes 3, 6, and 9 contain PCR products from amplification with primers directed toward the cry3A gene (Fig. V.1, B2-B1). Lanes 4, 7, and 10 contain PCR products from amplification with primers directed toward the nptII gene (Fig V.1, N2-N1). Lanes 11-15 are empty.

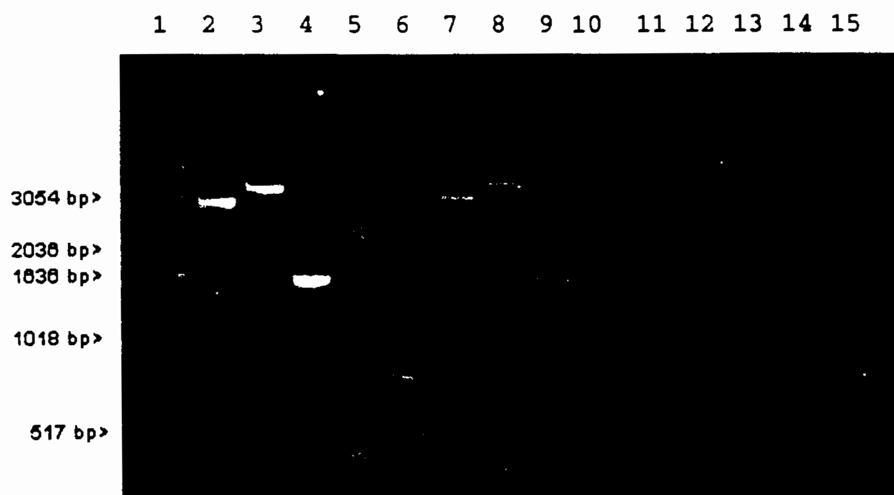

FIGURE V.4. PCR ANALYSIS OF GENETIC ELEMENT LINKAGE AND ELEMENTS OUTSIDE THE BORDERS IN LINE RBMT21-129.

Photograph of gel electrophoresis of PCR products following amplification of genomic DNAs using specific primers to test linkage between integrated genetic elements and presence of sequences flanking the borders. Lane 1 contains molecular weight markers. Lanes 2-6 contains the PCR product from amplification of control genomic DNA from nontransformed tissue plus PV-STMT21 plasmid. Lanes 7-11 contain the PCR product from amplification of line RBMT21-129 genomic DNA. Lanes 2 and 7 contain PCR products from amplification with primers to ascertain linkage between the *PLRVrep* and *cry3A* genes (Fig V.1, PB1 -B1). Lanes 3, 4, 8, and 9 contain PCR products from amplification with primers to ascertain linkage between the *cry3A* and *nptII* genes (Fig V.1, B2-SS1 and SS2-N1). Lanes 5 and 10 contain PCR products from amplification with primers directed toward *aad* specific sequences (Fig V.1, O1-O2). Lanes 6 and 11 contain PCR products from amplification with primers directed toward *oriV* specific sequences (Fig V.1, S1-S2). Lanes 12-15 are empty.

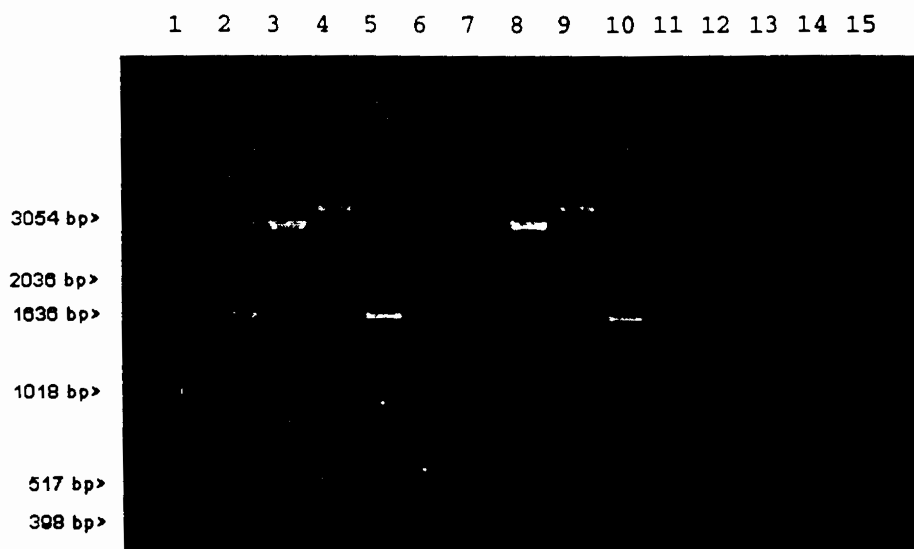

FIGURE V.5. PCR ANALYSIS OF GENETIC ELEMENT LINKAGE AND ELEMENTS OUTSIDE THE BORDERS IN LINES RBMT21-152 AND RBMT21-350.

Photograph of gel electrophoresis of PCR products following amplification of genomic DNAs using specific primers to test linkage between integrated genetic elements and presence of sequences flanking the borders. Lane 2 contains molecular weight markers. Lanes 3-7 contain the PCR products from amplification of RBMT21-152 genomic DNA. Lanes 8-12 contain the PCR products from amplification of RBMT21-350 genomic DNA. Lanes 3 and 8 contain PCR products from amplification with primers to ascertain linkage between *PLRVrep* and *cry3A* genes (Fig V.1, PB1-B1). Lanes 4, 5, 9, and 10 contain PCR products from amplification with primers to ascertain linkage between *cry3A* and n*ptIII* genes (Fig V.1, B2-SS1 and SS2-N1). Lanes 6 and 11 contain PCR products from amplification with primers directed toward *aad* specific sequences (Fig V.1, S1-S2). Lanes 7 and 12 contain PCR products from amplification with primers directed toward *oriV* specific sequences (Fig V.1, O1-O2). Lanes 1 and 13-15 are empty.

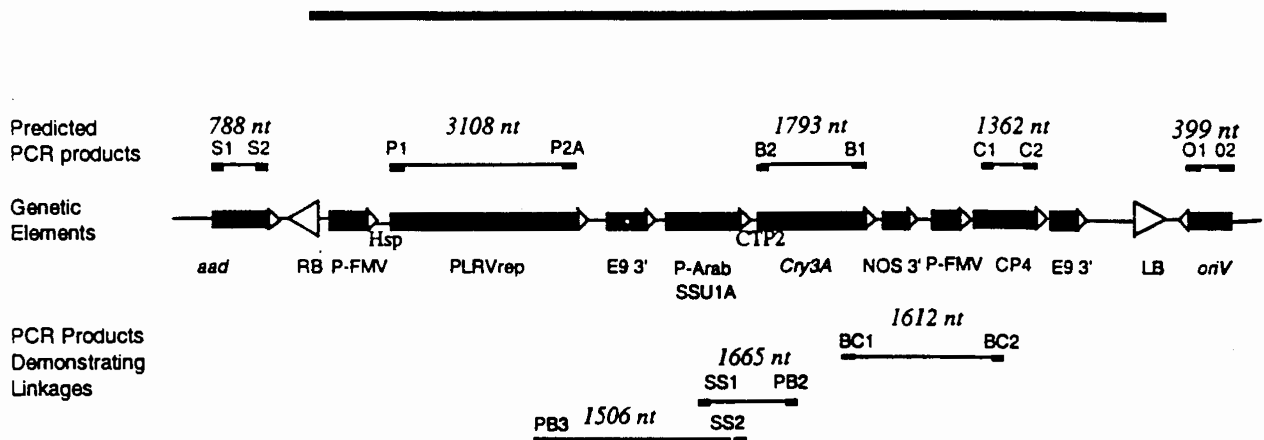

FIGURE V.6. SCHEMATIC REPRESENTATION OF PREDICTED PCR PRODUCTS FROM LINES RBMT22-82, RBMT22-186, RBMT22-238 AND RBMT22-262 TRANSFORMED WITH PV-STMT22.

Detailed map of the portion of the PV-STMT22 (Fig. IV.2) containing all the genetic elements (T-DNA) expected to be integrated as well as elements flanking the right border (*aad*) and left border (*oriV*). The expected T-DNA delineated by the right and left borders is indicated by the line above the map. Directly above the map is a representation of PCR products expected using primer sets designed to amplify specific elements. Below the map is a representation of PCR products from reactions designed to assess linkages between genetic elements. The italicized values are the expected size of the PCR product in nucleotides (nt). Hsp and CTP2 (Table IV.1) are leader sequences for the *PLRVrep* and *Cry3A* genes, respectively. Figure not to scale.

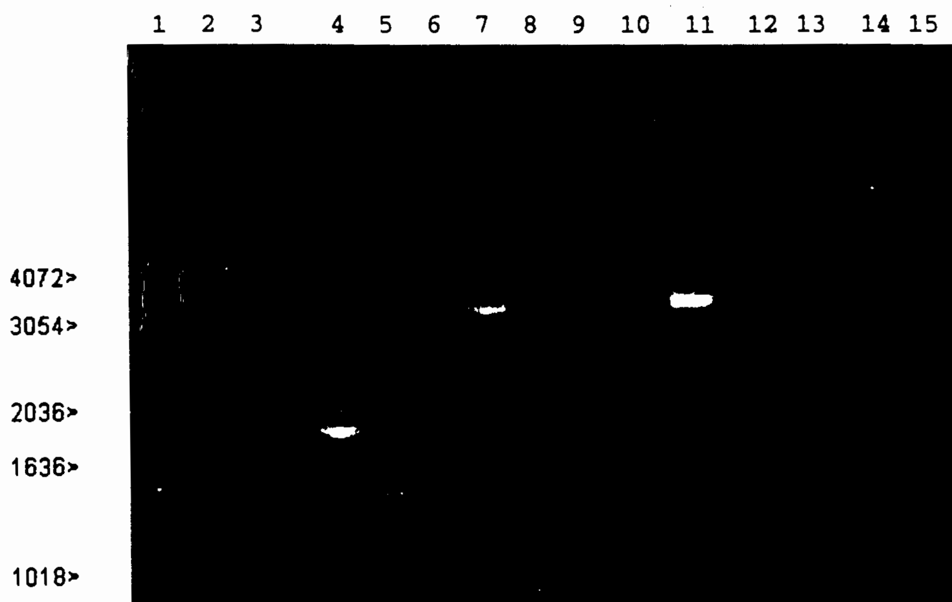

FIGURE V.7. PCR ANALYSIS OF INTEGRATED GENETIC ELEMENTS IN LINES RBMT22-82 AND RBMT22-186. Photograph of agarose gel electrophoresis of PCR products following amplification of genomic DNAs using primers for specific genetic elements. Lane 1 contains molecular weight markers. PCR products from amplification of genomic DNA from nontransformed tissue plus PV-STMT22 plasmid DNA (Lanes 3-5), RBMT22-82 (Lanes 7-9), RBMT22-186 (Lanes 11-13). Lanes 3, 7, and 11 contain PCR products directed toward the *PLRVrep* gene (Fig V.6, P1-P2A). Lanes 4, 8, and 12 contain PCR products directed toward the *cry3A* gene (Fig V.6, B2-B1). Lanes 5, 9, and 13 contain PCR products directed toward the CP*4* gene (Fig V.6, C1-C2). Lanes 2, 6, 10, 14 and 15 are empty.

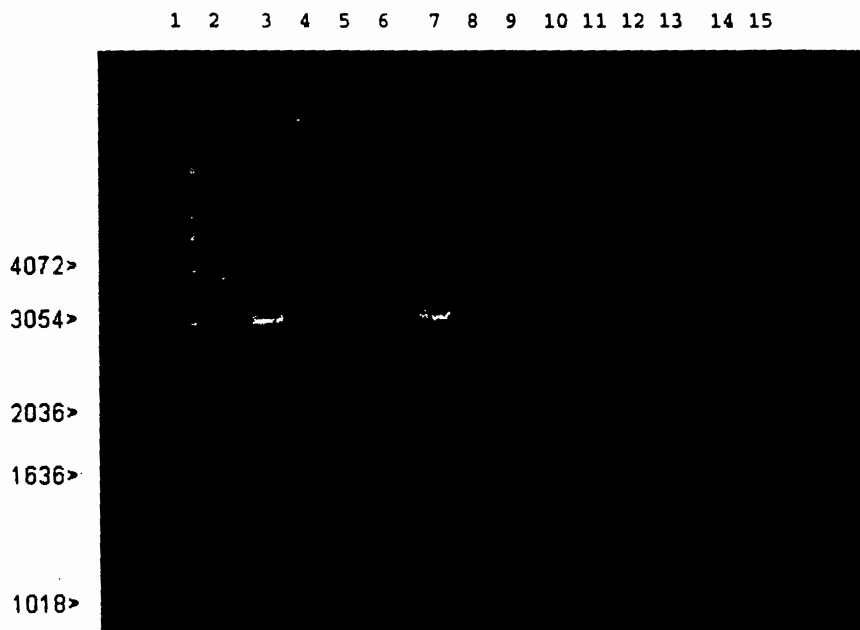

FIGURE V.8. PCR ANALYSIS OF INTEGRATED GENETIC ELEMENTS IN LINES RBMT22-238 AND RBMT22-262. Photograph of agarose gel electrophoresis of PCR products following amplification of genomic DNA using genetic element specific primers. Lane 1 contains molecular weight markers. PCR products from amplification of genomic DNA from RBMT22-238 (Lanes 3-5), RBMT22-262 (Lanes 7-9) and from nontransformed tissue (Lanes 11-13). Lanes 3, 7, 11 contain PCR products from amplification with primers directed toward the *PLRVrep* gene (Fig V.6, P1-P2A). Lanes 4, 8, and 12 contain PCR products from amplification with primers directed toward the *cry3A* gene (Fig V.6, B2-B1). Lanes 5, 9, and 13 contain PCR products from amplification with primers directed toward the *CP4* gene (Fig V.6, C1-C2). Lanes 2, 6, 10, 14, and 15 are empty.

FIGURE V.9. PCR ANALYSIS OF GENETIC ELEMENT LINKAGE AND ELEMENTS OUTSIDE THE BORDERS IN LINE RBMT22-82.

Photograph of gel electrophoresis of PCR products following amplification of genomic DNAs using specific primers to test linkage between integrated genetic elements and presence of sequences flanking the borders. Lane 1 contains molecular weight markers. PCR products from amplification of genomic DNA from nontransformed tissue plus PV-STMT22 plasmid DNA (Lanes 2-6) and RBMT22-82 genomic DNA (Lanes 7-11). Lanes 2, 3, 7, and 8 contain PCR products from amplification with primers directed toward *PLRVrep/cry3A* linkage (Fig V.6, PB3-SS2 and SS1-PB2). Lanes 4 and 9 contain PCR products from amplification with primers directed toward *cry3A*/CP*4* linkage (Fig V.6, BC1-BC2). Lanes 5 and 10 contain PCR products from amplification with primers directed toward the *aad* gene (Fig V.6, S1-S2). Lanes 6 and 11 contain PCR products from amplification with primers directed toward the *oriV* sequence (Fig V.6, O1-O2). Lanes 12-15 are empty.

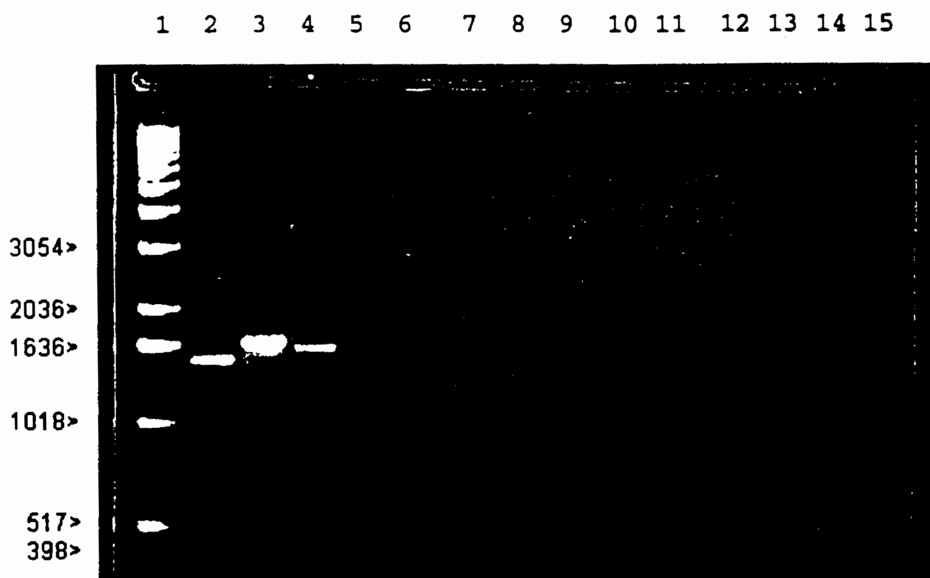

FIGURE V.10. PCR ANALYSIS OF GENETIC ELEMENT LINKAGE AND ELEMENTS OUTSIDE THE BORDERS IN LINE RBMT22-262.

Photograph of agarose gel electrophoresis of PCR products following amplification of genomic DNAs using specific primers to test linkage between integrated genetic elements and presence of sequences flanking the borders. Lane 1 contains molecular weight markers. Lanes 2-6 contain the PCR products from amplification of genomic DNA from line RBMT22-262. Lanes 2 and 3 contain PCR products directed toward *PLRVrep/cry3A* linkage (Fig V.6, PB3-SS2 and SS1-PB2). Lane 4 contains the PCR product directed toward *cry3A*/*CP4* linkage (Fig V.6, BC1-BC2). Lane 5 contains the PCR product directed toward the *aad* gene (Fig V.6, S1-S2). Lane 6 contains PCR products directed toward *oriV* specific sequences (Fig V.6, O1-O2). Lanes 7-15 are empty.

FIGURE V.11. PCR ANALYSIS OF GENETIC ELEMENT LINKAGE AND ELEMENTS OUTSIDE THE BORDERS IN LINES RBMT22-186 AND RBMT22-238.

Photograph of agarose gel electrophoresis of PCR products following amplification of genomic DNAs using specific primers to test linkage between integrated genetic elements and presence of sequences flanking the borders. Lane 1 contains molecular weight markers. Lanes 2-6 contain the PCR products from amplification of genomic DNA from line RBMT22-186. Lanes 7-11 contain the PCR products from amplification of line RBMT22-238 genomic DNA. Lanes 2, 3, 7, and 8 contain PCR products directed toward the *PLRVrep/cry3A* linkage (Fig V.6, PB3-SS2 and SS1-PB2). Lanes 4 and 9 contain PCR products directed toward the *cry3A*/CP*4* linkage (Fig V.6, BC1-BC2). Lanes 5 and 10 contain PCR products directed toward the *aad* gene (Fig V.6, S1-S2). Lanes 6 and 11 contain PCR products directed toward the *oriV* specific sequences (Fig V.6, O1-O2). Lanes 12-15 are empty.

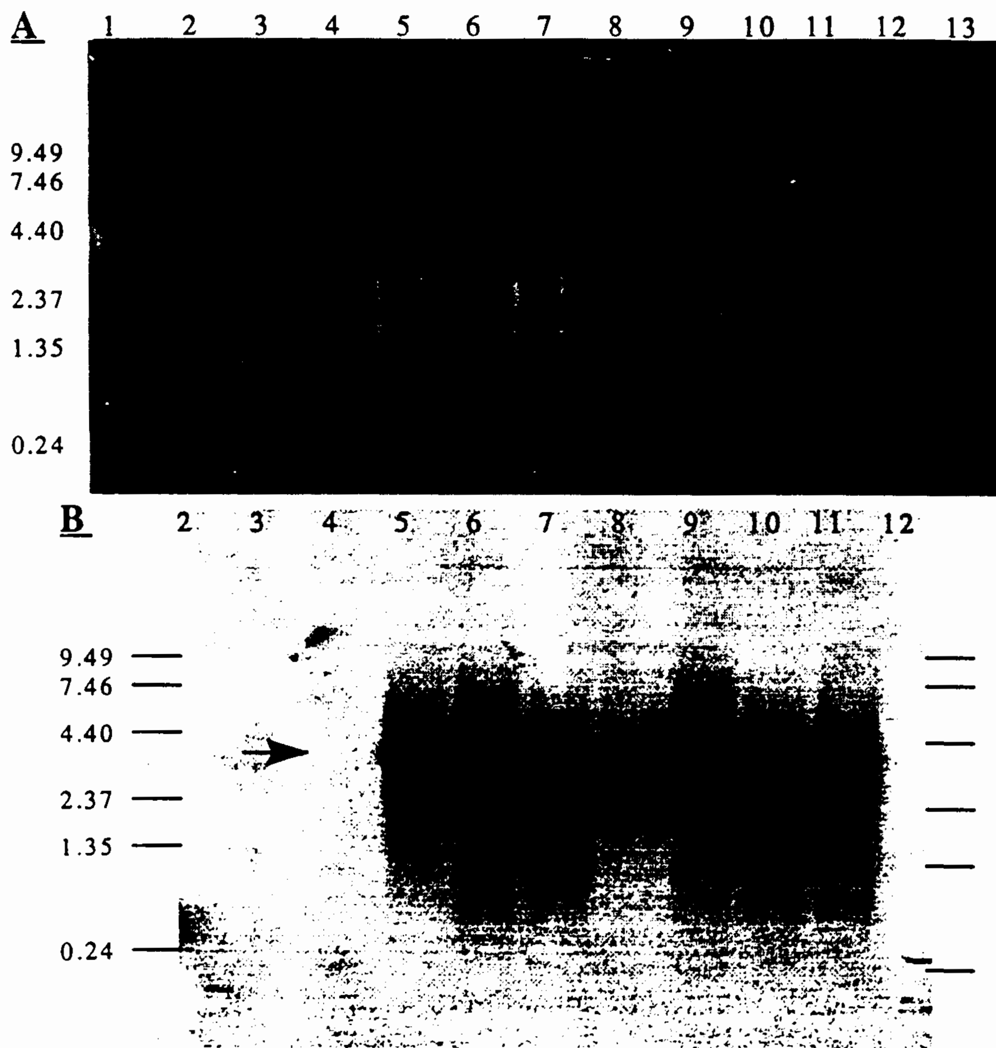

FIGURE V.12. NORTHERN ANALYSIS OF *PLRVREP* TRANSGENE MRNA.
Lanes: **1**, RNA molecular weight markers from BRL; **2**, empty; **3**, Russet Burbank control line; **4**, empty; **5**, line RBMT22-82; **6**, line RBMT21-152; **7**, line RBMT21-129; **8**, line RBMT22-238; **9**, line RBMT22-262; **10**, line RBMT21-350; **11**, line RBMT22-186; **12**, empty; 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.

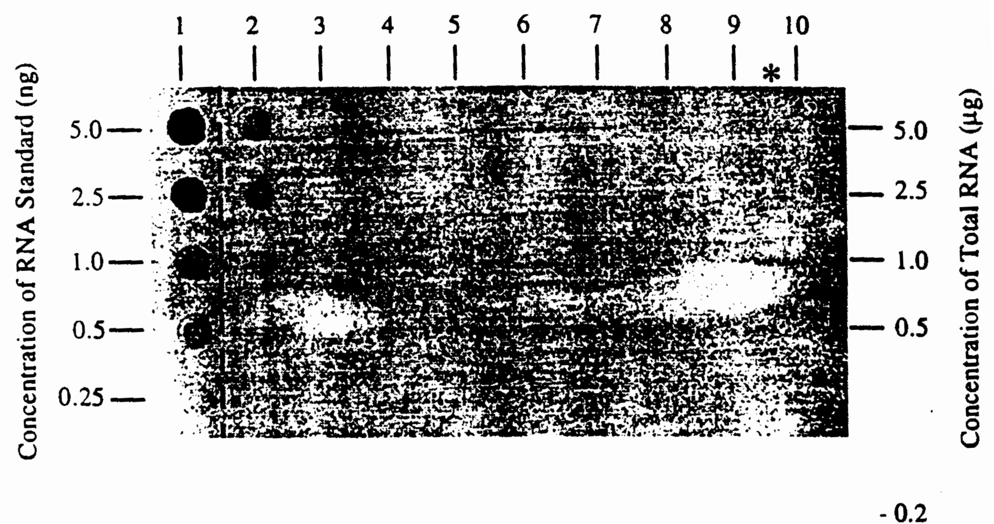

FIGURE V.13. LEAF RNA DOT HYBRIDIZATION ANALYSIS.
Lane 1 contains sense *PLRVrep* standard spiked into total RNA from control non-infected Russet Burbank plants. Lane 2 contains RNA from control Russet Burbank plants infected with PLRV. Lane 3 contains RNA from control uninfected Russet Burbank plants. Lanes 4 through 10 contain RNA from Transgenic lines RBMT21-129, RBMT 21-152, RBMT 21-350, RBMT 22-82, RBMT 22-186, RBMT 22-238 and RBMT 22-262, respectively. 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.

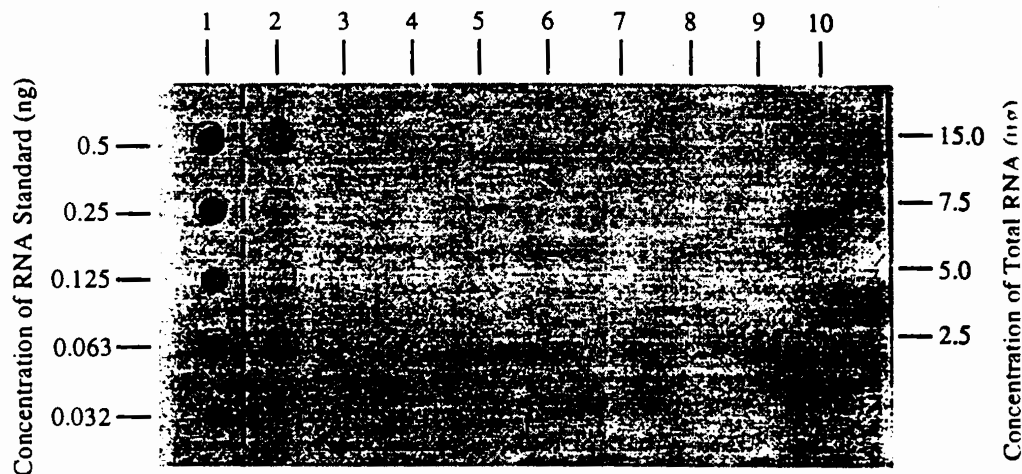

FIGURE V.14. TUBER RNA DOT HYBRIDIZATION ANALYSIS.
Lane 1 contains sense *PLRVrep* standard spiked into total RNA from control non-infected Russet Burbank plants. Lane 2 contains RNA from control Russet Burbank plants infected with PLRV. Lane 3 contains RNA from control uninfected Russet Burbank plants. Lanes 4 through 10 contain RNA from Transgenic lines RBMT21-129; RBMT 21-152, RBMT 21-350, RBMT 22-82, RBMT 22-186, RBMT 22-238 and RBMT 22-262, respectively. 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 lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 were evaluated in the field from 1994 - 1997 under USDA permits 93-362-01r, 94-217-02R, 94-342-01r, 96-277-01r, and 97-017-03r (in progress). The lines selected for commercialization are agronomically comparable to unmodified Russet Burbank potatoes and are additionally highly resistant to the Colorado potato beetle and the potato leafroll virus (see USDA Final Reports, Appendix 7; and Certification of NewLeaf Plus Russet Burbank Potatoes in the U.S. and Canadian Seed Certification Programs Appendix 2). During field trial evaluating of the transgenic potato lines, the potato plants were monitored for any unusual susceptibility to potato insect pests or pathogens (Table V.4.). 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 U.S. Leafhoppers were seen but mostly in the midwestern states (MN and WI). However, except for the intended PLRV and CPB resistance traits in the transgenic lines, no differences in susceptibility to insect pests or diseases between the transgenic and control lines were noted.

TABLE V.4. INSECTS AND DISEASE SYMPTOMS WHICH WERE LOOKED FOR DURING MONTHLY SCOUTING OF TRANSGENIC POTATO FIELD TRIALS.

Organism or Pathogen	Disease or Symptoms
Insect	
Emposasca fabae (Potato leafhopper)	Leaf feeding damage
Epitrix species (Flea Beetle)	Shotholes in leaves
Leptinotarsa decemlineata (Colorado Potato Beetle)	Defoliation
Limonius californicus (Wireworm)	Bored holes in tubers and shoots
Ostrinia nubilalis (European Corn Borer)	Sever vine wilting above point of injury
Paratrioza cockerelli (Potato Psyllid)	Yellows
Phthorimaea operculella (Tuberworm)	Foliar and tuber damage
Various aphid species	Leaf suckling damage
Virus and Virus-Like	
Aster Yellows MLO	Purple top disease
Potato Leafroll Virus	Rolling of leaves and net necrosis
Potato Spindle Tuber Viroid	Potato spindle tuber disease
Potato Virus A,M,X,Y	Mosaic symptoms
Tobacco Rattle Virus	Stem mottling
Bacteria and Fungi	
Erwinia carotovora	Blackleg, Aerial stem rot and Tuber soft rot
Corynebactium sepedonicum	Bacterial ring rot
Phytophthora infectans	Late blight
Verticillium spp.	Early dying
Sclerotinia sclerotiorum	Sclerotinia stalk rot
Rhizoctonia solani	Canker
Streptomyces scabies	Scab
Fusarium sp.	Dry rot
Phytophthora and Pythium	Water rot
Nematodes	
Globodera rostochiensis	Cysts
Meloidogyne ep.	Root knot
Paratrichodorus sp.	Stubby root
Pratylenchus sp	Root lesions

C. Compositional Analysis

Monsanto is consulting with FDA on the compositional analysis of potato tubers produced from the seven transformed lines. 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.4. GLYCOALKALOID CONTENT OF TUBERS FROM SEVEN 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.[1]

Line	Total Glycoalkaloids, mg/100g FW
RBMT21-129	7.8
RBMT21-152	6.7
RBMT21-350	8.2
RBMT22-82	9.0
RBMT22-186	7.9
RBMT22-238	9.4
RBMT22-262	6.6
RB Control	8.3
SE (mean)	2.93
SE (difference)	0.79
Literature Reported Ranges	3.1 to 16.1[2]

[1]Statistical analyses and combined least square means were obtained using a randomized complete block model. Underlined values are significantly different from the control at the 5% level ($p < 0.05$).

[2]Data obtained from tubers grown in Aberdeen, ID and is the averages from six individual trials (Pavek *et al.*, 1980-1992). Sinden and Webb (1972) reported a range for Russet Burbank of 3 - 39 mg/100g.

VI. ENVIRONMENTAL AND POTENTIAL ADVERSE CONSEQUENCES OF INTRODUCTION

NewLeaf Plus potatoes are resistant to the Colorado potato beetle and the Potato leafroll virus. The ecological consequences of both traits will be discussed.

A. Weediness of Colorado Potato Beetle and PLRV resistant Potatoes

NewLeaf Plus potatoes have been field test under USDA permits since 1993. As reported in the field data reports (Appendix 7), these plants have not exhibited any weedy tendencies compared to non-transgenic control plants. As discussed in section II above, potatoes plants do not have any inherent weediness. Although potatoes may arise as volunteers from tuber remaining in the field after harvest, these potatoes are easily managed and are not considered to be weeds. The stacked engineered traits of Colorado potato beetle resistance and potato leafroll virus resistance do not change the weediness of potatoes. NewLeaf potatoes which contain only the CPB resistant trait have been grown commercially in the U.S. and Canada since 1995. There have been no reports of increased weediness in those lines. Farmers typically applied insecticides to control damage from CPB and PLRV in the same field. There are no reports that control of both pests results in the plant becoming more "weedy".

B. Impact of Colorado Potato Beetle Resistance

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. NewLeaf potato lines express between 7.7 and 40 ppm of Cry3A (Table V.2.). This level is 24 - 125-fold and 9.6 - 50-fold higher than the level required to provide complete protection against neonate and adult CPB, respectively. As expected, Monsanto has seen no damage from CPB in any NewLeaf potato fields during the two years of commercial production. In field trials of NewLeaf Plus potatoes, lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 have performed equally as well. Monsanto has not observed any damage by CPB in any NewLeaf Plus field trial (Appendix 7).

Overall Safety and Impact on Non-target Organisms

The Cry3A protein has been reviewed in previous submissions to the USDA and EPA. USDA, APHIS came to a Finding Of No Significant Impact to the Environment for the potato plants expressing the Cry3A protein (USDA, 1995; 1996). Similarly, the Environmental Protection Agency has granted an Exemption from the Requirement of a Tolerance for the Cry3A protein (EPA, 1995b). In data evaluated by the EPA and USDA in those previous submissions, Monsanto demonstrated that Cry3A protein is non-toxic to non-target organisms including larval and adult honeybee, ladybird beetle, green lacewing, parasitic wasp, Collembola spp., earthworm, mice and bobwhite quail.

Impact on the Agriculture and the Environment

Use of NewLeaf Plus for CPB control alone is expected to dramatically reduce the overall amount of pesticides applied to potato fields. In commercial of NewLeaf potatoes (CPB resistant trait only), the total number of all pesticide treatments, active ingredient per acre, and formulation lb. per acre were all reduced compared on non-

NewLeaf potatoes (Table VI.1.). Monsanto also showed a secondary benefit of potatoes expressing Cry3A to control CPB. In field trials, beneficial predaceous and parasitic arthropods such as big-eyed bugs, damsel bugs, minute pirate bugs, hymenoptera spp., and spiders were significantly more abundant in the NewLeaf potato plots than in those treated with conventional chemical insecticides to control the Colorado potato beetle (USDA Petition 94-257-01p). As insect resistance management continues to be a high priority for Monsanto. We continue to consult with the EPA to ensure that biotechnology for insect resistance is used responsibly and will remain as a viable tool for agriculture in the future.

TABLE VI.1. 1996 COMPARATIVE ANALYSIS OF INSECTICIDE USAGE IN COMMERCIAL NEWLEAF POTATO FIELDS.

Farms using NewLeaf program[3]	Farms	NewLeaf[1]			Non-NewLeaf[2]			Reduction %		
		Trts[4]	ai/acre[5]	Form. lb/acre[6]	Trts[4]	ai/acre[5]	Form. lb/acre[6]	Trts[4]	ai/acre[5]	Form. lb/acre[6]
Avg. all regions	35	1.77	1.91	8.07	3.12	2.43	8.90	1.35	0.52	0.83
MN, NB	3	1.67	1.29	2.62	3.33	1.89	5.63	1.67	0.60	3.01
MN, ND, WI	15	2.58	1.73	3.46	3.85	1.60	4.12	1.27	-0.12	0.66
Idaho	14	0.53	1.58	13.62	1.56	3.25	16.00	1.03	1.67	2.38
OR, WA	4	3.25	4.53	13.23	4.00	6.16	24.09	0.75	1.63	10.86

[1]NewLeaf Russet Burbank variety.
[2]Traditional non-engineered Russet Burbank variety.
[3]Farms which grew non-engineered Russet Burbank and NewLeaf Russet Burbank using management practices as recommended by Monsanto/NatureMark.
[4]The average number of treatments per field.
[5]The active ingredient of insecticide used per acre.
[6]The pounds of formulated insecticide used per acre.

C. *Impact of Potato Leafroll Virus Resistance*

Control of PLRV

In addition to the Colorado potato beetle resistance trait, the NewLeaf Plus potato lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 are highly resistant to the potato leafroll virus. NewLeaf Plus potato lines 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 VI.2). 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. 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. The control Russet Burbank plants averaged 67.5% leafroll infection in four location-year test sites. PLRV symptoms were distinct and severe on all infected controls. PLRV infection was verified on a smaller sub-set of these samples by either ELISA testing of greenhouse or field plant-back material. In all cases, symptoms of PLRV read in the field matched the plant-back ELISA results. None of the transgenic lines tested exceeded the 10% PLRV symptom criteria set for viable commercial clones at the beginning of the study.

	Percent of plants infected with PLRV				
	Parma, ID		Prosser, WA		Ave. 4
Clone	1994	1995	1994	1995	Loc-Yrs
21-129	0	0	0	8	2.0
21-152	5	3	0	0	2.0
21-350	5	0	0	3	1.9
22-082	5	0	0	3	1.9
22-186	0	0	0	0	0.0
22-238	20	0	10	5	8.8
22-262	0	0	15	3	4.5
R. Burbank	61	99	47	63	67.5

Overall Safety and Impact on Non-target Organisms

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. Monsanto is consulting with FDA on the nutritional qualities of the potatoes. Data show that the potato are substantially equivalent to non-engineered Russet Burbank potatoes.

Impact on Agriculture and the Environment

Pesticide Usage

The potential environmental benefits of planting NewLeaf Plus potatoes, particularly in the pacific northwestern U.S., is substantial. Typically, potato growers in the pacific northwest apply 4 applications of insecticides per year

to Russet Burbank potatoes to control the aphids which spread PLRV. Schreiber and Guenthner (Appendix 1) predict that up to 265,000 acres of NewLeaf Plus Russet Burbank potatoes could be planted by farmers resulting in as much as 509,000 fewer acre-treatments of insecticides and 704,000 fewer pounds of insecticides applied to potato in the U.S alone. This estimate assumes a 50% marker share, therefore, the potential is actually twice the estimated acreage.

Plant Pest Issues

For virus-resistant transgenic plants, three main issues related to risk have been identified: transencapsidation, synergism and recombination (Robinson, 1996; Miller *et al.*, 1997). Monsanto has also requested the opinion of Dr. Allen Miller, a luteovirus researcher, and Dr. Josef Bujarski to provide their expert opinion on the ecological/plant pest risk of introducing PLRV replicase-mediated virus resistance potato plants into agriculture. Dr. Bujarski co-wrote the following "Recombination" section with Monsanto. Dr. Miller's opinion is stated in Section VI.D. below.

1. Transencapsidation

Transencapsidation requires that the inserted gene encodes for a viral coat protein. Progeny genomes of the incoming virus are assembled into particles in which some or all of the protein subunits are derived from the transgene. NewLeaf Plus potato lines do not contain any part of the PLRV coat protein gene; therefore, transencapsidation by the replicase transgene of another virus is not an issue.

2. Synergism

Synergy is when one virus or product from a transgene from a virus potentiates the effect of another incoming virus on the host plant. Synergy is not known to occur between potato leafroll virus and any other potato virus (OECD, 1996). Therefore, it is reasonable to assume that no synergy would occur between a component of PLRV, such as the replicase transgene, and any other potato virus.

3. Recombination

This section was written in cooperation with Professor Jozef J. Bujarski, Northern Illinois University.

a) Summary

NewLeaf® Plus potatoes were developed to be highly resistant to potato leafroll virus (PLRV) and under field conditions, no infection by PLRV was detected. In this section, we provide evidence that the likelihood of recombination between the constitutively expressed transgenic PLRV mRNA and other known potato viruses is extremely unlikely, and a recombination event, if it occurred, would likely result in a less fit recombinant virus.

The following characteristics make recombination between known potato viruses including other PLRV isolates and the transgenic mRNA improbable. The basis for each summary point is discussed in the accompanying text.

- Recombination by template switching involving the PLRV replicase protein is involved is not likely to occur with the transgene mRNA. The transgenic replicase mRNA does not contain the 5' or 3' ends of the PLRV genome and therefore is not likely a substrate for PLRV replicase.

- Recombination by template switching where any plant viral replicase is not likely to occur with the PLRV replicase mRNA. The 5' and 3' untranslated ends and coding sequence of the PLRV transgenic mRNA have no significant nucleotide sequence homology with any other potato viruses other than PLRV itself.

- Even if the PLRV replicase enzyme is produced in the transgenic plants, it is unlikely to participate in the replication or the production of plus sense or minus sense RNA from any virus or plant gene that might serve as templates for recombination. The 5' and 3' 150 nucleotides of the PLRV genome have no significant homology with any known potato viruses and thus the PLRV replicase is not expected to bind to and replicate other viral genomes.

- Recombination by breakage and religation of the PLRV replicase gene contained in the transgene mRNA would require a double crossover event in order to integrate into another PLRV genome or any other viral genome. The efficiency of this potential recombination mechanism is less than the template switching model, and has not been confirmed to operate during recombination in RNA viruses.

- The constitutive expression of the PLRV transgene in potato does not increase the risk for recombination above that which currently occurs in potato plants infected with multiple viruses.

- Cell to cell movement, systemic plant infection and vector transmission are important determinants of plant virus diseases In the unlikely event that recombination should occur, the PLRV replicase gene or gene products is not known to contribute to these traits.

In conclusion, there is a very minimal or no likelihood of viral recombination above that which might be expected to occur naturally between the PLRV replicase mRNA in transgenic potato plants expressing the PLRV replicase gene and other viral RNAs. Therefore, there is no anticipated plant pest risk associated with plants that contain these sequences.

b) Background

Recombination and Virus Evolution

RNA recombination is a rare event in plant virus replication that involves the exchange of RNA templates during virus replication and results in a replication product that represents the union of two previously distinct RNA

templates (Allison *et al.*, 1996). RNA recombination is thought to have been a factor in the evolution of viruses, including luteoviruses, based largely on sequence comparisons of numerous plant and animal RNA viruses (Holland *et al.*, 1982; Makino *et al*, 1986;Robinson *et al.*, 1987; Strauss and Strauss, 1988; Allison *et al.*, 1989; Gibbs and Cooper, 1995). Luteoviruses have been divided into two distinct subgroups due to differences in genome organization, coat protein and replicase sequences thought to have arisen during evolutionary recombination events (Gibbs and Cooper, 1995; Martin *et al.* 1990; Gibbs, 1995). The polymerase genes of subgroup I are thought to have arisen from a recombination event with a dianthovirus, while those from subgroup II from recombination with a sobemovirus (Habili and Symons, 1989). The result is that subgroup I and II polymerases are quite distinct, but the agronomic consequence of such an evolutionary event is difficult to determine since both subgroups generally overlap in host range and contain representative viruses of economic importance. Also, recombination with other viruses has been implicated for differences in genome organization and coat protein origin between subgroup I and II luteoviruses in addition to the differences in the replicase gene.

The selection pressure during these natural RNA recombination events is not known although such factors as virulence, transmissibility, virus concentration, etc., might contribute to the observed recombinant profiles. Falk and Bruening (1994) argue that natural selection pressure is quite low and that viruses already have evolved to be highly fit competitors in the environment. Mixed virus infections in agricultural settings provide a continuous opportunity for recombination, especially when one considers that mixed infections can constantly occur in inoculated cells but go undetected due to the lack of virus spread (Benda, 1956; Hamilton and Nichols, 1977). Therefore, it is reasonable to assume that viruses from different groups can and do replicate in the same cells; yet, new virus diseases are typically minor variants of known viruses. Therefore recombination may play a role in viral evolution, but such events that lead to new pathogenic viruses appear to be rare in nature and must compete with an array of already highly fit viruses.

The Mechanism of RNA Recombination: Homologous and Nonhomologous Crossovers

Recombination between RNA viruses involves unique mechanisms because the replication of RNA viruses is limited to the cytosol and does not involve DNA intermediates. Formally, there are two types of RNA recombination, homologous and nonhomologous (for a review, see Simon and Bujarski, 1994;Bujarski and Nagy, 1994). As described by Lai (1992), homologous recombination may occur between related viral RNAs at corresponding sites and leads to regeneration of wild-type or close to wild type RNAs. In contrast, nonhomologous recombination may occur between unrelated RNA molecules at non-corresponding sites (Bujarski and Nagy, 1994). Using such definitions, homologous RNA recombination occurs among strains of the same virus or viruses of the same group and leads to related variants of the virus. On the other hand, nonhomologous recombination occurs among different, unrelated viruses and leads to the emergence of new viral species. A more molecular definition of homologous and nonhomologous recombination has been made possible due to the results obtained in Bujarski's laboratory (Bujarski and Dzianotti, 1991; Nagy and Bujarski, 1992, 1993, 1995, 1997). Namely, they have studied the mechanism of both types of recombination in brome mosaic virus (BMV), an RNA virus. They found that

homologous recombination occurs at special stretches of homology between recombining RNA substrates (with certain composition of AU-rich and GC-rich sequences) while nonhomologous recombination can be promoted by stretches of complementary regions among the recombining RNAs. Therefore, Bujarski proposed that homologous recombination be defined as that driven by homologous RNA sequences while nonhomologous recombination be defined as facilitated by other types of RNA sequences.

Between two possible mechanisms of recombination, 'breakage and re-ligation' and 'template switching (or copy-choice)' the results from several laboratories strongly suggest that the second mechanism is responsible for both homologous and nonhomologous crossover events (Kirkegaard and Baltimore, 1986; Cascone *et al*, 1993; Bujarski and Nagy, 1994; Nagy *et a.*, 1995). Breakage and re-ligation has yet to be confirmed to operate during recombination of RNA viruses and its role is highly hypothetical. Template switching involves the viral replicase, the enzyme which makes new viral RNA molecules. The replicase changes RNA templates during RNA synthesis. Thus, properties of both viral RNAs and viral replicase must be responsible for the efficiency of RNA crossovers. Rao and Hall (1993), Ishikawa *et al.* (1991) and Nagy and Bujarski (unpublished results) have shown that nonhomologous recombination can occur during minus strand synthesis, and recent data suggest that homologous recombination can occur mostly during plus strand synthesis (Nagy and Bujarski, 1997). Nagy *et al* (1995) found that mutations in certain regions of replicase proteins can affect the frequency of crossovers, the location of crossovers or the precision of crossovers.

Greene and Allison (1994) showed that a viral transgene which included the viral 3' replicase binding region and 338 nucleotides of identical sequence could complement via recombination a defective infecting virus. When Allison *et al.* (1996) made several different deletions of the viral 3' replicase binding region included in the transgene, none of the 479 transgenic plants challenged with the defective virus supported a recombination event. These results are supported by Ishikawa *et al.* (1991) and Cascone *et al.* (1993) who found that eliminating replication by altering the 3' replicase binding region resulted in the lack of detectable recombinants. Schoelz and Wintermantel (1993) also implicated template switching as the mechanism responsible for recombination in a DNA virus that replicates through an RNA intermediate (caulimoviruses). Together these reports lead to the conclusions that replicase-mediated template switching is the most likely mechanism responsible for recombination between plant RNA viruses. As indicated above, template switching depends upon sequence homology and/or replicase binding and RNA synthesis. The PLRV replicase transgene mRNA is not predicted to be involved in template-switching recombination, the most frequent mechanism cited in recombination-based viral evolution. The transgene does not pose a significant risk to giving rise to new viruses through recombination. The PLRV replicase gene used in NewLeaf Plus potato lines to provide resistance to PLRV infection has no homology to any other genes in GenBank and does not contain any known viral replicase binding regions. This argument will be expanded upon below.

c) Recombination in Transgenic Potato Plants Expressing the PLRV Replicase Gene

The *PLRVrep* mRNA Lacks the Properties Known to be Important for Template Switching

As described above, several researchers have shown that replicase binding and sequence identity are key factors involved in recombination. The seven transgenic potato lines contain the FMV promoter, soybean heat shock protein 17.9 leader, PLRV replicase sequence, an intergenic region, and the E9 terminator. The FMV promoter is a constitutive promoter which would predictably drive the transcription of the replicase mRNA in all tissues in the potato plant. The mRNA transcribed from this gene includes approximately the 3' 70 nucleotides of the FMV promoter, the soybean HSP 17.9 (77 nucleotides), the full-length native PLRV replicase gene and intergenic region (3389 nucleotides), and the 5' 234 nucleotides of the E9 terminator, and a poly-A tail of unknown length (Fig VI.1). The full-length mRNA from the *PLRVrep* transgene was shown to be produced in all seven transgenic lines (see section V.A.2. above). The transcribed mRNA lacks the 5' and 3' ends of the PLRV genome which are expected to serve as the replication complex initiation sites for the PLRV replicase. Because the predicted 3' viral replicase binding sequence is not present in the mRNA transcript, no minus-strand RNA can be synthesized by PLRV replicase enzyme, even if present in the plant. Furthermore, extensive sequence comparisons have shown that the E9 terminator sequences at the 3' end of the replicase transgene message has no primary sequence homology with any known plant virus (Appendix 3). Therefore, based on primary nucleotide sequence homology, the replicase message contains no known binding region for any plant viral replicase. The primary criteria for recombination, replicase binding and subsequent template switching, would not appear to be possible. With respect to sequence homology, the transgenic replicase mRNA contains no homology to other viruses that infect potato other than isolates of PLRV, so the sequence homology requirement for recombination also is not met (see the section below entitled 'Homology between the PLRV Replicase mRNA and Other Plant Viruses is Very Low').

Theoretically, it is possible that if the transgenic DNA was inserted adjacent to an endogenous plant promoter, then this may allow for synthesis of antisense PLRV replicase mRNA directed by this plant promoter. In this case, the subgenomic binding site located at the 3' end of the replicase gene would be available for binding by PLRV replicase leading potentially to positive sense RNA. The production of an antisense message to the entire expression cassette would produce a replicase-binding region and would theoretically allow for template switching recombination. However, the production of such a minus-strand from a serendipitous neighboring plant promoter is extremely unlikely. Both the PV-STMT21 and PV-STMT22 construct inserts contain one or more polyadenylation signal sequences which cause the termination of the synthesis of mRNA followed by the addition of 3' terminal adenosine residues (Appendix 4). Also, the resultant positive sense RNA made from the subgenomic promoter region and available for recombination would not include any of the PLRV replicase sequence because the replicase-mediated RNA synthesis would start downstream of the replicase gene.

Homology between the PLRV Replicase mRNA and Other Plant Viruses is Very Low

Monsanto compared the mRNA sequence homology to that of other virus in GenBank and especially the sequences of viruses known to infect potato in order to evaluate the likelihood of recombination occurring by studied mechanisms. PLRV is a subgroup II luteovirus and is most closely related to sobemoviruses and carmoviruses (Koonin and Dolja 1993). Luo and Taylor (1990) evaluated the requirement for template switching, the proposed mechanism for recombination between transgenes and viral RNA, using an *in vitro* system and an animal retrovirus. One factor studied was the amount of sequence homology required for template switching. They found that the extent of overlap between the donor and acceptor templates affected the amount of recombination. With 100 nucleotides of overlap, there was a detectable amount of template switching involving reverse transcriptase (retroviruses begin replication by making an antisense DNA copy to the positive sense RNA genome, unlike plant RNA viruses which have no DNA intermediates). With only 10 nucleotides of overlap, no template switching was observed. Regions of homology also have been implicated as a factor for template switching in plant RNA virus replication (Nagy and Bujarski, 1995; Allison *et al.*, 1996) although again this is not the only factor involved. Even with significant sequence homology, no recombination between a CCMV transgene and invading virus was detected in transgenic plants when the transgene contained deletions in the 3' replicase binding region (Allison *et al.*, 1996). Sequence comparison of the transgenic PLRV replicase mRNA and GenBank viral sequences found significant homology only with PLRV isolates (TableVI.1). A much smaller amount of homology was found with other luteoviruses, and the non-luteoviruses pea enation enamovirus and cocksfoot mottle sobemovirus. However, none of these viruses are known to be potato pathogens. A specific comparison was made between viruses known to infect potatoes systemically (USDA Web site). The homology found was comparable to that of a randomized control sequence (Table VI.2; Appendix 5 for raw data). Because the PLRV replicase transgene does not contain regions of significant sequence homology to other viruses (other than PLRV isolates), it seems highly unlikely that this message would be any more prone to recombine virus than the probability of this event occurring with any other random RNA sequence.

The Risks Associated with Homologous Recombination of the PLRV Transgene and Other PLRV Isolates is not Significant

Homology is implicated as a requirement for homologous recombination between RNA viruses (Simon and Bujarski, 1994). The only homology of the transgenic mRNA is to the homologous PLRV isolate and other isolates of PLRV. No other luteoviruses are known to infect potato. A distinction has been made between subgroup I and subgroup II luteoviruses including one difference is in the viral replicase gene (Martin *et al.*, 1990), however, only members of subgroup II luteoviruses have been reported in potato. Therefore, the risk of homologous recombination giving rise to a new virus is minimal since these interactions already can occur through mixed infections of various PLRV isolates. The replicase sequences of known PLRV isolates are highly homologous; therefore recombination, if it occurred, would not contribute to increased diversity of PLRV or necessarily impart a competitive advantage to a recombinant. In summary, PLRV isolates are the only known luteoviruses to infect

potato and the risk of homologous recombination between these isolates and the transgene giving rise to a new pathogenic virus is not significant in the transgenic plant.

Selection Pressure and Competitive Advantage for Nonhomologous Recombination Involving the transgenic mRNA are Low

Recent articles report that recombination to restore function can occur under high selection pressure between genetically modified viruses and homologous viral transgene mRNA (Greene and Allison, 1994, Allison *et al.*, 1996; Schoelz and Wintermantel 1993). However, it is important to note that the plants used in these experiments were not engineered or selected for resistance to virus infection. These articles suggested that relatively long stretches of about 100 nucleotides of identical nucleotide sequence and the ability of the viral replicase enzyme to bind the transgenic mRNA are key elements for recombination. In each of these studies, recombination was required for the defective infecting virus to produce systemic symptoms, so selection pressure was very high.

Under weak or no selection pressure, no recombination was detected between pairs of replicating viruses or between infecting virus and transgenic viral messages (Angenent, 1989; and Cooper *et al.*, 1994). Expression of the full length functional AL1 gene of TGMV in transgenic tobacco plants complemented AL1 defective viruses and did not result in recombination to restore function to the mutant (Hanley-Bowdoin *et al.*, 1990). The expression of functional viral genes in transgenic plants are more likely to be inhibitory or complementary than to lead to recombination with wild-type viruses. The use of a full-length PLRV replicase gene confers a greater frequency of recovery of PLRV resistant potato plants compared to a truncated replicase gene (Thomas *et al.*, 1995). It has been suggested (Falk and Bruening, 1994) and data shows (de Jong and Ahlquist, 1992; Schoelz and Wintermantel, 1993) that recombinant viruses are less fit and thus less able to compete with the wild type virus. Therefore any argument contemplating the risk of recombination between unrelated viruses and the PLRV replicase transgenic message must consider the fact that selection pressure on such an event would be very low. The incoming virus already contains a functional replicase gene encoding an enzyme evolved to efficiently replicate that virus. In addition, it is known that most viruses already are capable of replicating in most plant cells, so it is difficult to imagine how a competitive advantage or an extension of host range would be imparted by gaining the PLRV replicase function. Since the PLRV replicase enzyme is not involved in cell to cell movement or in insect transmission, there would be little or no competitive advantage in virus spread for a recombinant virus.

The Constitutive Expression of the PLRV Transgene in Potato does not Increase Risk

It has been suggested that by using a constitutive promoter to drive expression of luteoviral transgene sequences in cells where the virus is thought not to occur represents a potential risk for recombination with other viruses. However, in NewLeaf Plus potatoes, this does present a significant risk for several reasons. First, as stated above, the transgene message is no more likely to recombine with other viruses than is any other RNA sequence in the host plant because of the lack of any significant sequence identity with other viruses that infect potato (Table VI.2) and the lack of sequence homology with 5' and 3' terminal viral sequences (Appendix 3). It is reasonable to assume

that the concentration or level of template would have a statistical correlation with recombination frequency. In Section V, data is presented to demonstrate that the level of transgenic PLRV replicase mRNA is significantly lower than the level of PLRV RNA during a natural infection. Therefore the level of template available for recombination is significantly higher in a naturally infected potato plant than in the the transgenic plants. Unlike the transgene mRNA, this natural template has the features implicated as required for recombination. Also, the PLRV replicase transgene represents only 1 of 5 genes of the native virus. A transgenic PLRV resistant potato plant expressing the replicase gene mRNA from a constitutive promoter is apparently not the same at the molecular level as having cells infected with the virus. Less genetic information as modules are available for recombination and the mRNA from the transgene does not contain the sites most likely to be involved in recombination.

An effort was made to use phloem-specific promoters to provide resistance to PLRV infection. However, from our experience in plant gene expression, few, if any, promoters are truly tissue-specific but rather tissue-enhanced. Therefore, alternatives to constitutive promoters were not obvious. The promoter of glutamine synthetase from pea (Edwards *et al.*, 1990), which was shown to cause a high level of phloem-enhanced expression in tobacco, was evaluated as a promoter to drive expression of PLRV coat protein in potato. The resulting transgenic potato plants were not resistant to PLRV infection (Appendix 6). Conversely, constitutive expression of PLRV coat protein did result in a reduced level of infection and accumulation by PLRV (Appendix 6). One possible conclusion regarding the failure of the very strong, phloem-enhanced promoter to provide resistance to PLRV is that PLRV is not limited to phloem tissues.

Lastly, peer-reviewed papers have been published showing that PLRV is not strictly limited to phloem tissues (van den Heuvel *et al*, 1995; Barker, 1987). Since PLRV occurs in the same tissues as other potato viruses, constitutive expression of a PLRV transgene does not create an opportunity for recombination between PLRV mRNA and other potato viruses that has not previously existed in nature. PLRV is thought to primarily infect phloem-associated cells, however, it is <u>not</u> <u>limited</u> to sieve elements and companion cell complexes. Van den Heuvel *et al.* (1995) found that PLRV was not exclusively limited to the phloem tissue in infected potato plants. Barker (1987) observed that 0.2% of mesophyll protoplasts isolated from PLRV-infected *N. clevelandii* leaves contained PLRV antigen. He later found that *N. clevelandii* plants co-infected with PLRV and one of several sap-transmissible 'helper' viruses (*e.g.* potato virus Y) that the proportion of PLRV-infected mesophyll cells increased about 10-fold (Barker, H. 1989). Therefore in a mixed infection, the second virus serves as a helper virus in the sense that it is able to provide movement functions to PLRV, and therefore PLRV is not strictly limited to certain cell types in nature. Taken together, the conclusion is that the risk from recombination is not increased in transgenic potato plants expressing the PLRV replicase gene.

<u>Risks Associated with Satellite RNA, Satellite Viruses and Defective RNAs of Plant Viruses.</u>

PLRV is a subgroup II luteovirus and is related to sobemoviruses and carmoviruses (Koonin and Dolja 1993). In addition to PLRV, the members of subgroup II luteoviruses include BWYV, BYDV-RPV, BYDV-RGV, and

BYDV-RMV (Gibbs and Cooper, 1995). The more distantly related members of the sobemovirus group include southern bean mosaic virus, sowbane mosaic virus turnip rosette virus , rice yellow mottle virus, blueberry shoestring virus, lucerne transient streak virus among others, none of which are known to infect potato.

Satellite RNAs and satellite viruses are small RNAs that use components of the host virus for parts of its life cycle. They generally have little or no sequence homology with the host virus and are essentially parasites of the host virus. Some satellite RNAs may encode a gene product which aids in it's replication, and satellite viruses may encode one or more gene products which provide virus specific functions. They are dependent on one or more gene products of a helper virus to propagate or aid in the spread of these viral RNAs. Recombination between various satellite RNAs has been observed and some of the nucleotide or structural requirements necessary for these nonrandom recombination events have been identified (Cascone *et al.*, 1993). There are no known satellite RNAs or satellite viruses of PLRV. While there are known satellites of other subgroup II luteoviruses, there is no evidence that helper functions extend from one virus to another with regard to the satellites.

Defective interfering (DI) RNAs are derived from viruses during host infection and are homologous to these viruses. They may contain an open reading frame, but often this ORF is defective, containing deletions, nonsense codons, frameshifts, and truncations. It has been shown for various viruses that the production of DI RNA molecules requires the presence of the 5' and 3' replicase binding regions of the virus from which the DI RNAs are derived. These terminal sequences are essential for the accumulation of DI RNA molecules in plants (Pogany *et al.*, 1995). DI RNAs have not been found to be associated with PLRV. The PLRV replicase transgene does not contain the 5' and 3' genomic ends of the virus nor is there significant homology with any 5' and 3' sequences, currently in GenBank, of any other viruses known to infect potato (Appendix 5). Therefore, there is no reason to believe that the expression of PLRV replicase as a transgene in potato would produce DI RNAs.

<u>Recombination with Another Virus would Lead to a Less Fit Virus</u>

As stated above, the likelihood of recombination between unrelated viruses is extremely unlikely. However, it may be useful to address the probable outcome if recombination did occur.

Recombination between the transgene mRNA and PLRV strains is not likely to increase the diversity of PLRV in the environment. The transgene mRNA does not contain sufficient sequence homology to other viruses for template switching recombination. Incorporation of the mRNA via an event that included double crossover nonhomologous recombination would likely cause deletions or insertional disruptions of gene(s) in the receiving genome. This would produce a defective, less-fit recombinant. If the replicase portion of the mRNA became incorporated into the genome of the invading virus, it would have to be integrated in a translational reading frame to produce an active replicase protein. The receiving virus would also have to be able to package the additional amount of RNA resulting from the recombination event. The function of PLRV replicase is to replicate the RNA genome of potato leafroll virus. All viruses known to infect potatoes contain their own replicase which has evolved with the virus to

efficiently and specifically replicate its own genome. PLRV replicase is not likely to replicate the genome of other invading potato viruses even if a recombination event did occur because replication requires recognition of sequences in the template by the replicase. A GenBank FASTA search using the 3' or 5' 150 nt of PLRV showed significant homology only to other PLRV strains (Appendix 3). No significant homology was even found with other non-PLRV luteoviruses. A specific comparison between this sequence and known potato viruses found no sequence homology with their 3' ends thus the PLRV replicase gene likely would not bind to and efficiently replicate any other plant virus and not impart any type of advantage. Therefore, the most likely outcome of any recombination event is a less fit or dysfunctional virus.

Other viruses may infect potato plants but lack the ability to move from the inoculated cell and infect the plant systemically. Although unlikely, even if recombination did occur with another virus, it would not have an effect on the systemic movement of the virus in potato. The PLRV replicase gene products does not play a direct role in virus movement; therefore, it would not be predicted to function in this capacity for any other virus.

d) Conclusions

The following characteristics are inconsistent with recombination between mRNA of the transgene in NewLeaf Plus potatoes and other plant virus RNAs.

- The transgenic mRNA lacks the 5' and 3' ends of the PLRV genome, and does not contain any primary sequence homology to any other potential plant viral replicase binding region. Because the transgenic replicase mRNA will not likely serve as a template for any known viral replicase, the likelihood of recombination is extremely low because replication is required for recombination.

- The transgenic mRNA has no significant nucleotide sequence homology with any known non-PLRV potato viruses; therefore, the homology requirement for recombination is not met with this transgenic mRNA.

- The transgenic replicase cannot generate minus-strand mRNA from the transgene mRNA due to lack of sequence homology needed for recognition. Likewise, any replicase protein produced would not bind to and generate plus or minus-strands from any other known virus that infects potato.

- Incorporation of the transgene mRNA into other viral genomes by homologous and nonhomologous recombination events would likely cause deletions or insertional disruptions of gene(s) in the receiving genome. This would produce defective, less fit recombinants.

- If a recombination event did occur with another luteovirus, the new RNA would not support systemic movement of the recombinant.

- The risk is not significantly increased by using a constitutive promoter to drive the transgene. The level of replicase mRNA in a transgenic plant is much lower in total than the level of viral RNAin a naturally infected potato. Evidence shows that PLRV is not phloem-limited in single or mixed infections.

- Satellite and DI RNAs do not pose a recombination risk in plants that express the PLRV replicase gene.

The risk from recombination of the transgene PLRV mRNA and any other virus is negligible, especially when compared to the risk of continued infection and subsequent crop loss from planting PLRV-susceptible potatoes (*e.g.* Russet Burbank). Additionally, the overall risk of viral recombination is expected to be reduced when planting PLRV-resistant, NewLeaf Plus potatoes. The worst consequences of transgenic resistance from the use of NewLeaf Plus potatoes would be less damaging than the PLRV outbreaks with which we currently live (Miller *et al.*, 1997).

D. Expert Opinion on the Plant Pest Risks Associated with the PLRV Resistance Trait in NewLeaf Plus Potatoes

OPINION ON POTENTIAL RISKS ASSOCIATED WITH TRANSGENIC "NewLeaf® Plus" POTATOES TRANSFORMED WITH ORFS 1 AND 2 OF POTATO LEAFROLL LUTEOVIRUS

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I was invited by Monsanto Company to include my expert opinion on risks of their transgenic potatoes that show immunity to PLRV. I have 13 years experience in studying the replication and gene expression of barley yellow dwarf luteoviruses (BYDVs). My lab also works on transgenic resistance to BYDVs and associated risks. One hundred percent of my research effort is devoted to luteoviruses. I have authored several invited review articles and book chapters on luteovirus replication, evolution and gene expression (Miller, 1994; Miller *et al.*, 1997a; Miller *et al.*, 1995; Miller and Rasochova, 1997; Miller and Young, 1995), including one on potential risks associated with transgenic resistance to luteoviruses (Miller *et al.*, 1997b).

Brief Introduction to Luteovirus ORFs 1 and 2.
Based on genome organizations, RNA-dependent RNA polymerase (replicase) gene sequence homologies, and cytopathological properties, luteoviruses have been divided into two subgroups (Mayo and Ziegler-Graff, 1996; Miller *et al.*, 1995). Potato leafroll virus (PLRV), beet western yellows virus (BWYV), beet mild yellowing virus (BMYV), cucurbit aphid-borne yellows virus (CABYV), and the RPV barley yellow dwarf virus (BYDV-RPV) are in subgroup II. The PAV barley yellow dwarf virus (BYDV-PAV) is a member of subgroup I. An RNA (ST9a RNA) associated with the ST9 strain of BWYV is not a virus, but it encodes a functional subgroup I-like polymerase. ORF1 of subgroup II luteoviruses is believed to encode a protease (Demler and de Zoeten, 1991; Miller *et al.*, 1995) and is known to encode the genome-linked protein (VPg) (van der Wilk et al., Virology, in press). ORF1 of subgroup I lacks both of these functions and has no homology with its counterpart in subgroup II. ORF2 of both subgroups is known to be expressed only as a fusion with ORF1 by ribosomal frameshifting (Kujawa *et al.*, 1993; Prufer *et al.*, 1992). It encodes the catalytic domain of the RNA-dependent RNA polymerase. The polymerase genes (ORF2) of subgroup II luteoviruses are most closely related to those of the sobemoviruses, while the polymerase genes of subgroup I are totally unrelated to subgroup II, being most closely related to those of the diantho, carmo and tombusvirus groups.

Risks of transgenic potato plants expressing PLRV ORFs 1 and 2.

1. Recombination.

A. Subgenomic promoter. I have proposed that recombination has occurred between subgroup I-related and subgroup II-related viruses at subgenomic RNA promoters (Miller *et al.*, 1995; 1997). Thus, these sequences should be avoided in transgenic constructs. No known or proposed subgenomic RNA promoters are present in the NewLeaf® Plus construct. The (+) strand complement of the subgenomic mRNA promoter is almost certainly present at the 3' end of ORF2 (the pol gene) in the transgene, but this is not the strand recognized by the replicase according to current theory. If PLRV RNA behaves like other known subgenomic RNA-producing plant viruses, the replicase recognizes the subgenomic RNA promoter in the (-) strand only. To express the (-) strand, the transgene would have to have integrated adjacent to an endogenous plant promoter that fortuitously transcribes the transgene (-) strand. This would be unlikely, owing to polyadenylation sites in the (-) strand (identified by K. Reding, Monsanto) that would be expected to terminate (-) strand transcription before the subgenomic promoter is reached.

Even if an invading virus did initiate RNA synthesis at the subgenomic promoter, the resulting (+) transcript produced by transcription from the subgenomic promoter would encompass a short stretch of viral intergenic RNA and the adjacent cryIIIA and CP4 genes at the 3' end of the construct. It is difficult to envision how this would lead to a new, fit virus by recombination. Other mechanisms of subgenomic RNA synthesis are known. Subgenomic RNAs of members of the mammalian coronavirus group are generated by recombination between the 5' end of the genome and the subgenomic promoter. If this or any other known or proposed subgenomic RNA synthesis mechanism applied to PLRV, none would impose a risk, owing to the lack of the 5' and 3'-termini of the genome.

It should be noted that recombination has not been observed directly between luteoviruses. It has taken place recently on an evolutionary time scale. Thus, our proposed model on recombination at subgenomic promoters in luteovirus evolution has not been proven experimentally. In fact, based on so many differences in *cis* acting translation and replication signals that my lab has uncovered (e.g. Mohan *et al.*, 1995; Wang *et al.*, 1997), I doubt that recombination between a subgroup I and subgroup II luteovirus could produce a viable virus in a single step.

A more likely possibility is intra-subgroup recombination. This may have occurred in the evolution of beet mild yellowing virus (BMYV) which seems to have been generated by a recombination between ancestors of cucurbit aphid-borne yellows virus (CABYV) and BWYV at the subgenomic RNA promoter. BMYV and BWYV are highly homologous in their 3' halves, but the ORFs upstream of the subgenomic RNA promoter (ORFs 0,1 and 2) diverge and those of BMYV are more similar to their homologues in CABYV (Guilley *et al.*, 1995). All of these viruses are in subgroup II. My lab has found that the severe (in oats) PAV129 strain of BYDV differs substantially from other PAV isolates in ORFs 1 and 2, suggesting divergent evolution, followed by a more recent recombination at the sgRNA1 promoter to maintain the much higher homologies of ORFs 3,4 and 5 between PAV129 and other PAV isolates. Finally, the severe RPV-Mex1 and RPV-CA isolates of BYDV show no homology in the 5' halves of their genomes to the 5' half of the RPV-NY genome based on northern blot hybridization (Miller and Rasochova, 1997), but the 3' halves (ORFs 3,4, and 5) show high homology, again suggesting intra-subgroup recombination at the sgRNA1 promoter.

In contrast to the above viruses, all sequenced isolates of PLRV show very high homology to each other. Canadian, Scottish and Dutch isolates of PLRV differ at less than 3% of the bases (Keese *et al.*, 1990). However, ORF2 of an Australian isolate differs by 11.6% from the Canadian isolate and a 600 base region in this ORF differs by 22% (Keese *et al.*, 1990). However, ORFs 1 of these two isolates are 97% identical. Because the NewLeaf® Plus potatoes include all of ORFs 1 and 2, the high homology of ORF1 may be sufficient to confer resistance even to the Australian isolate. I have been informed that, despite much effort, Monsanto has been unable to find a strain of PLRV that overcomes the immunity conferred in the NewLeaf® Plus potatoes. In this case it would be difficult to imagine how recombination between a strain of PLRV and the transgene could occur. Even if it did, by acquiring sequence homologous to the transgene, the recombinant virus would now have homology to the transgenic RNA and the plant would be instantly resistant to the recombinant virus, preventing spread of the virus to other cells or plants.

B. Replication origins. Recombination has been detected between PLRV and a host mRNA. Mayo and Jolly (1991) found that a fraction of the PLRV genomes in Scottish isolates had acquired a 119 nt sequence in the 5' untranslated region (UTR) by recombination with a chloroplast RNA. The presence of these molecules in three different isolates suggests that they are viable genomes.

TABLE VI.1. 1996 COMPARATIVE ANALYSIS OF INSECTICIDE USAGE IN COMMERCIAL NEWLEAF POTATO FIELDS.

Farms using NewLeaf program[3]	Farms	NewLeaf[1]			Non-NewLeaf[2]			Reduction %		
		Trts[4]	ai/acre[5]	Form. lb/acre[6]	Trts[4]	ai/acre[5]	Form. lb/acre[6]	Trts[4]	ai/acre[5]	Form. lb/acre[6]
Avg. all regions	35	1.77	1.91	8.07	3.12	2.43	8.90	1.35	0.52	0.83
MN, NB	3	1.67	1.29	2.62	3.33	1.89	5.63	1.67	0.60	3.01
MN, ND, WI	15	2.58	1.73	3.46	3.85	1.60	4.12	1.27	-0.12	0.66
Idaho	14	0.53	1.58	13.62	1.56	3.25	16.00	1.03	1.67	2.38
OR, WA	4	3.25	4.53	13.23	4.00	6.16	24.09	0.75	1.63	10.86

[1]NewLeaf Russet Burbank variety.
[2]Traditional non-engineered Russet Burbank variety.
[3]Farms which grew non-engineered Russet Burbank and NewLeaf Russet Burbank using management practices as recommended by Monsanto/NatureMark.
[4]The average number of treatments per field.
[5]The active ingredient of insecticide used per acre.
[6]The pounds of formulated insecticide used per acre.

C. *Impact of Potato Leafroll Virus Resistance*

Control of PLRV

In addition to the Colorado potato beetle resistance trait, the NewLeaf Plus potato lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 are highly resistant to the potato leafroll virus. NewLeaf Plus potato lines 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 VI.2). 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. 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. The control Russet Burbank plants averaged 67.5% leafroll infection in four location-year test sites. PLRV symptoms were distinct and severe on all infected controls. PLRV infection was verified on a smaller sub-set of these samples by either ELISA testing of greenhouse or field plant-back material. In all cases, symptoms of PLRV read in the field matched the plant-back ELISA results. None of the transgenic lines tested exceeded the 10% PLRV symptom criteria set for viable commercial clones at the beginning of the study.

	Percent of plants infected with PLRV				
	Parma, ID		Prosser, WA		Ave. 4
Clone	1994	1995	1994	1995	Loc-Yrs
21-129	0	0	0	8	2.0
21-152	5	3	0	0	2.0
21-350	5	0	0	3	1.9
22-082	5	0	0	3	1.9
22-186	0	0	0	0	0.0
22-238	20	0	10	5	8.8
22-262	0	0	15	3	4.5
R. Burbank	61	99	47	63	67.5

Overall Safety and Impact on Non-target Organisms

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. Monsanto is consulting with FDA on the nutritional qualities of the potatoes. Data show that the potato are substantially equivalent to non-engineered Russet Burbank potatoes.

Impact on Agriculture and the Environment

Pesticide Usage

The potential environmental benefits of planting NewLeaf Plus potatoes, particularly in the pacific northwestern U.S., is substantial. Typically, potato growers in the pacific northwest apply 4 applications of insecticides per year

The replication origins of PLRV are predicted to be at the 3' termini of the genomic (+) and (-) strands, based on our understanding of most other RNA viruses. Indeed, the above chloroplast RNA sequence insertion was in the 5' UTR (the complement of the 3' end of (-) strand), of the PLRV genome. The PLRV terminal sequences are not present on the construct in NewLeaf® Plus potatoes.

C. Unpredictable recombination sites. Recombination can take place potentially anywhere in a sequence. There is no way to guarantee that a given sequence is not a recombination hot spot (or "warm" spot). For example, Gibbs and Cooper identified likely recombination sites in ORF5 of luteoviruses far from any subgenomic promoters (Gibbs and Cooper, 1995). Gibbs *et al.* (1997) propose that recombination is more likely to be detected at boundaries of functional domains in genes. Several domains exist in the ORF1-2 transgene, as it has putative protease, VPg, and polymerase functions. However, the recombination sites at domain boundaries would be detected preferentially, not because of the inherent tendency of the replicase to switch strands at these boundaries, but because recombination *within* protein domains are less likely to yield a functional protein than those that occur *between* protein domains. In summary, we do not know enough about RNA virus replication or recombination to rule out the possibility of recombination at any particular site in the genome. Thus, any viral transgene, including those already approved for coat protein-mediated resistance, has a potential to recombine with an invading virus.

D. New opportunities for recombination? It has been argued that recombination to produce a more virulent, evolutionarily fit pathogen would not occur between a virus and transgenic RNA because it would have already occured in natural mixed infections (Falk and Bruening, 1994). However, it has been suggested that this argument would not apply in these transgenic plants expressing PLRV RNA, because the transgenic RNA is now present in all cells of the plant, instead of just the phloem cells to which naturally infecting PLRV is confined. This counterargument states that viruses that normally infect mesophyll and other cells would not normally have opportunities to recombine with PLRV RNA because they would rarely encounter PLRV RNA even in mixed infections with both viruses in the same plant, owing to their different cell tropism. I don't think this argument is strong for several reasons. Over evolutionary time, the viruses would likely have had plenty of chances to encounter each other. The confinement of PLRV to phloem (phloem parenchyma, companion cells) may not be as strict as we think. Barker (1987) detected PLRV in mesophyll cells of *Nicotiana clevelandii*, and showed that co-infection with PVY increased the number of mesophyll cells infected by PLRV, suggesting that PVY may facilitate movement of PLRV into the mesophyll. Cells would have to be infected with both viruses for this complementation to occur. However, Barker (1987) was unable to detect PLRV outside of the phloem in PLRV or PLRV+PVY-infected potato. In contrast, van den Heuvel *et al.* (1995) showed that PLRV can be found in mesophyll cells in natural infections by PLRV in potato.

A second argument suggesting interaction of PLRV with other viruses in natural infections is that all viruses are transported throughout the plant via the phloem, so it is quite possible that nonphloem-limited viruses (e.g. PVX, PVY) could co-infect a PLRV-infected phloem cell. Thirdly, luteoviruses can replicate to high levels in non-phloem cells (Young *et al.*, 1989; and work in my own lab). Presumably, they just lack the ability to move from cell-to-cell outside of the phloem cells which have specialized plasmodesmata. Given that luteoviruses are aphid transmitted, it is likely that a few individual epidermal and perhaps mesophyll cells are infected with luteoviruses regularly. When aphids feed, they first probe the outer cells with their stylets to test the plant. During such feeding it is quite possible that these cells could be infected with luteoviruses, but that the virus would not

move to the neighboring cells owing to lack of an appropriate movement protein. This kind of probing is sufficient for transmission of nonpersistently transmitted viruses such as potyviruses. Thus, it is possible, and even likely over evolutionary time, that an individual aphid would transmit more than one type of virus (e.g. PLRV and one of the many aphid-transmitted nonluteovirus potato viruses, which bind different sites in the aphid), into the same cell. These viruses would replicate to high levels intracellularly and have much more opportunity to recombine than would be the case with a partial viral genome espressed at the lower transgenic levels.

2. **Enhancement of susceptibility to other viruses.**

A. Synergy. We predicted that the products of ORFs 1 and/or 2 may be involved in synergistic interactions between subgroup II and subgroup I or subgroup I-like infectious RNAs (Miller *et al.*, 1997b). Thus, it is possible that the PLRV transgene could synergistically enhance replication of an invading subgroup I (-like) luteovirus. This is a low risk from both sides of the risk equation (probability of event x consequences of event = risk):

i. Probability of event. PLRV is subgroup II and no subgroup I-like viruses or RNAs are known that infect potatoes or interact with PLRV.

ii. Consequences of event. Even if synergy did occur, it would cause losses only in transgenic potatoes. These potato genotypes could be removed from future production. Monsanto would lose sales revenue. Thus, the market would control the losses and no government regulation would be needed to remove the offending potato variety from production. This is in contrast to the risk of recombination which would result in new, potentially uncontrollable viruses.

B. Replication of RNAs *in trans*. If the construct actually expresses significant amounts of wildtype replicase, it could possibly replicate other RNAs. For example, defective RNAs that contain mutations or deletions in essential coding regions, but not in the origins of replication, could now be replicated *in trans* by the replicase.

i. Satellite RNAs A satellite RNA could potentially be replicated by the transgenic replicase, possibly obviating the need for a helper virus. The satellite RNA of BYDV-RPV would be a good candidate, as it also is replicated by BWYV (also subgroup II) (Rasochova *et al.*, 1997). The satellite RNA is not known to occur in the field, and is known only to reduce viral RNA accumulation and attenuate disease symptoms. Thus it is not likely to be a risk. However, sobemoviral satellites are more common, and given the similarity to the BYDV satellite and the homology between sobemoviral and subgroup II (e.g. PLRV) luteoviral replicase genes, it is possible that the PLRV replicase could copy a sobemoviral satellite. At least one of these, e.g. lucerne transient streak virus satellite RNA is known to exist in northwestern North America (AbouHaidar and Paliwal, 1988).

ii. ST9a RNA. Another possibility would be *trans*-replication of an associated RNA like the ST9a RNA associated with BWYV. This RNA is replicated to high levels in the presence of BWYV genomic RNA, it enhances replication of BWYV itself by an order of magnitude, and greatly exacerbates disease symptoms (Falk and Duffus, 1984; Sanger *et al.*, 1994). The synergistic increase in RNAs is at the level of RNA replication and not due to increased movement, because the increase is seen in infected protoplasts (Sanger et al., 1994; Rasochova et al., 1997). ST9a RNA encodes its own replicase and it can replicate independently in protoplasts, yet it depends on BWYV for invasion of plants (Passmore *et al.*, 1993), probably because BWYV provides the movement protein and coat protein.

Because transgenic potato plants expressing PLRV replicase would not contribute these functions, an ST9a RNA-like agent may not be able to invade these transgenic plants any better than nontransgenic plants.

Another potential concern is revealed by the interaction between ST9aRNA and BWYV RNA. Corresponding with the increase in each RNA, additional subgenomic forms of each RNA appear in infected cells. One possibility is that the replicase of each RNA fortuitously recognizes a subgenomic promoter on the other RNA. Whether this is the reason for the enhanced replication is unknown. However, if these additional RNAs are made by such a mechanism, it implies that transgenically expressed functional PLRV replicase could potentially recognize a sequence on a host or other viral RNA that resembles a subgenomic RNA promoter and copy it. While this doesn't seem to be a risk, *per se*, it allows for the possibilities of strand switching to needed to permit recombination.

iii. DI RNAs. Defective forms of the viral genome that still contain replication origins, such as DI RNAs, could potentially be replicated by the transgenic replicase without the need for helper virus. Transgenically expressed replicases of alfalfa mosaic virus (Taschner *et al.*, 1991) and cymbidium ringspot tombusvirus (Kollar and Burgyan, 1994) have been observed to replicate incomplete genomes or DI RNAs *in trans*. However, we have been unable to detect replication of defective BYDV-PAV (subgroup I) genomes in *trans* by viable BYDV-PAV RNA co-inoculated in protoplasts (Mohan et al., 1995; and unpublished data). This includes RNAs with numerous different small and large deletions in various parts of the genome. Similar negative results have been found with the subgroup II BWYV RNA (V. Ziegler-Graff, personal communication). Thus, it is possible that luteovirus RNA replication is *cis*-preferential (Weiland and Dreher, 1993), i.e. the replicase copies only the RNA from which it is translated. This would reduce the likelihood of *trans*-replication. However, extremely efficient satellite RNA replication shows that this *cis*-preferential replication, if it exists, can be overcome.

iv. de novo generation of replicons. Unlike in a normal virus infection, NewLeaf® Plus plants would be expressing replicase (if it is indeed expressed), in the absence of a replication template. A replicase without its natural template may copy a host RNA with some very low efficiency. Given the high error rate of RNA-dependent RNA polymerases, after enough rounds of very inefficient replication, mutations would be selected to make the RNA a better template. It is conceivable that a good replicase template could eventually arise (Miller *et al.*, 1997b). This has been proposed to explain the origin of satellite RNAs (Francki, 1985), and the origin of variant RNAs that accumulate in transformed *E. coli* cells expressing the Qβ replicase (van Duin, 1988). However, such RNAs would, if anything, be expected to cause an undesired phenotype in uninfected potato plants, preventing them from being released in the first place. (Transgenic expression of Qβ replicase greatly reduces growth of *E. coli* cells (van Duin, 1988).) The PLRV replicase would be unable to compete with that of an invading virus for that virus' RNA, so a PLRV replicon would not evolve on the RNA of another virus. Recombination between the *de novo*-arising replicon and useful genes (movement protein, coat protein) of the other virus, combined with acquisition of the PLRV replicase by additional recombination events would be required. Thus, the probability of producing a new virus this way is remote.

v. Movement. Allison *et al.* (1996) and others have pointed out that any kind of defective RNA that might arise would not likely be able to move from the initially infected plant cell, given that no movement protein is present. The movement

protein(s) of luteoviruses is encoded by ORFs 4 and/or 5 (Chay *et al.*, 1996; Tacke *et al.*, 1993; Ziegler-Graff *et al.*, 1996). Transcripts lacking these ORFs are infectious in protoplasts but not in plants. ORFs 1 and 2 are insufficient for systemic movement of virus in plants (Chay *et al.*, 1996; Ziegler-Graff *et al.*, 1996). Thus, even if the transgenically expressed replicase could occasionally amplify any of the above types of RNAs in the absence of helper virus, the damage would likely be confined to the single cell in which that RNA entered or arose.

Finally if, despite the unlikelihood of each of the necessary events described above, the NewLeaf® Plus plants actually were more susceptible to another virus or RNA, only transgenic plants would be affected; no new uncontrolled virus would be released. The grower could control the problem, at the growers' and subsequently Monsanto's loss, as described for synergy risks above.

C. Other functions encoded in ORFs 1 and 2. Based on sequence comparisons, ORF1 seems to harbor the amino acids that form the catalytic triad of a serine protease (Mayo and Ziegler-Graff, 1996; Miller *et al.*, 1995). This protease, if it is expressed in these transgenic potatoes, seems not to affect host cell proteins, because the transgenic plants are indistinguishable phenotypically from nontransformed potato plants. Such a protease could be imagined to enhance processing of polyproteins of a virus such as PVY, but much more likely would either have no effect or cause deleterious cleavage of the invading viral protein. ORF1 was also shown recently to code for the viral genome-linked protein (VPg) (van der Wilk *et al.*, *Virology*, in press). This is probably intimately involved in RNA replication initiation and there is no obvious way such a protein would affect replication of other viruses. ORFs 1 and 2 may harbor other functions that may affect replication of other viruses in unpredictable ways. This is true for all viral gene products expressed in transgenic plants, including coat protein.

Worst case scenario. For transgenic expression of functional replicase to be a true risk, one would have to argue that the above opportunities for *trans* replication could increase the likelihood of recombination between transgene and RNA. This would require two strand-switching events to allow the *trans*-replicated RNA to acquire the replicase transgene, or transgenic RNA to acquire terminal replication origins from the invading RNA. Whether the resulting recombinant virus retains a single RNA component, or is bipartite like pea enation mosaic virus (Demler *et al.*, 1993), it is difficult to imagine how an invading virus would have replication origins recognized by the transgenic replicase, or how it would gain an advantage by acquiring the transgenic replicase. After all that happens, then the recombinant virus has to be fit enough to be a competitive virus, and, finally, it has to cause undesirable disease symptoms, for there to be a problem.

Unanswered questions
The above discussion assumes that functional replicase is expressed at high levels in all cells. This may not be the case.

- What is the expression level of transgenic mRNA? (Total and in various tissues.)

  If mRNA expression is low or undetectable, then all the above concerns are greatly reduced.

- What is expression level of both ORF1 and the ORF1-2 (polymerase) frameshift product in transgenic plants?

  If transgenic protein expression is low or undetectable, then the *trans*-replication or complementation worries are reduced. It is possible that ORF1 but not ORF1-2 would be

expressed. In the case of BYDV-PAV (which has completely different frameshiftsignals), a sequence 4,000 bases downstream of the frameshift site is needed for significant ribosomal frameshifting to translate ORF2 (Wang and Miller, 1995; and C. Paul, unpublished). If this were the case with PLRV, no polymerase would be produced in these transgenic plants. Finally, even if polymerase is expressed, we don't know if it is functional.

- How many different strains of PLRV are known? How much do they differ in symptomatology, ability to infect various potato varieties, and in nucleotide sequence?

  I suggest consulting with people who really know PLRV to answer this. In addition to Pete Thomas (USDA, Prosser, WA), I suggest Mike Mayo or Hugh Barker at the Scottish Crops Research Institute.

Summary

The above represents a list of every possible risk I could think of, no matter how low the probability. The main thing to remember is that it takes a long chain of rare events before a new, competitive and disease-causing pathogen is released as described in the Worst Case Scenario. Obviously there is some risk. Because commercial use of transgenic crops expressing viral genes is in its infancy, we cannot rule out the occurrence of unforeseen events. Thus, transgenic crops should be monitored for the occurrence of new or unusual viruses and disease symptoms. However, I think that the risk of deploying transgenic plants expressing PLRV replicase is no greater than that of transgenic plants, already approved for commercial release, expressing viral coat protein genes. The facts that coat protein *(i)* is involved in long distance movement and in some cases cell-to-cell movement, *(ii)* is often a symptom or host range determinant, and *(iii)* determines vector transmissibility, suggest that coat protein expression may be a greater risk than expression of replicase. Certainly, the risks of expression of PLRV ORFs 1 and 2 in transgenic potatoes are lower than the risks that potato growers currently face, which include a high probability of PLRV infection, an expensive seed certification program, and extensive use of pesticides to control the virus' vector.

References

Allison, R.F., Schneider, W.L., and Greene, A.E. (1996) Recombination in plants expressing viral transgenes. *Sem. Virol.* 7, 417-422.

AbouHaidar, M.G. and Paliwal, Y.C. (1988) Comparison of the nucleotide sequences of the viroid-like satellite RNAs of the Canadian and Australian strains of lucerne transient streak virus. *J. Gen. Virol.* **69**, 2369-2373.

Barker, H. (1987). Invasion of non-phloem tissue in Nicotiana clevelandii by potato leafroll luteovirus is enhanced in plants also infected with potato Y potyvirus. *J. Gen. Virol.* **68**, 1223-1227.

Chay, C. A., Gunasinge, U. B., Dinesh-Kumar, S. P., Miller, W. A., and Gray, S. M. (1996). Aphid transmission and systemic plant infection determinants of barley yellow dwarf luteovirus-PAV are contained in the coat protein readthrough domain and 17-kDa protein, respectively. *Virology* **219**, 57-65.

Demler, S.A. and deZoeten, G.A. (1991) The nucleotide sequence and luteovirus-like nature of RNA 1 of an aphid non-transmissible strain of pea enation mosaic virus. *J. Gen. Virol.* **72**, 1819-1834.

Demler, S.A., Rucker, D.G., and deZoeten, G.A. (1993) The chimeric nature of the genome of pea enation mosaic virus: The independent replication of RNA 2. *J. Gen. Virol.* **74**, 1-14.

Falk, B. W., and Bruening, G. (1994). Will transgenic crops generate new viruses and new diseases? *Science* **263**, 1395-1396.

Falk, B. W., and Duffus, J. E. (1984). Indentification of single and double-stranded RNAs associated with severe symptoms in beet western yellows virus-infected *Capsella bursa-pastoris*. *Phytopathology* **74**, 1224-1229.

Francki, R. I. B. (1985). Plant virus satellites. *Annu. Rev. Microbiol.* **39**, 151-174.

Gibbs, M. J., and Cooper, J. I. (1995). A recombinational event in the history of luteoviruses probably induced by base-pairing between the genomes of two distinct viruses. *Virology* **206**, 1129-1132.

Gibbs, M. J., Waterhouse, P. M., and Weiller, G. F. (1997). Analysis of natural viral recombination may assist the design of new virus resistant traits. In: *Commercialisation of Transgenic Crops: Risk, Benefit and Trade Considerations*, (McLean, G.D., Waterhouse, P.M., Evans, G., Gibbs, M.J. ed.s) Proceedings of a workshop, 11-13 March 1997, Cooperative Research Centre for Plant Science & Bureau of Resource Sciences, Canberra, Australia. pp. 159-172.

Guilley, H., Richards, K. E., and Jonard, G. (1995). Nucleotide sequence of beet mild yellowing virus RNA. *Arch. Virol.* **140**, 1109-1118.

Keese, P., Martin, R. R., Kawchuk, L. M., Waterhouse, P. M., and Gerlach, W. L. (1990). Nucleotide sequences of an Australian and a Canadian isolate of potato leafroll luteovirus and their relationships with two European isolates. *J. Gen. Virol.* **71**, 719-724.

Kollar, A., and Burgyan, J. (1994). Evidence that ORF1 and 2 are the only virus-encoded replicase genes of cymbidium ringspot virus tombusvirus. *Virology* **201**, 169-172.

Kujawa, A. B., Drugeon, G., Hulanicka, D., and Haenni, A. L. (1993). Structural requirements for efficient translational frameshifting in the synthesis of the putative viral RNA-dependent RNA polymerase of potato leafroll virus. *Nucleic Acids Res* **21**, 2165-2171.

Mayo, M. A., and Jolly, C. A. (1991). The 5'-terminal sequence of potato leafroll virus RNA: Evidence of recombination between virus and host RNA. *J. Gen. Virol.* **72**, 2591-2595.

Mayo, M. A., and Ziegler-Graff, V. (1996). Molecular biology of luteoviruses. *Adv. Virus Res.* **46**, 413-460.

Miller, W. A. (1994). Luteoviruses. *In* "Encylopedia of Virology" (R. G. Webster, and A. Granoff, Eds.), Vol. 2, pp. 792-798. 3 vols. Academic Press, London.

Miller, W. A., Brown, C. M., and Wang, S. (1997a). New punctuation for the genetic code: luteovirus gene expression. *Seminars in Virology* **8**, (in press).

Miller, W. A., Dinesh-Kumar, S. P., and Paul, C. P. (1995). Luteovirus gene expression. *Critic Rev Plant Sci* **14**, 179-211.

Miller, W. A., Koev, G., and Mohan, B. R. (1997b). Are there risks associated with transgenic resistant to luteoviruses? *Plant Disease* **81**, 700-710.

Miller, W. A., and Rasochova, L. (1997). Barley yellow dwarf viruses. *Annu. Rev. Phytopathol.* **35**, 167-190.

Miller, W. A., and Young, M. J. (1995). Prospects for genetically engineered resistance to barley yellow dwarf viruses. *In* "Barley Yellow Dwarf: 40 Years of Progress" (C. J. D'Arcy, and P. A. Burnett, Eds.), pp. 345-369. APS Press, St. Paul.

Mohan, B. R., Dinesh-Kumar, S. P., and Miller, W. A. (1995). Genes and *cis*-acting sequences involved in replication of barley yellow dwarf virus-PAV RNA. *Virology* **212**, 186-195.

Passmore, B. K., Sanger, M., Chin, L., and Falk, B. W. (1993). Beet western yellows virus-associated RNA: An independently replicating RNA that stimulates virus accumulation. *Proc. Natl. Acad. Sci. USA* **90**, 10168-10712.

Prufer, D., Tacke, E., Schmitz, J., Kull, B., Kaufmann, A., and Rohde, W. (1992). Ribosomal frameshifting in plants: a novel signal directs the -1 frameshift in the synthesis of the putative viral replicase of potato leafroll luteovirus. *EMBO J* **11**, 1111-1117.

Sanger, M., Passmore, B., Falk, B. W., Bruening, G., Ding, B., and Lucas, W. J. (1994). Symptoms severity of beet western yellows virus strain ST9 is conferred by the ST9-associated RNA and is not associated with virus release from the phloem. *Virology* 200, 48-55.

Tacke, E., Schmitz, J., Pruefer, D., and Rohde, W. (1993). Mutational analysis of the nucleic acid-binding 17 kDa phosphoprotein of potato leafroll luteovirus identifies an amphipathic alpha -helix as the domain for protein/protein interactions. *Virology* 197, 274-282.

Taschner, P. E., van der Kuyl, A. C., Neeleman, L., and Bol, J. F. (1991). Replication of an incomplete alfalfa mosaic virus genome in plants transformed with viral replicase genes. *Virology*, 445-450.

van den Heuvel, J. F. J. M., and al., e. (1995). Localization of potato leafroll virus in leaves of secondarily-infected potato plants. *Eur. J. Plant Pathol.* 101, 567-571.

van Duin, J. (1988). Single-stranded RNA bacteriophages. *In* "The bacteriophages" (R. Calendar, Ed.), Vol. 1, pp. 117-167. Plenum, New York.

Wang, S., and Miller, W. A. (1995). A sequence located 4.5 to 5 kilobases from the 5' end of the barley yellow dwarf virus (PAV) genome strongly stimulates translation of uncapped mRNA. *J Biol Chem* 270, 13446-13452.

Wang, S. P., Browning, K. S., and Miller, W. A. (1997). A viral sequence in the 3' untranslated region mimics a 5' cap in facilitating translation of uncapped mRNA. *EMBO J.* 16, 4107-4116.

Weiland, J. J., and Dreher, T. W. (1993). Cis-preferential replication of the turnip yellow mosaic virus RNA genome. *Proc. Natl. Acad. Sci. USA* 90, 6095-6099.

Young, M. J., Larkin, P. J., Miller, W. A., Waterhouse, P. M., and Gerlach, W. L. (1989). Infection of Triticum monococcum protoplasts with barley yellow dwarf virus. *J Gen Virol* 70, 2245-2251.

Ziegler-Graff, V., Brault, V., Mutterer, D., Simonis, M.-T., Herrbach, E., Guilley, H., Richards, K. E., and Jonard, G. (1996). The coat protein of beet western yellows luteovirus is essential for systemic infection but the viral gene products P29 and P19 are dispensable for systemic infection and aphid transmission. *Molec. Plant-Microbe Interact.* 9, 501-510.

Disclaimer

I serve as a paid consultant to Monsanto on their research on transgenic resistance to BYDV. I receive no grant funds from Monsanto and have no financial interest in Monsanto Company or its subsidiaries. The decision by the USDA on commercial release of NewLeaf® Plus Potatoes will have no affect on my research funding or personal income. It will have an effect on the research direction of all labs, commercial and academic, that are pursuing replicase-mediated transgenic resistance to plant viruses. The above discussion represents my honest, objective scientific opinion.

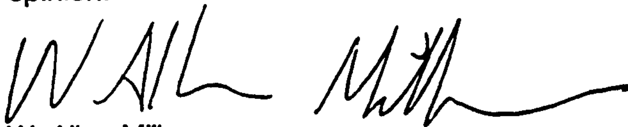

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Table VI.1. Comparison of transgenic PLRV Replicase mRNA[1] (3795 n.t.) and related viral sequences in GenBank.

Virus	GenBank Accession #	Size (n.t.)	% Identity/ n.t. overlap[2]	Longest stretch of identity[2] (n.t.)	Infects Potatoes Systemically?
Potato leafroll virus (Polish isolate) RNA sequence	X74789	5882	98.3/3373	> 200	Yes
Potato leafroll luteovirus (strain 1 from Scotland) genomic RNA	X14600	5987	98.0/3373	> 200	Yes
Potato leafroll virus genomic RNA (strain Wageningen)	Y07496	5882	97.6/3373	> 200	Yes
Potato leafroll luteovirus (Canadian isolate) genomic RNA, complete sequence	D13954; D00734	5883	97.2/3372	> 200	Yes
Potato leafroll luteovirus (Australian isolate) genomic RNA, complete sequence	D13953; D00733	5882	91.5/3372	180	Yes
Potato leafroll virus (Japanese isolate) genomic RNA for RNA-dependent RNA polymerase, partial cds.	AB001894	1882	98.0/1883	> 200	Yes
Barley yellow dwarf virus - RPV (isolate NY) complete genome	L25299	5723	66.5/1611	20	No
Beet western yellows virus (isolate 2ITB) genomic RNA	X83110	5722	64.5/1776	26	No
Beet western yellows virus (BWYV-FL1) genomic RNA	X13063	5641	65.2/1725	29	No
Cucumber yellows virus (isolate N) genomic RNA	X76931	5669	65.3/1627	27	No
Pea enation mosaic virus (strain WSG) ORF 1, complete cds; ORF 2, complete cds; ORF 3, 3' end cds; coat protein gene, complete cds; ORF 5, 3' end cds.	L04573	5706	56.6/852	11	No
Cocksfoot mottle virus (isolate Russia) ORF1, proteinase, RNA-dependent RNA polymerase, and coat protein	L40905	4083	63.7/248	13	No
Transgenic PLRV replicase mRNA Randomized (- Control)	-	3795	75/24	7	-

[1]The replicase portion of the PLRV replicase transgene mRNA is identical to the PLRV strain LR7 replicase.
[2] Sequences were analysis using FASTA program from GCG Version 9.0. FASTA Performs a Pearson and Lipman search for similarity between a query sequence and any group of sequences. FASTA answers the question "What sequences in the database are similar to my sequence?"

TABLE VI.2. COMPARISON OF TRANSGENIC PLRV REPLICASE MRNA[1] (3795 N.T.) AND SEQUENCES FROM GENBANK OF COMMON VIRUSES IN POTATOS

Virus[2]	GenBank Accession #	Size (n.t.)	Overall[3] Similarity(%)	BestFit[4] Similarity (% Identity/nt overlap)	Longest Stretch of Identity for BestFit
Potato Virus A	z21670	9585	37.7	81.8/34	9
Potato Virus M	X53026	8535	37.2	85/20	11
Potato Virus S	D00461	3552	37.0	82.6/23	11
Potato Virus X	X55802	6432	38.9	64.2/68	6
Potato Virus Y	A08776	9705	39.8	70/40	7
Tobacco Rattle Virus RNA1	D00155	6791	37.3	73.5/34	7
Tobacco Rattle Virus RNA2	X03955	3389	37.3	73.5/34	7
Tomato Spotted Wilt Virus M RNA	S48091	4821	38.9	65.6/62	8
Tomato Spotted Wilt Virus S RNA	D13926; D00821	2837	37.7	75/50	8
Tomato Spotted Wilt Virus L RNA	D10066; D01230	8897	37.2	86.9/23	10
Tomato Spotted Wilt Virus S RNA	D00645	2916	37.2	64.3/99	8
Transgenic PLRV replicase mRNA Randomized (- Control)	-	3795	36.3	71.4/35	9

[1]The replicase portion of the PLRV replicase transgene mRNA is identical to the PLRV strain LR7 replicase.
[2]Virus List obtained from USDA Web site: http://www.aphis.usda.gov/biotech/cropvir.html
[3]GAP from GCG Version 9.0 uses the algorithm of Needleman and Wunsch to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps.
[4]BestFit from GCG Version 9.0 creates an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman.

VII. CONCLUSIONS

Damage by CPB and PLRV results in severe crop losses per year. Schreiber and Guenthner (Appendix 1) estimate that the total insecticide material and application cost savings at more than $10,000,000 for the growers who choose to plant NewLeaf Plus potatoes. Estimated benefits to consumers could exceed $100,000,000. The use of CPB and PLRV-resistant potatoes will also aid in the development of biointensive integrated pest management programs for sustainable agriculture. Figure VII.1 illustrates the benefits of NewLeaf Plus for pest control compared to conventional methods. Commerial use of NewLeaf Plus seed potatoes will be closely monitored in U.S. and Canadian seed certification programs (Appendix 2) as is customary for commercial seed potatoes. In addition to a toll-free number, Monsanto/NatureMark maintains close contact with potato growers through sales representatives, crop service partners and crop consultants to ensure that the best management practices are in place to receive the maximum benefits from using NewLeaf Plus potatoes.

Figure VII.1.

Sustainability in Action

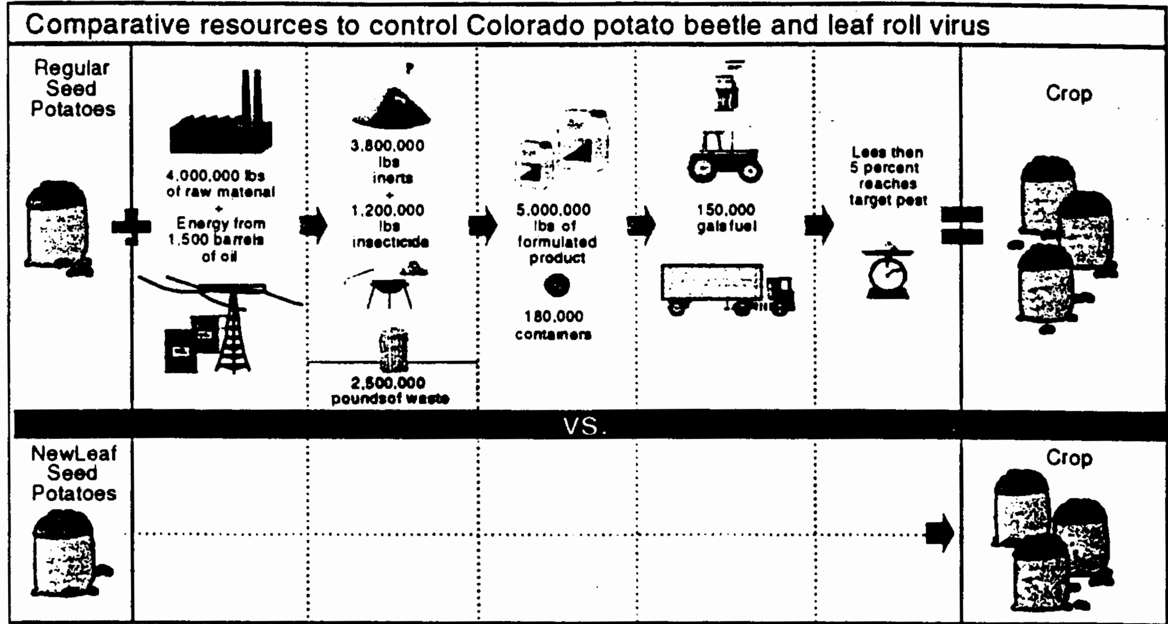

Figure VII.1. Benefits of using NewLeaf Plus potatoes.

VIII. REFERENCES

Adiwilaga, K.D. and Brown, C.R. 1991. Use of 2n pollen-producing triploid hybrids to introduce tetraploid Mexican wild species germplasm to cultivated tetraploid potato gene pool. Theor. Appl. Genet. 81:645-652.

Allison, R.F., M. Janda, and P. Ahlquist. 1989. Sequence of cowpea chlorotic mottle virus RNAs 2 and 3 and evidence of a recombination event during bromoviral evolution. Virology. 172:321-330.

Allison, R.F., W.L. Schneider, and A.E. Greene. 1996. Recombination in plants expressing viral transgenes. Seminar Virology. 7:417-422.h

Allison, R.F., M. Janda, and P. Ahlquist. 1989. Sequence of cowpea chlorotic mottle virus RNAs 2 and 3 and evidence of a recombination event during bromoviral evolution. Virology. 172:321-330.

Almeida, E. R. P., Gossele, V., Muller, C. G., Dockx, J., Reynaerts, A., Botterman J., Krebbers, E. and Timko, M. P. 1989. Transgenic Expression of Two Marker Genes Under the Control of an Arabidopsis rbcS Promoter: Sequences Encoding the Rubisco Transit Peptide Increase Expression Level. Mol. Gen. Genet. 218, 78-86.

Angenent, G.C., E. Posthumus, F.Th. Brederode, and J.F. Bol. 1989. Genomic structure of tobacco rattle virus strain PLB: further evidence on the occurrence of RNA recombination among tobraviruses. Virology 171:271-274.

Bachmann, B. G. 1987. Derivatives and Genotypes oof Some Mutant Derivatives of Escherichia coli K-12. In Escherichia coli and Salmonella pyphimurium, Neidhardt, F. C., Ed., Vol.2, American Society for Microbiology, Washington, DC, pp 1190-1219.

Banttari, E.E., P.J. Ellis and S.M.P. Khurana. 1993. Management of diseases caused by viruses and viruslike pathogens, In: R. C. Rowe (ed.), Potato Health Management. APS Press, St. Paul, Minnesota.

Barker, H 1989. Specificity of the effect of sap-transmissible viruses in increasing the accumulation of luteoviruses in co-infected plants. Annuals of Applied Biology 115:71-78.

Barker, R. F., Idler, K. B., Thompson, D. V. and Kemp, J. D. 1983. Nucleotide Sequence of the T-DNA Region from the Agrobacterium tumefaciens Octopine Ti Plasmid pTi15955. Plant Molecular Biology 2:335-350.

Barker, H. 1987. Invasion of non-phloem tissue in Nicotiana clevelandii by potato leafroll luteovirus is enhanced in plants also infected with potato Y potyvirus. Journal of General Virology 68:1223-1227

Barker, H. 1992. Potato Leafroll, In: H.S. Chaube, J. Kumar, A.N. Mukhopadhyay, and U.S. Singh (eds.), Plant Diseases of International Importance, Volume II: Diseases of Vegetables and Oil Seed Crops, Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

Barkley, G.M. and Schrage, W.W.M. 1993. Potato varieties in Canada, 5th Edition. New Brunswick Department of Agriculture, Fredericton, NB, Canada.

Barry, G., Kishore, G., Padgette, S., Taylor, M., Kolacz, K., Weldon, M., Re D., Eichholtz., Fincher, K., and Hallas, L. 1992. ?Inhibitors of amino acid biosynthesis: Strategies for imparting glyphosate tolerance to crop plants. In Biosynthesis and Molecular Regulation of Amino Acids in Plants. Eds Singh, B. K., Flores, H. E., and Shannon, J. C. American Society of Plant Physiologists, Rockville, Maryland 139-145.

Bavyko, N.F. 1978. Geographic ranges and local names of the primitive cultivated potato species of South America. Trudy po Prik. Bot. Gen. i Selek. 62:102-114.

Beck, E., Ludwig, G., Auerswald, E. A., Reiss, B. and Schaller, H. 1982. Nucleotide Sequence and Exact Localization of the Neomycin Phosphotransferase Gene from Transposon Tn5. Gene 19:327-336.

Benda, G.T.A. 1956. Infection of Nicotiana glutinosa L. following injection of two strains of tobacco mosaic virus into a single cell. Virology 2: 820-827.

Benson, L. 1959. Plant classification. D.C. Health and Company, Lexington, Mass., pp. 226.

Bevan, M., Barnes, W. M., Chilton, M. 1983. Structure and Transcription of the Nopaline Synthase Gene Region of T-DNA. Nucleic Acids Res. 11:369-385.

Boiteau, G., C. Misener, R. P. Singh and G. Bernard. 1992. Evaluation of a vacuum collector for insect pest control in potato. Am. Pot. J. 69: 157-166.

Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L. and Boyer, H. W. 1977. Construction and Characterization of New Cloning Vehicles II. A Multipurpose Cloning System. Gene 2:95-113.

Broadbent, L., G.D. Heathcote, N. McDermott, and C.E. Taylor. 1957. The effect of date of planting and of harvesting potatoes on virus infection and on yield. Annuals of Applied Biology. 45:603.

Brown, C.R. 1988. Characteristics of 2n pollen producing triploid hybrids between Solanum stoloniferum and cultivated diploid potatoes. Am. Pot. J. 65:75-84.

Brown, C.R. and Adiwilaga, K.D. 1990. Introgression of Solanum accale germplasm from the endosperm balance number 2 gene pool into the cultivated endosperm balance number 4 potato gene pool via triploids. Genome 33:273-278.

Bujarski, J.J. and Dzianotti, A.M. 1991. Generation and analysis of nonhomologous RNA-RNA recombinants in brome mosaic virus: sequence complementarities at crossover sites. J. Virol. 65: 4153-4159.
Bujarski, J.J. and Nagy, P.D. 1994. Genetic RNA-RNA recombination in positive-stranded RNA viruses of plants. In: J. Paszkowski (ed), Homologous recombination in plants. Kluwer Academic Publisher, Dordrecht, The Netherlands, pp. 1-24.

C.A.B., International. 1991. Distribution maps of pests. Map no. 139 (2nd revision). London.

Camadro, E.L. and Peloquin, S.J. 1980. The occurrence and frequency of 2n pollen in three diploid Solanums from northwest Argentina. Theor. Appl. Genet. 56:11-15.

Cantelo, B. Personal communication with S. Love.

Carr, J.P., Marsh, L.E., Lomonossoff, G.P., Sekiya, M.E., Zaitlin, M. 1992. Resistance to tobacco mosaic virus induced by the 54-kDa gene sequence requires expression of the 54-kDa protein. Mol. Plant-Microbe Interact. 5:397-404.

Carr, J.P., Gal-On, A., Palukaitus, P., Zaitlin, M. 1994. Replicase-mediated resistance to cucumber mosaic virus in transgenic plants involves suppression of both virus replication in the inoculated leaves and long-distance movement. Virology 199:439-447.

Casagrande, R.A. 1987. The Colorado potato beetle: 125 years of mismanagement. Bull. Entomol. Soc. 33:142-150.

Cascone, P.J., Haydar, T.F., Simon, A.E. 1993. Sequences and structures required for recombination between virus-associated RNAs. Science 260: 801-805.

Cole, D. J. 1985. Mode of Action of Glyphosate - A Literature Analysis. In The Herbicide Glyphosate. Grossbard, E. and Atkinson, D., Eds., Butterworths, Boston, pp. 48-74.

Cooper, J.I. Edwards, M.L., Rosenwasser, O., and Scott, O.W. 1994. Transgenic resistance genes from nepoviruses: efficacy and other properties. New Zeal. J. Crop Hort. 22: 129-137.

Correll, D.S. 1962. The potato and its wild relatives. Texas Research Foundation, Renner, Texas.

Coruzzi, G., Broglie, R., Edwards, C. and Chua, N-H. 1984. Tissue-specific and Light-regulated Expression of a Pea Nuclear Gene Encoding the Small Subunit of Ribulose-1,5-bisphosphate Carboxylase. EMBO J. 3:1671-1679.

Dale, P.J., McPartlan, H.C., Parkinson, R., MacKay, G.R. and Scheffler, J.A. 1992. Gene dispersal from transgenic crops by pollen. In Proceedings of the Second International Symposium on the Biosafety results of Field Tests of Genetically Modified Plants and Microorganisms, Goslar, Germany, May 11-14, 1992, pp 73-78.

Dean, D.A. 1993. Development and Validation of an Enzyme-Linked Immunosorbent Assay (ELISA) for Detection and Quantitation of Bacillus thuringiensis subsp. tenebrionis (B.t.t.) insecticidal protein in Colorado Potato Beetle Resistant Potatoes. Monsanto Technical Report, MSL No. 12341.

De Jong, W. and Ahliquist, P. 1992. A hybrid plant RNA virus made by transferring the noncapsid movement protein from a rod-shaped to an icosahedral virus is competent for systemic infection. Proc. Natl. Acad. Sci. USA 89: 6808-6812.

DeBlock, M., Herrera-Estrella, L., Van Montagu, M., Schell, J., and Zambryski, P. 1984. Expression of foreign genes in regenerated plants and in their progeny. EMBO J. 3:1681-1689.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H. M. 1982. Nopaline Synthase: Transcript Mapping and DNA Sequence. J. Molec. Appl. Genet. 1:561-573.

Edwards, J.W., Walker, E.L., Coruzzi, G.M. 1990. Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase. Proc. Natl. Acad. Sci. USA 87: 3459-3463.

EPA. 1995b. Plant Pesticide Bacillus thuringiensis Cry3A Delta-Endotoxin and the Genetic Material Necessary for Its Production; Tolerance Exemption. Federal Register 60: 47871-47874.

EPA. 1997. Replicase Protein of Potato Leaf Roll Virus and the Genetic Material Necessary for Its production; Exemption from the requirement of a tolerance. Federal Register 62: 43650-43653.

Falk, B.W. and Bruening, G. 1994. Will transgenic crops generate new viruses and new diseases? Science 263: 1395-1396.

Ferro, D.N. and Boiteau, G. 1992. Management of major insect pests of potato. In Plant Health Management in Potato Production (R. C. Rowe ed.) pp. 209-234, Am. Phytopath. Soc. Press, St. Paul, Minnesota.

Ferro, D.N., Morzuch, B.J., and Margolies, D. 1983. Crop loss assessment of the Colorado potato beetle (Coleoptera: Chrysomelidae) on potatoes in western Massachusetts. J. Econ. Entomol. 76:349-356.

Fling, M., Kopf, J. and Richards, C. 1985. Nucleotide Sequence of the Transposon Tn7 Gene Encoding an Aminoglycoside-Modifying Enzyme, 3'(9)-O-Nucleotidyltransferase. Nucleic Acids Res. 13:7095-7106.

Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S., Adams, S. P., Bittner, M. L. Brand, L. A., Fink, C. L., Fry, J. S., Galluppi, G. R., Goldberg, S. B., Hoffmann, N. L. and Woo, S. C. 1983. Expression of Bacterial Genes in Plant Cells. Proc. Natl. Acad. Sci. USA 80:4803-4807.

Fraley, R.T., Horsch, R.B., Matzke, A., Chilton, M.-D., Chilton, W.S. and Sanders, P.R. 1984. In vitro transformation of petunia cells by an improved method of co-cultivation with A. tumefaciens strains. Plant Mol. Biol. 3:371-378.

Grace, A. M. and Lavrik, P. B. 1996. Validation of a polyclonal antibody based enzyme linked immunosorbent assay (ELISA) for measurement of CP4 5-enoylpyruval-shikimate-3-phosphate synthase in potato plant tissues. Monsanto Technical Report, MSL No. 14906.

Grace, A.M. and Rogan, G.J. 1995. Development and validation of a monoclonal antibody based enzyme linked immunosorbent assay (ELISA) for detection and quantitation of neomycin phosphotransferase II in Colorado potato beetle resistant potatoes. Monsanto Technical Report, MSL No. 13758.

Gibbs, M.J. and Cooper, J.I. 1995. A recombinational event in the history of luteoviruses probably induced by base-pairing between the genomes of two distinct viruses. Virology 206:1129-1132.

Gibbs, M.J. 1995. The luteovirus supergroup: rampant recombination and persistent partnerships. In: Molecular Basis of Viral Evolution, pp. 351-368, A.J. Givvs, C.H. Calisher, and F. Garcia-Arenal, eds. Cambridge University Press, Cambridge.

Greene, A.E. and Allison, R.F. 1994. Recombination between viral RNA and transgenic plant transcripts. Science 263: 1423-1425.

Groden, E., F. A. Drummond, R. A. Casagrande, and D. L. Haynes. 1990. Coleomegilla maculata (Coleoptera: Coccinellidae): its predation upon the Colorado potato beetle (Coleoptera: Chrysomelidae) and its incidence in potatoes and surrounding crops. J. Econ. Entomol. 83: 1306-1315.

Habili, N. and Symons, R. H. 1989. Evolutionary relationship between luteovirus and other RNA plant viruses based on sequence motifs in their putatuive RNA polymerase and nucleic acid helicases. Nucl. Acids Res. 17:9543-9555

Hamilton, R.I. and Nichols, C. 1977. The influence of bromegrass mosaic virus on the replication of tobacco mosaic virus in Hordeum vulgare. Phytopathology 67: 484-489.

Hanley-Bowdoin, L., Elmer, J.S., Rogers, S.G. 1990. Expression of functional replication protein from tomato golden mosaic virus in transgenic tobacco plants. Proc. Natl. Acad. Sci. 87: 1446-1450.

Hanneman, R.E. Jr. and Bamberg, J.B. 1986. Inventory of tuber-bearing Solanum species. Wisconsin Agric. Exp. Sta. Bull. 533.

Harcourt, D. G. 1971. Population dynamics of Leptinotarsa decemlineata (Say) in eastern Ontario. III. Major population processes. Can. Entomol. 103:1149-1161.

Hawkes, J.G. 1990. The Potato: Evolution, Biodiversity and Genetic Resources. Smithsonian Institution Press, Washington, D.C.

Hawkes, J.G. 1978. Biosystematics of the potato. In The Potato Crop (P.M. Harris ed.). Halsted Press, New York, pp 15-69.

Herrnstadt, C., Soares, G.C., Wilcox, E.R. and Edwards, D.L. 1986. A new strain of Bacillus thuringiensis with activity against Coleopteran insects. Biotechnology 4:305-308.

Holland, J., K. Spindler, F. Horodyski, E. Gradau, S. Nichol, and S. van de Pol. 1982. Rapid evolution of RNA genomes. Science. 215:1577-1585.

Horsch, R.B., Fraley, R.T., Rogers, S.G., Sanders, P.R., Lloyd, A., and Hoffmann, N. 1984. Inheritance of functional foreign genes in plants. Science 223:496-498.

Howard, H.W. 1970. Genetics of the potato. Springer-verlag New York, Inc., New York, pp 20-23.

Howe, A.R., Tamayo, F., Brown, S., Armstrong, C., Fromm, M., Hart, J., Padgette, S., Parker, G. and Horsch, R. 1992. Development of Glyphosate as a Selectable Marker for the Production of Fertile Transgenic Corn Plants. In Vitro Cell. Dev. Biol. 28:124A.

Howe, A.R., Tamayo, F., Brown, S., Armstrong, C., Fromm, M., Hart, J., Padgette, S., Parker, G. and Horsch, R. 1992. Development of Glyphosate as a Selectable Marker for the Production of Fertile Transgenic Corn Plants. In Vitro Cell. Dev. Biol. 28:124A.

Huttner, S. L., Arntzen, C., Beachy, R., Breuning, G., Nester, E., Qualset, C. and Vidaver, A. 1992. Revising oversight of genetically modified plants. Bio/Technology 10, 967-971.

Ishikawa, M., Kroner, P., Ahlquist, P., Mseshi, T. 1991. Biological activities of hybrid RNAs generated by 3'-end exchanges between tobacco mosaic and brome mosaic viruses. J. Virol. 65: 3451-3459.

Jackson, M.T., Rowe, P.R. and Hawkes, J.G. 1978. Crossability relationships of Andean potato varieties of three ploidy levels. Euphytica 27:541-551.

Kaniewski, W. 1997. Personal communication.

Kaniewski, W., Lawson, C., Loveless, J., Thomas, P., Mowry, T., Reed, G., Mitsky, T., Zalewski, J., and Muskopf, Y. 1994. Expression of potato leafroll virus (PLRV) replicase genes in Russet Burbank potatoes provide immunity to PLRV. Proceed. 3rd EFPP Conference, M. Manka ed. J. Phytopath. 289-292.

Kirkegaard, K. and Baltimore, D. 1986. The mechanism of RNA recombination in poliovirus. Cell 47: 433-443.

Klee, H.J., Y.M. Muskopf, and C.S. Gasser. 1987. Cloning of an Arabidopsis thaliana gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. Mol. Gen. Genet. 210:437-442.

Klee, H.J. and Rogers, S.G. 1989. Plant gene vectors and genetic transformation: plant transformation systems based on the use of Agrobacterium tumefaciens. Cell Culture and Somatic Cell Genetics of Plants. 6:1-23.

Koncz, C. and Schell, J. 1986. The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of Agrobacterium binary vector. Mol. Gen. Genet. 204:383-396.

Koonin, E. V., and V.V. Dolji. 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Critic. Rev. Biochem. Molec. Biol. 28:375-430.

Krieg, A. Huger, A.M., Langenbruch, G.A., and Schnetter, W. 1983. Bacillus thuringiensis var. tenebrionis, a new pathotype effective against Coleoptera. Z. Angew. Ent. 96:500-508.

Lai, M.M-C. 1992. RNA recombination in animal and plant viruses. Microbiol. Rev. 56:61-79.
Lawson, H.M. 1983. True potato seeds as arable weeds. Potato Res. 26:237-246.

Levin, J. G. and Sprinson, D. B. 1964. The Enzymatic Formation and Isolation of 3-Enolpyruvylshikimate-5-phosphate. J. Biol. Chem. 239:1142-1150.

Lommel, S. and Z. Xiong (1991). "Reconstitution of a functional red clover necrotic mosaic virus by recombinational rescue of the cell-to-cell movement gene expressed in a transgenic plant." J. Cell Biochem. **15A**: 151.

Love, S. 1996. Personal Communication.

Luo, G. and Taylor, J. 1990. Template switching by reverse transcriptase during DNA synthesis. J. Virol. 64: 4321-4328.

MacIntosh, S.C., Stone, T.B., Sims, S.R., Hunst, P.L., Greenplate, J.T., Marrone, P.G., Perlak, F.J., Fischhoff, D.A., and Fuchs, R.L. 1990. Specificity and efficacy of purified Bacillus thuringiensis proteins against agronomically important insects. J. Invert. Path. 56:258-266.

Makino, S., J.G. Keck, S.A. Stohlman and M.M-C. Lai. 1986. High frequency RNA recombination of murine coronaviruses. J. Virol. 57:729-737.

Manzer, F.E., D.C. Merriam, R.H. Storch, and G.W. Simpson. 1982. Effect of time of inoculation with potato leafroll virus on development of net necrosis and stem-end browning in potato tubers. American Potato Journal. 59:337.

Martin, R.R., Keese, P.K., Young, M.J., Waterhouse, P.M., Gerlach, W.L. 1990. Evolution and molecular biology of luteoviruses. Annu. Rev. Phytopathol. 28: 341-363.

Mayo, MA. And V. Ziegler-Graf. 1996. Molecular biology of luteoviruses. Advances in Virus Research. 46:413-460.

McPherson, S.A., Perlak, F.J., Fuchs, R.L., Marrone, P.G., Lavrik, P.B. and Fischhoff, D.A. 1988. Characterization of the coleopteran-specific protein of Bacillus thuringiensis subsp. tenebrionis. Bio/Technol. 6:61-66.

Miller, W.A., Dinesh-Kumar, S.P. and Paul, C.P. 1995. Luteovirus Gene Expression, Critical Review in Plant Sciences 14:179-211.

Miller, W.A., G. Koev, and B.R. Mohan. (1997). Are there risks associated with transgenic resistance to luteoviruses? Plant Disease. 81:700-710.

Mitsky, T.A., Hemenway, C.L. and Tumer, N.E. 1996. Plants Resistant to Infection by PLRV. United States Patent Assigned to Monsanto Co. Patent No. 5,510,253.

Moyer, D. D. 1992. Fabrication and operation of a propane flamer for Colorado potato beetle control. Cornell Coop. Extension Bulletin, 7p.

Moyer, D., Kujawski, R., Derksen, R., Moeller, R., Sieczka, J. B. and Tingey, W. M. 1991. Development of a propane flamer for Colorado potato beetle control. Mimeo (also on videotape), Cornell Cooperative Extension, Suffolk County. Riverhead, New York.

Murant, A.R., et al., 1995. Genus Umbravirus. In "Virus Taxonomy-classification and Nomenclature of Viruses. Sixth Report of the International Committee on the Taxonomy of Viruses" (F.A. Murphy, C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martelli, M.A. Mayo, and M. D. Summers, eds.), pp. 388-391. Springer-Verlag, Vienna.

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A. and Summers, M.D. (Eds). 1995. Virus Taxonomy: Classification and Nomenclature of Viruses. Sixth Report on the Intenational Committee on Taxonomy of Viruses, Springer-Verlag, New York, pp 379-383.

Nagy, P.D., Dzianott, A., Ahlquist, P., Bujarski, J.J. 1995. Mutation of the helicase-like domain of of protein 1a alters the sites of RNA-RNA recombination in brome mosaic virus. J. Virol. 69: 2547-2556.

Nagy and Bujarski, unpublished results referenced in Simon, A.E. and J.J. Bujarski. 1994. RNA-RNA recombination and evolution in virus-infected plants. Ann. Rev. Phytopathol. 32:337-362.

Nagy, P.D. and Bujarski, J.J. 1992. Genetic recombination in brome mosaic virus: effect of sequence and replication of RNA on accumulation of recombinants. J Virol. 66: 6824-6828.

Nagy, P.D. and Bujarski, J.J. 1993. Targeting the site of RNA-RNA recombination in brome mosaic virus with antisense sequences. Proc. Natl. Acad. Sci. USA 90: 6390-6394.

Nagy, P.D. and Bujarski, J.J. 1995. Efficient system of homologous RNA recombination in brome mosaic virus: sequence and structure requirements and accuracy of crossovers. J. Virol. 69: 131-140.

Nagy, P.D. and Bujarski, J.J. 1997. Engineering of homologous recombination hotspots with AU-rich sequences in brome mosaic virus. J. Virol. 71: 3799-3810.

National Potato Council. 1996. Potato Statistical Yearbook. Englewood, Colorado, pp. 56..

National Potato Council. 1992. Potato Statistical Yearbook. Englewood, Colorado, pp. 26-27.

National Potato Council. 1993. Potato Statistical Yearbook. Englewood, Colorado, p. 10.

Newell, C. A., Rozman, R., Hinchee, M. A., Lawson, E. C., Haley, L., Sanders, P., Kaniewski, W., Tumer, N. E., Horsch, R. B. and Fraley, R. T. 1991. Agrobacterium-mediated transformation of Solanum tuberosum L. cv. Russet Burbank. Plant Cell Rep. 10:30-34.

Novy, R.G. and Hanneman, R.E. 1991. Hybridization between Gp. tuberosum haploids and 1 EBN wild potato species. Am. Pot. J. 68:151-169.

Olkowski, W., Saiki, N., and Daar, S. 1992. IPM options for Colorado potato beetle. The IPM Practitioner 16:1-21.

Passmore, B.K., M. Sanger, L. Chin, and B.W. Falk. 1993. Beet western yellows virus-associated RNAL An independently replicating RNA that stimulates virus accumulation. Proc. Natl. Acad. Sci. USA 90:10168-10172.

Pavek, J. Personal communication.

Perlak, F.J., Stone, T.B., Muskopf, Y.M., Petersen, L.J., Parker, G.B., McPherson, S.A., Wyman, J., Love, S., Reed, G., Biever, D. and Fischhoff, D.A. 1993. Genetically Improved Potatoes: Protection from Damage by Colorado Potato Beetles. Plant Molecular Biology **22,** 313-321.

Peters, D. 1970. Potato Leafroll Virus. CMI/AAB Descriptions of Plant Viruses, No.36

Pogany, J., J. Romero, Q. Huang, J-Y. Sgro, H. Shang, and J. J. Bujarski. 1995. De novo generation of defective interfering -like RNAs in broad bean mottle bromovirus. Virology 212:574-586.

Potato Growers of Michigan, Inc., and the Michigan Potato Industry Commission. 1992. December 1991 CPB Survey Results: Crop Years 1989-1991.

Rao, A.L.N. and T.C. Hall. 1993. Recombination and polymerase error facilitate restoration of infectivity in brome mosaic virus. J. Virol. 67:969-979.

Raschke,E., G. Baumann,. and F. Schoffl. 1988. Nucleotide sequence analysis of soybean small heat shock protein genes belonging to two different multigene families. J. Mol. Biol. 199:549-557

Rasochova, L., B.K. Passmore, B.W. Falk, and W.A. Miller. 1997. Satellite RNA of Barley Yellow Dwarf Virus-RPV is supported by Beet Western Yellow Virus in dicotyledonous protoplasts and plants. Virology 231 182-191.

Richins, R., Scholthof, H., and Shepard, R. 1987. Sequence of figwort mosaic virus DNA (caulimovirus group). Nucleic Acids Research, 15:8451-8466.

Rick, C.M. 1979. Biosystematic studies in Lycopersicon and closely related species of Solanum. In The Biology and Taxonomy of the Solanaceae. Eds. Hawkes, Lester and Skelding.

Rochester, D.E. and P.B. Lavrik. 1997. Molecular characterization of potato leafroll virus and Colorado potato beetle resistant Russet Burbank potato lines transformed with plant vector PV-STMT21, Study No. 96-01-37-20.

Robinson, D.J., Hamilton, W.D.O., Harrison, B.D., Baulcombe, D.C. 1987. Two anomalous tobravirus isolates: evidence for RNA recombination in nature. J. Gen. Virol. 68: 2551-2561.

Robinson, D. J. 1996. Environmental risk assessment of releases of transgenic plants containing virus-derived inserts. Transgenic Research 5:359-362.

Roush, R. T., and W. M. Tingey. 1993. Strategies for management of insect resistance to synthetic and microbial insecticides. In press. In Advances in Potato Pest Management. American Phytopathological Society Press, Minneapolis.

Schoelz, J. E. and Wintermantel, W.M. 1993. Expansion of viral host range through complementation and recombination in transgenic plants. The Plant Cell 5: 1669-1679.

Shields, E.J. and Wyman, J.A. 1984. Effect of defoliation at specific growth stages on potato yields. J. Econ. Entomol. 77:1194-1199.

Sijen, T., Wellink, J., Hendriks, J., Verver, J., van Kammen, A. 1995. Replication of cowpea mosaic virus RNA1 or RNA2 is specifically blocked in transgenic Nicotiana benthamiana plants expressing the full-length replicase or movement protein genes. Mol. Plant-Microbe Interact. 8:340-347.

Simon, A.E. and J.J. Bujarski. 1994. RNA-RNA recombination and evolution in virus-infected plants. Ann. Rev. Phytopathol. 32:337-362.

Sims, S.R. 1993. Sensitivity of Selected Insect Species to the Colorado Potato Beetle Active Protein from Bacillus thuringiensis subsp. tenebrionis. Study No. 92-01-37-05. EPA MRID No. 42932207, an unpublished study conducted by Monsanto Company.

Slack, S.A. 1993. Seed certification and seed improvements programs, In: R. C. Rowe (ed.), Potato Health Management .APS Press, St. Paul, Minnesota.

Slaney, A.C., Robbins, H.L., and English, L. 1992. Mode of action of Bacillus thuringiensis toxin Cry3A: An analysis of toxicity in Leptinotarsa decemlineata (Say) and Diabrotica undecimpunctata Howardii Barber. Insect Biochem. Molec. Biol. 22:9-18.

Smith, H.B. 1927. Chromosome counts in the varieties of Solanum tuberosum and allied wild species. Genetics 12:84-92.

Stalker, D. M., Thomas, C. M. and Helinski, D. R. 1981. Nucleotide Sequence of the Region of the Origin of Replication of the Broad Host Range Plasmid RK2. Mol. Gen. Genetics 181:8-12.

Stevenson, F.J. 1951. The potato - its origin, cytogenetic relationship, production, uses and food value. Econ. Bot. 5:153-171.

Strauss, J.H. and E.G. Strauss. 1988. Evolution of RNA viruses. Ann. Rev. Microbiol. 42:657-683.

Sutcliffe, J. G. 1978. Complete nucleotide Sequence of the Escherichia coli Plasmid pBR322. Symposia on Quantitative Biology 43:77-103.

Tenllado, F., Garcia-Luque, I., Serra, M.T., Diaz-Ruiz, J.R. 1995. Nicotiana benthamiana plants transformed with the 54-kDa region of the pepper mild mottle tobamovirus replicase gene exhibit two types of resistance responses against viral infection. Virology 211:170-183.

Thomas, P.E. 1986. PLRV Isolate LR-7 supplied by U.S. Department of Agriculture, Agricultural Research Service, Prosser, WA.

Thomas P., W. Kaniewski, G. Reed, and C. Lawson. 1995. Transgenic resistance to potato leafroll virus in Russet Burbank potatoes, p. 551-554. In: Environmental Biotic Factors in Integrated Plant Disease Control. Proceed. 3rd EFPP Conference.

Tynan, J.L., Williams, M.K. and Conner, A.J. 1990. Low frequency of pollen dispersal from a field trial of transgenic potatoes. J. Genet. and Breed. 44:303-306.

USDA Web Site. Http://www.aphis.usda.gov/biotech/cropvir.html.

USDA. 1996. Monsanto Company; Availability of Determination of Nonregulated Status for Potato Lines Genetically Engineered for Insect Resistance. 61:24755-24757

USDA. 1991. Potato Facts Fall/Winter, Agricultural Marketing Service, USDA.

USDA. 1995. Availability of Determination of Nonregulated Status for Gentically Engineered Potato Lines. 60:13108-13109.

Vaitukatus, J.L. 1982,Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections. In: H. Van Vunakis and J.J. Langone (ed.), Methods In Enzymology, 73:46-52.Academic Press: San Diego, CA.

Van der Wilk, F., Huisman, M.J., Cornelissen, B.J.C., Huttinga, H. and Goldbach, R. 1989. Nucleotide Sequence and Organization of Potato Leafroll Virus Genomic RNA. FEBS Letters 245:51-56.

Van den Heuvel, J.F.J.M., et al. 1995. Localization of potato leafroll virus in leaves of secondarily-infected potato plants. European Journal of Plant Pathology 101:567-571.

Vance, V. B. 1991. Replication of potato virus X RNA is altered in coinfections with potato virus Y. Virolog. 182:486-494.

Waterhouse, P.M., and A. F. Murant. 1983. Further evidence on the nature of the dependence of carrot mottle virus on carrot red leaf virus for transmission by aphids. Annals App. Biol. 103:455-464.

Whitson, T.D., Burrill, L.C. Dewey, S.A., Cudney, D.W., Nelson, B.E., Lee R.D., and Parker,R. 1991. Weeds of the west. Western Society of Weed Science and University of Wyoming, pp. 574-583.

Wong, E. Y., Hironaka, C. M. and Fischhoff, D. 1992. Arabidopsis thaliana Small Subunit Leader and Transit Peptide Enhances the Expression of Bacillus thuringiensis Proteins in Transgenic Plants. Plant Molec. Biol. 20:81-93.

Yerk, G.L. and Peloquin, S.J. 1990. Performance of haploid x wild species, 2x hybrids (involving five newly evaluated species) in 4x x 2x families. Am. Pot. J. 67:405-417.

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Appendices

Appendix 1. Biologic and Economic Assessment of Genetically Modified CPB- and PLRV-Resistant Potatoes

Biologic and Economic Assessment of Genetically Modified CPB- and PLRV-Resistant Potatoes

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Abstract

The changes in insecticide use patterns on U.S. potatoes following anticipated introduction of potatoes genetically modified to resist Colorado potato beetle and potato leaf roll virus were estimated. Results suggest that introduction of pest-resistant potatoes will significantly reduce insecticide use on potatoes. An estimated 184,000 to 265,000 acres of CPB- and PLRV-resistant Russet Burbank potatoes could be expected to be planted by growers, resulting in as much as 362,000 to 509,000 fewer acre-treatments of insecticides and 486,000 to 704,000 fewer pounds of insecticides applied. This represents a 13.6% to 19.7% reduction in insecticide use on potatoes in the U.S. Total insecticide material and application cost savings are estimated at more than $10,000,000 for the growers who choose to plant CPB- and PLRV-resistant potatoes. Estimated benefits to consumers could exceed $100 million. Use of pest-resistant potatoes will also aid in the development of biointensive integrated pest management programs.

Introduction

Potato, *Solanum tuberosum*, the most commonly consumed vegetable in the U.S., is attacked by a variety of insect pests. Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, and aphids of various species that vector potato leaf roll virus (PLRV), potato virus Y (PVY) and other potato viruses, are the most important insect pests nationally. Green peach aphid, *Myzus persicae*, is the most important viral vector in potatoes. Insecticides are the primary method of control for CPB and aphids. National Agricultural Statistics Service (NASS) surveys indicate 88% of the U.S. potato crop is treated with insecticides totaling 3.6 million pounds annually, and over 50% of all insecticides applied to potatoes are for the control of CPB and aphids that vector PLRV.

CPB is a foliage feeder that can cause severe reduction in yields if not controlled. Adult CPB emerge in the spring and lay eggs on young potato plants, giving rise to the first larval generation. CPB larvae and adult beetles feed on foliage, reducing yield. Defoliation can occur throughout the growing season. Control is achieved through application of both systemic and foliar insecticides. Management of CPB is hampered in many states by widespread occurrence of insecticide resistance.

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Although aphids can cause yield losses by removing sap, their primary damage is transmission of viral diseases. Yield losses due to PLRV can be significant, but the most consequential losses due to this virus are from reduced quality in stored potatoes. Tubers from plants infected with PLRV sometimes exhibit a condition known as net necrosis, which is the leading source of pest-related losses in U.S. potatoes. Losses due to net necrosis not only result in losses to growers, but also result in significant losses to the potato processing industry. Treatment thresholds do not exist for aphids that are viral vectors, thus precluding development of biointensive IPM programs. To prevent unacceptable losses from aphid-vectored diseases, growers are required to make frequent applications of insecticides.

The first potato genetically modified to be resistant to a pest (CPB) was commercialized in May of 1995 (EPA, 1995). The next genetically modified potato product under review at EPA is resistant to both CPB and PLRV. Potatoes genetically altered to resist both CPB and PLRV have the potential to significantly reduce reliance on conventionally applied insecticides, offering the potential for development of more sophisticated IPM programs.

We have examined current pesticide use practices and the performance of CPB - and PLRV-resistant potatoes and have developed estimates of expected biological and economic impacts of the technology on the U.S. potato industry.

Methods

Biologic Assessment

Market Share

Estimates of grower reliance on CPB- and PLRV-resistant potatoes were needed to quantify impacts. Since there are no previous examples of genetically modified seed potatoes, a market share model was not developed. Instead, the authors made market share assumptions based on their knowledge of the industry and discussion with research and extension specialists and other individuals associated with the potato industry. The assumptions are for approximately three to four years after market introduction.

Many factors could influence the use of genetically-modified potatoes, so market share assumptions were made in ranges. Analysis was limited to the eight states that produce a significant amount of Russet Burbanks -- the variety that has been genetically modified to be resistant to CPB and PLRV. Data from the National Potato Council Potato (NPC) Statistics Yearbook (1994) was used to determine current acreage of Russet Burbank and all potatoes. Although variety mix continually changes, and the introduction of CPB- and PLRV-resistant potatoes may cause some growers to change varieties, the 1994 data were used as a base for assumptions.

Reduction in Acre-Treatments

NASS (1995) data on insecticide applications to potatoes served as the basis for determining

expected shifts in use patterns following introduction of CPB- and PLRV-resistant potatoes. Based on current insecticide use pattern data provided by university potato research and extension specialists, acre-treatments of insecticide applications to Russet Burbanks were segregated from total amounts of insecticides applied to potatoes. Acre-treatments of insecticide usage on Russet Burbanks for control of CPB and aphids vectoring PLRV were further segregated from total amounts of insecticides applied to Russet Burbanks.

Typically, insecticides used to control CPB and aphids vectoring PLRV in Russet Burbank production are not used to control other insect pests, making segregation of insecticide usage by target pest a relatively straight forward process. Where insecticides are applied to control other insect pests of Russet Burbanks as well (in Midwestern states and Maine), the relative portion of insecticide used for each pest was determined by data from NASS (1995) and information provided by university specialists.

Reduction in Pounds of Insecticides Applied

To determine pounds of insecticides not expected to be applied as a result of reliance on CPB- and PLRV-resistant potatoes, the estimated total of acre-treatments of each insecticide not expected to be applied was multiplied by the average rate of application as calculated by NASS (1995).

Pesticide Containers Not Used

The number of pesticide containers not used as a result of CPB- and PLRV-resistant potatoes was calculated for nine of the insecticides having the greatest reduction in pounds applied. The aggregate amount of these pesticides accounts for 95% of potential use reduction. Container type was determined by surveying three pesticide dealers in major potato growing regions of Washington and Oregon on the typical package sizes of insecticides sold to potato growers. The relative frequency of package size for each insecticide was calculated. The total of the formulated pounds of each active ingredient expected not to be applied was divided by package sizes used by potato growers.

Economic Assessment

Economic impacts were estimated for potato growers as well as for consumers. Grower impacts consisted of insecticide costs avoided and break-even prices for CPB- and PLRV-resistant seed potatoes. Potential benefits of enhanced yield and quality were estimated for grower revenue and consumer potato expenditures.

Reduced Insecticide Costs

Insecticide costs avoided were calculated based on the market share assumptions in Table 1. The midpoint of the ranges in Table 1 as well as the midpoints in Table 2 and Table 3 were used to estimate quantities of the pesticides that would be replaced. Insecticide prices were obtained from the USDA Agricultural Prices (1994) and Patterson et al. (1995). Insecticide application costs

were estimated at $5.00 per acre, which is the average cost of potato pesticide application in Patterson, et al. (1995).

Break-even prices of pesticides were estimated to show the premium that growers could afford to pay for CPB- and PLRV-resistant seed potatoes as a replacement for current pesticide practices. The procedure was to divide per acre insecticide savings by the typical seed potato planting rate in each state. This did not account for any yield benefits or quality benefits from planting CPB- and PLRV-resistant potatoes.

Yield and Quality Assumptions

Yield impacts have been assessed in other studies. USDA Agriculture Handbook No. 291 (1965) estimates annual average yield losses due to PLRV at 3% and losses due to "foliage feeding insects," of which CPB is the main pest, also at 3%. Hammond (1981) and Kirpes, et al. (1982) found that potato yields are reduced one percent for each percent of PLRV infection. More recent studies have analyzed the impact of potato growers losing access to insecticides. Wyman (1990) found that potato yields in North Dakota would be reduced by 25% if the number of potato insecticides were reduced by half. Future yield and quality impacts of CPB- and PLRV-resistant potatoes would be influenced by the availability and effectiveness of alternative control methods for CPB and PLRV.

In this study specific yield and quality impacts are not estimated. Instead, impacts were estimated under three different assumptions: increases of 2%, 4% and 6% in both yield and quality. Quality is in terms of higher grower revenue due to processor contract quality incentives, reduced rejections and improved fresh market packouts. It was assumed that CPB- and PLRV-resistant potatoes would reduce the amount of undersized potatoes and potatoes with net necrosis.

Market Adjustment

A market adjustment was used to capture the influence of increased yields on long-run prices. Guenthner (1987) found that each 1% increase in U.S. potato supply leads to a long-run decline in price of 1.2%, after allowing for a series of grower production responses and market price responses. Consumer benefits were calculated with the use of marketing margin data published by NPC (1994) for fresh potatoes and USDA (1994) for processed potatoes. This simple average of the two measures was 22%, indicating that the farm value of potatoes is 22% of retail. Consumer benefits were calculated as the grower market adjustment (explained above) times the inverse of the 22% or 4.545. An additional benefit to consumers would be enhanced quality, but that was not estimated.

Results and Discussion

Biologic Assessment

Market share

An estimated 184,000 to 265,000 acres of CPB- and PLRV-resistant potatoes, comprising 31% to 45% of the U.S. Russet Burbank market (Table 1), could be expected to be planted by growers. Attaining or exceeding this level of market share will depend on at least four factors. 1) Growers must have sufficient access to the genetically modified seed. 2) Reliance on pest- resistant potatoes will be influenced by the availability of other effective alternatives for control of CPB or aphids. The development of resistance to a major control alternative, such as imidacloprid, would have a significant positive influence on the market share of pest-resistant potatoes. If resistance develops to imidacloprid or another major alternative, the market share for pest-resistant potatoes will increase beyond the estimates provided in this analysis. 3) The prices of CPB- and PLRV-resistant potatoes will determine the extent to which the product is used by growers. The price is expected to be comparable to alternative control programs based on use of insecticides such as imidacloprid and aldicarb. A significant price differential between CPB- and PLRV-resistant potatoes and imidacloprid or aldicarb based control programs would strongly influence market share. 4) Processors or purchasers of processed potatoes may attempt to influence use of genetically modified potatoes. Attempts by processors to provide incentives or disincentives for grower use of modified potatoes could influence market share.

Because CPB- and PLRV-resistant potatoes will be used only by growers producing Russet Burbank potatoes, the greatest reliance is expected to be in the primary Russet Burbank producing states of Idaho, Washington and Oregon. Additionally, control of CPB and PLRV-vectoring aphids accounts for the vast majority of insecticide applications in this region; hence, the greatest incentive to use the technology exists in the Pacific Northwest (PNW). Based on these two factors, CPB- and PLRV-resistant potatoes can be expected to attain 35% to 50% of the Russet Burbank market in the PNW. An estimated 95% of CPB- and PLRV-resistant acreage is expected to be in the three Northwestern states. Total CPB- and PLRV-resistant potato acreage in the PNW is expected to be 175,000 to 251,000 acres.

Reliance on CPB- and PLRV-resistant potatoes is expected to be much less in Midwestern states, because growers need to control differing pest complexes such as CPB, leafhoppers and potato virus Y. Many Midwestern and Eastern growers will preferentially select control options such as potato varieties engineered to be resistant to CPB and PVY or foliar and systemic insecticides that control local pest complexes. For this reason, reliance on potatoes genetically modified to be resistant to CPB and PLRV would be limited to no more than approximately 5% to 10% in all states producing Russet Burbank potatoes outside of the PNW. Total CPB- and PLRV-resistant potato acreage, excluding the PNW, is expected to be in the range of 8,500 to 14,300 acres.

Reduction in Acre-Treatments

Due to the ability of genetically altered potatoes to resist CPB and PLRV, growers will not have

to apply insecticides for control of either CPB or aphids that vector PLRV. The extent to which insecticide usage shifts will be proportional to the amount of insecticides that would have been applied to conventional Russet Burbank potatoes had plantings not been substituted with pest-resistant potatoes. The greatest shifts in usage will be in the areas with the most acreage of pest-resistant potatoes and the most intensive CPB and PLRV pressure.

Total acre-treatments of insecticides not applied due to reliance on CPB- and PLRV-resistant potatoes were estimated to be 362,000 to 509,000 (Table 2). Based on these estimates, employment of CPB-and PLRV-resistance technology would reduce acre-treatments of insecticides applied to potatoes in the U.S. by nearly 12% to 16%, based on NASS (1995) estimates of 3.1 million acre treatments of insecticides to the 11 primary potato producing states in the U.S. Of this amount, 94% of the reduced number of acre-treatments will be in the three PNW states. Total reduction of acre-treatments for the PNW is expected to be 339,000 to 467,000.

The greatest reduction in acre-treatments will be in Washington (195,000 to 263,000). This represents a potential reduction of 34% to 47% in insecticide use on potatoes in Washington. This is due to widespread planting of Russet Burbank potatoes and high numbers of insecticide applications for control of PLRV-vectoring aphids. Idaho would have the second largest reduction of acre treatments (96,000 to 135,000). This represents a potential reduction of 26% to 37% in insecticide use on potatoes in that state. Although Idaho has the most extensive plantings of Russet Burbank and is expected to have the greatest acreage of genetically modified potatoes, the insect pressure is lower in Idaho than in Oregon and Washington; therefore, less potential exists for reducing insecticide usage. Oregon will have the third largest reduction in acre-treatments (48,000 to 69,000). This represents a potential reduction of 35% to 50% in insecticide use on potatoes in Oregon. The state ranks third in Russet Burbank production and has CPB and PLRV pressure intermediate between that of Washington and Idaho. Use of CPB- and PLRV-resistant potatoes is expected to result in an average reduction of five, one and four applications of insecticides per acre, respectively, for Washington, Idaho and Oregon.

The use of CPB- and PLRV-resistant potatoes will be limited in Midwestern and Eastern states, because Russet Burbank is not a predominant variety, and other insect pest complexes are primary targets for control. Relative importance of Russet Burbank in the remaining five states ranges from 32% in Wisconsin to 17% in Maine and Michigan. The limited acreage of CPB- and PLRV-resistant potatoes and the reduced need to control PLRV-vectoring aphids in Eastern and Midwestern states is expected to result in relatively modest overall decreases in insecticide usage in these areas. Total reduction of acre-treatments for states outside the PNW is expected to be 22,000 to 42,000. The state of Maine is expected to account for the greatest amount of this reduction in acre-treatments, due to the larger number of acres planted with Russet Burbanks and to heavy CPB infestations.

Pounds of Insecticides Not Applied

In general, shifts in use of insecticides, in terms of pounds applied, is approximately proportional

to shifts in acre-treatments. However, because rates of application for each active ingredient are different, changes in acre-treatments are not directly related to changes in pounds of insecticides applied. Compounds applied at relatively low rates (e.g. pyrethroids) or high rates (e.g. cryolite) skew the relationship between pounds applied and acre-treatments.

Total estimated pounds of insecticides not applied due to reliance on CPB- and PLRV-resistant Russet Burbank potatoes is 486,000 to 704,000 (Table 3). Based on these estimates, employment of CPB-and PLRV-resistance technology would reduce pounds of insecticides applied to all U.S. potatoes by 13.6% to 19.7%. This is based on NASS (1995) estimates of 3.57 million pounds of insecticides applied in the 11 primary potato producing states in the U.S.

Of this reduction in pounds of insecticides applied, 95% would be in the three PNW states. Total reduction of pounds for the PNW is expected to be 464,000 to 665,000. The greatest reduction in pounds will be in Washington (211,000 to 300,000). Idaho would have the second largest reduction (199,000 to 287,000), and Oregon would have the third largest reduction (54,000-78,000). Total reductions in pounds of insecticides applied in the five remaining primary Russet Burbank producing states are expected to total 22,000 to 40,000 pounds.

Shifts in Usage Patterns

The insecticide with the greatest reduction in pounds applied would be phorate, with a potential reduction of 230,000 to 327,000 pounds and 85,000 to 120,000 acre-treatments. This reduction would represent nearly half of all reduction in pounds applied and 23% of reductions in acre-treatments. Methamidophos would have the greatest reduction in acre-treatments, with a potential reduction of 136,000 to 190,000 acre-treatments and 94,000 to 132,000 pounds applied. This reduction would represent 37% of all reduction in acre-treatments and nearly 20% of the reduction in pounds applied. Other insecticides with significant reductions in pounds applied would include carbofuran (44,000 to 67,000), disulfoton (43,000 to 69,000), oxamyl (25,000 to 37,000) and propargite (17,000 to 25,000). Collectively, these active ingredients would comprise more than 90% of the pounds of insecticides replaced by CPB- and PLRV-resistant potatoes.

Pesticide Containers Not Used

The reduction of insecticide use will result in a reduction in pesticide containers used by growers. Up to an estimated 40,000 to 60,000 pesticide containers would not be needed, due to the overall reduction in insecticide applications. Phorate containers, which represented nearly half of all estimated reduced use, accounted for 62% of the containers. The common containers likely to have the greatest reduction in use were 2.5-gallon containers for several liquid products and 45-pound containers for phorate.

Additional Considerations

Particularly in the PNW, insecticide applications for CPB and aphids vectoring PLRV are thought to control other insects, due to the broad spectrum nature of insecticides applied. It is possible that if insecticides are no longer applied for control of CPB or aphids, insecticides may have to be applied to control insect pests that would have otherwise been controlled. Therefore,

the estimates of reductions in acre-treatments and pounds of pesticides applied should be considered potential maximum reductions. The actual net reductions will be the amounts of insecticides applied for unforeseen insects infestations subtracted from the maximum potential amount of insecticides avoided due to use of pest-resistant potatoes.

Our estimates of insecticide usage are based on 1994 NASS (1995) data. Insecticide use patterns have shifted since that time, most notably due to the introduction of imidacloprid. Reliance on imidacloprid for control of CPB and aphid pests has resulted in some decrease in insecticide usage. Further decreases in usage are expected in the PNW with re-introduction of aldicarb in 1996. Therefore, our estimates should be used as an indication of the potential for reduction of insecticide usage.

Economic Assessment

The results of the economic assessment are in Tables 5, 6 and 7. Total insecticide material and application cost savings are estimated at more than $10,000,000 for the growers who choose to plant CPB- and PLRV-resistant potatoes. Cost savings ranged from about $100,000 in Michigan to well over $5,000,000 in Washington.

Grower Benefits

The cost savings calculated in Table 5 are also shown in Table 6, where they were converted to dollars per acre. The break-even prices in Table 6 are estimates of how much premium growers could afford to pay for CPB- and PLRV-resistant seed potatoes (in addition to underlying price of the seed) as a replacement for insecticides. The break-even ranges from $0.97 per cwt in Idaho to $9.31 per cwt in Michigan. The average for the eight states is $2.24 per cwt.

The economic assessments of enhanced yield and quality under three different assumptions are in Table 7. The market adjustment accounts for the price impact of increased supply from yield enhancement. Grower benefits for the eight states were extrapolated to the entire crop produced in the eight states during the 1992-1994 period.

Consumer Benefits

Consumer benefits were in the form of lower prices for potato products. This is calculated by accounting for the 22% farm share of retail potato expenditures. The market adjustment for reduced grower prices (due to higher yield) would expand to a much higher value at the consumer level, assuming the market spread would remain the same. At each yield and quality assumption, consumers would benefit by a much larger amount than would growers.

Qualitative Assessment

Impact on Potato IPM Programs
Currently, most of the compounds applied to potatoes for insect pests are broad spectrum organophosphate, carbamate and pyrethroid insecticides. The use of CPB- and PLRV-resistant potatoes would virtually eliminate the use of these insecticide products on potatoes genetically modified to be resistant to CPB and PLRV. Large-scale field trials in Oregon and Washington, using closely related CPB-resistant potatoes, have demonstrated that, in the absence of broad spectrum insecticides, populations of beneficial arthropods increase significantly (Reed, 1994; 1995; Feldman, et al., 1994). The use of CPB- and PLRV-resistant potatoes is expected to further increase the potential for incorporation of beneficial organisms into potato IPM programs. Use of CPB- and PLRV-resistant potatoes, in combination with judicious use of selective insecticides, is expected to form the basis of future potato IPM programs. (Garrell Long, Washington State University, personal communication).

Two-spotted spider mite, *Tetranychus urticae*, is a secondary pest of potatoes in the PNW. Outbreaks of the pest in potatoes are caused by applications of insecticides, particularly pyrethroid insecticides, for control of CPB and aphids (Garrell Long, Washington State University, personal communication). NASS (1995) indicated that 52,800 acre-treatments of pyrethroid insecticides were applied to PNW potatoes, and 62,700 acre-treatments of propargite were used to control mite outbreaks. Secondary pest outbreaks of two-spotted spider mite would cease to occur on acreage planted with CPB- and PLRV-resistant potatoes.

Changes in Quality
Decreases in yield quality due to PLRV and the resulting net necrosis occur routinely, particularly in the PNW, and such incidents are increasing. Schreiber (1994) documented that the incidence of PLRV in seed potatoes used by Washington growers increased from 6% in the mid-1980s to 12% in the early 1990s In 1993, 1.2% of Washington Russet Burbank potatoes were rejected for being out of grade due to unacceptable occurrence of net necrosis. The value of this loss was estimated to be in excess of $36 million. In addition, losses from net necrosis in potatoes not considered out of grade are equal to or greater than losses due to outright rejections. Use of PLRV-resistant potatoes could eliminate up to 50% of the losses from net necrosis in the PNW.

Reduced Human and Environmental Exposure to Insecticides
The significance of the reduction in exposure to insecticides, as a result of the use of pest-resistant potatoes, is difficult to estimate. The nearly 14% to 20% reduction in insecticide use on U.S. potatoes is a considerable reduction. Many of the insecticides expected to decline significantly in usage due to reliance on CPB- and PLRV-resistant potatoes have the potential to pose some risk to human or environmental health (Table 4.), if exposure is excessive. Six of the insecticides with the greatest potential for exposure reduction, phorate, carbofuran, azinphos-methyl, oxamyl and disulfoton, are Toxicity Class 1 insecticides. Propargite usage, which could decline by as much as 25%, is in the EPA Special Review process due to concerns associated

with its classification as a B_2 carcinogen. Pyrethroid usage, which could decline by 38% to 60% in the PNW, is thought by the EPA to pose some risk to aquatic environments.

The reduced usage of insecticides will result in reduced number of applications, which will reduce mixer, loader, applicator and non-target exposure. Between 1990 and 1994, 47 individuals claimed exposure (poisoning) to methamidophos and azinphos-methyl aerially applied to potatoes in Washington (WSDA, 1995). Investigation of the incidents by the Washington State Department of Agriculture indicated that the likelihood of actual exposure for the 47 individuals ranged from definite to unlikely. The estimated potential reductions in the use of these insecticides in Washington are 50% to 60% (methamidophos) and (22% to 29%) (azinphos-methyl). These reductions in usage would be expected to reduce off-site human exposures.

Use of resistant potatoes will result in reduced types of other crop protection inputs. Growers will make fewer trips across their fields, due to the reduced need to apply insecticides. In addition to the reduced number of pesticide containers, growers will have fewer excess insecticides to dispose of.

Impact on Potato Seed Industry

The impact of CPB- and PLRV-resistant potatoes on the potato seed industry will be significant, although quantifying benefits to this sector of the potato production system is beyond the scope of this assessment. Due to the significant amount of insecticides applied to seed potatoes to ensure quality requirements and prevention of seed-borne disease, pest-resistant potatoes are expected to have considerable benefits to the seed industry. Benefits to the seed industry are expected to include reduced use of pesticides (not included in above calculations), lower costs associated with reduced removal (roguing) of diseased plants and reduced decertification of seed potatoes. Because most PLRV inoculum is believed to be seed-borne, elimination of PLRV from a substantial portion of Russet Burbank seed will benefit conventional potatoes indirectly, due to the generally lesser amount of disease inoculum available. The reduction in PLRV inoculum can be expected to substantially reduce PLRV infections and the resulting widespread reductions in yield quality.

Acknowledgment. The authors wish to thank Jim Dwyer, Jennifer Feldman, Garrell Long, Ed Plissy, Duane Preston, Gary Reed, Dick Storch, Mike Thornton, Robert Thornton and Sam Thornton for their input on insecticide use patterns.

Literature Cited

EPA. 1995. Pesticide Fact Sheet. Plant-pesticide *Bacillus thuringiensis* CryIII(A) delta endotoxin and the genetic material necessary for its production in potato. Conditional Registration. Office

of Prevention, Pesticides and Toxic Substances. U.S. Environmental Protection Agency

Feldman, J.G. Reed, J. Wyman, J. Stewart and T. Stone. 1994. Genetically modified Colorado potato beetle resistant potato plants, foliar applied microbial B.t.t., and conventional insecticides: comparative impacts on non-target arthropods. USDA petition for determination of unregulated status: potatoes producing the Colorado potato beetle control protein *Bacillus thuringiensis* subsp. *tenebrionis*.

Gianessi, L.P. and J.E. Anderson. 1995. Pesticide use in U.S. crop production: National Summary Report. National Center for Food and Agricultural Policy. 280 p.

Guenther, Joseph F. 1987. Acreage response: an econometric analysis of the United States potato industry. Unpublished Ph.D. dissertation, Department of Agricultural Economics, Washington State University, Pullman, Washington.

Hammond, David F. 1981. The dangers of using year-out or non-certified seed potatoes. Proceedings of the 1981 Idaho Potato School, University of Idaho, Moscow, Idaho.

Kirpes, Daniel J., Donnie M. Powell, Raymond J. Folwell, George Tamaki and John L. Baritelle. 1982. Costs of suppressing leafroll on potatoes by systemic soil insecticides. Research Circular XC 0642, Agricultural Research Center, Washington State University, Pullman, Washington.

Knutson, Ronald D., Charles R. Hall, Edward G. Smith, Samuel D. Cotner and John W. Miller. 1993. Economic impacts of reduced pesticide use on fruits and vegetables. American Farm Bureau Research Foundation.

NASS. 1995. Agricultural Chemical Usage 1994 Field Crops Summary. USDA. Natl. Agric. Stat. Serv. Ag Ch 1 (95). 106 p.

NPC. 1994. Potato Statistical Yearbook. Natl. Pot. Council. 69 p.

Patterson, Paul E., Robert L. Smathers, C. Wilson Gray and Neil R. Rimbey. 1995. 1995 Crop Inputs Cost Summary for Idaho. AE Extension Series No. 95-7, Department of Agricultural Economics, University of Idaho, Moscow, Idaho.

Reed, G. 1994. Pest management of potato insects and associated diseases. 1993 Research Progress Reports. Oregon State University. 17 p.

Reed, G. 1995. Pest management of potato insects and associated diseases. 1994 Research Progress Reports. Oregon State University. 33 p.

Schreiber, A.A. 1995. Impact of aldicarb loss to the Washington potato industry. Report to Washington State Potato Commission. 13 p.

United States Department of Agriculture. 1965. Losses in Agriculture. Agricultural Research Service Agricultural Handbook No. 291. Washington, D.C.

United States Department of Agriculture. 1994. Agricultural Statistics 1994. National Agricultural Statistics Service, Washington, DC.

United States Department of Agriculture. 1994. Agricultural Prices 1994 Summary. National Agricultural Statistics Service, Washington, DC.

WSDA. 1995. Pesticide Investigation Summary. Unpublished data. WA St. Dept. Agric. 10 p.

Wyman, J.A. 1990. Phorate use on potato. In The Biologic and Economic Assessment of Phorate and Terbufos. USDA Technical Bulletin Number 1785. Washington, D.C.

Table 1. Estimated market share for CPB- and PLRV-resistant Russet Burbank potatoes

STATE	PROPORTION OF ACRES GROWN TO RUSSET[1] BURBANK	TOTAL POTATO ACRES	RUSSET BURBANK POTATO ACRES	ESTIMATED[2] MARKET SHARE	MARKET SHARE BY ACRE
Idaho	0.883	410,000	362,000	0.35-0.5	127,000 - 180,000
Maine	0.176	780,000	14,000	0.35-0.5	5,000 - 7,000
Michigan	0.168	440,000	7,000	0.05-0.1	400 - 700
Minnesota	0.31	740,000	23,000	0.05-0.1	1,000 - 2,000
North Dakota	0.154	133,000	20,000	0.05-0.1	1,000 - 2,000
Oregon	0.637	535,000	34,000	0.35-0.5	12,000 - 17,000
Wisconsin	0.323	73,000	24,000	0.05-0.1	1,000 - 2,000
Washington	0.696	152,000	106,000	0.35-0.5	37,000 - 53,000
Total			590,000		184,000 - 264,000

[1] Based on Potato Statistics Yearbook (NPC, 1994)
[2] Assumed market share as derived by authors, based on market share 2-3 years post-introduction

Table 2. Acre-treatments avoided due to use of CPB- and PLRV-Resistant Russet Burbank potatoes by state and active ingredient

INSECTICIDE	IDAHO	MAINE	MICHIGAN	MINNESOTA	NORTH DAKOTA	OREGON	WISCONSIN	WASHINGTON	TOTAL
	Thousands of acre-treatments								
Azinphos-methyl		4.0-6.0	0.4-0.9		0.2-0.4	12.0-18.0	0.1-0.2	3.0-4.0	20.0-30.0
Bt			0.1						0.1
Carbaryl			0.1			3.0-4.0			3.0-4.0
Carbofuran	14.0-20.0		0.1	1.0-3.0	1.0-2.0	3.0-4.0	0.2-0.3	8.0-11.0	28.0-40.0
Cryolite		0.5-0.7	0.1-0.2						0.6-0.9
Dimethoate			0.1				0.3-0.6		0.3-0.6
Disulfoton						5.0-7.0		11.0-16.0	16.0-2.03
Endosulfan	7.0-10.0	3.0-4.0	0.2-0.5	0.2-0.4	0.5-1.0		0.8-2.0		12.0-18.0
Esfenvalerate	7.0-10.0	1.0-2.0	0.2-0.4	0.1-0.3	0.3-0.6		0.8-2.0		9.0-15.0
Methamidophos	8.0-11.0		0.4-0.7		0.2-0.3	14.0-21.0	0.9-2.0	113.0-146.0	136.0-190.0
Methyl parathion			0.2-0.4						0.2-0.4
Permethrin		0.3-0.7	0.1	0.8-2.0		1.0	0.7-1.0	15.0-22.0	18.0-27.0
Piperonyl butoxide		0.3-0.5	0.8-2						1.0-3.0
Phorate	60.0-84.0		0.1		0.2-0.4	4.0-6.0	0.1-0.2	20.0-29.0	85.0.0-120.0
Phosmet		1.0-2.0	0.7-1.0						2.0-3.0
Propargite						3.0-4.0		11.0-15.0	14.0-19.0
Oxamyl						3.0-4.0		14.0-20.0	17.0-24.0
Total	96.0-135.0	10.0-16.0	4.0-7.0	2.0-6.0	2.0-5.0	48.0-69.0	4.0-8.0	195.0-263.0	362.0-509.0

Table 3. Insecticide avoided due to use of CPB -and PLRV-resistant Russet Burbank potatoes (listed by state and active ingredient)

INSECTICIDE	IDAHO	MAINE	MICHIGAN	MINNESOTA	NORTH DAKOTA	OREGON	WISCONSIN	WASHINGTON	TOTAL
	Thousands of pounds								
Azinphos-methyl		2.0-3.0	0.4-0.9		0.2-0.4	0.5-0.7	0.1-0.2	1.0-2.0	4.0-7.0
Bt			0.1						0.1
Carbaryl			0.1			4.0-5.0			4.0-5.0
Carbofuran	28.0- 40.0		0.1	1.0-3.0	1.0-2.0	3.0-6.0	0.2-0.3	11.0-16.0	44.0-67.0
Cryolite		5.0-7.0	0.1						5.0-7.0
Dimethoate			0.1				0.3-0.6		0.4-0.7
Disulfoton						13.0-19.0		30.0-50.0	43.0-69.0
Endosulfan	5.0-7.0	2.0-3.0	0.2-0.5	0.2-0.4	0.5-1.0		0.8-2.0		9.0-14.0
Esfenvalerate	0.3- 1.0	0.1	0.2-0.4	0.1-0.3	0.3-0.6		0.8-2.0		2.0-4.0
Methamidophos	6.0-9.0		0.4-0.7		0.2-0.3	14.0-20.0	0.9-2.0	73.0-100.0	95.0-132.0
Methyl-parathion			0.2-0.4						0.2-0.4
Permethrin		0.1	0.1	0.9-2.0		0.1	0.7-1.0	2.0	4.0-5.0
Piperonyl-butoxide		0.1-0.2	0.8-2						0.9-2.0
Phorate	160.0-230.0		0		0.2-0.4	11.0-16.0	0.1-0.2	60.0-80.0	230.0-327.0
Phosmet		0.9-1.0	0.7-1						2.0
Propargite						3.0-4.0		14.0-21.0	17.0-25.0
Oxamyl						5.0-7.0		20.0-30.0	25.0-37.0
Total	199.0-287.0	10.0-14.0	4.0-7.0	2.0-6.0	2.0-5.0	54.0-78.0	4.0-8.0	211.0-300.0	486.0-704.0

Table 4. LD50s and LC50s for 10 common potato insecticides and potential use reduction due to reliance on CPB- and PLRV-resistant potatoes.

Insecticide	Reduction in Acre Treatments (000)	Reduction in Pounds (000)	% reduction[1] on potatoes	LD50 mammalian (mg/kg)	LC50 bird (mg/kg)	LC50 fish (mg/kg)
methamidophos	136-190	95-132	27-38	118[2]		51[3]
phorate	85-120	230-327	28-39	20-30[2]		13[3]
carbofuran	28- 40	44-67	9-12	11,200[2]	438[4]	0.28[3]
azinphos-methyl	20- 30	4-7	5-8	220[2]	639[4]	4.3[3]
permethrin	18- 27	4-5	8-11	>2,000[2]	15,500[4]	0.009[3]
oxamyl	17- 24	25-37	18-25	2960[2]	4.18[4]	4.2[3]
disulfoton	16- 23	43-69	23-34	10(rat)[2]	544[4]	1850[3]
propargite	14- 19	17-25	22-30	2940[2]		0.1[3]
endosulfan	12- 18	9-14	4-6	359[2]	805[4]	1.4[3]
esfenvalerate	9- 15	2-4	4-6	>2000[2]		

[1] Percent reduction of insecticide usage on potatoes is based on estimated reduction in acre treatments as portion of total amount of active ingredients on all U.S. fall potatoes.
[2] Rabbit (dermal)
[3] Rainbow trout
[4] Quail

Table 5. Insecticide costs avoided by use of CPB- and PLRV-resistant potatoes

Insecticide	ID	ME	MI	MN	ND	OR	WA	WI	Total
	Thousands of dollars								
Azinphos methyl[1]	-	66.8	14.1	-	6.5	85.0	42.6	3.3	218.2
Bt	-	-	3.2	-	-	-	-	-	3.2
Carbaryl	-	-	1.0	-	-	41.9	-	-	42.9
Carbofuran	520.2	-	1.8	35.6	26.7	64.1	220.3	4.5	873.1
Cryolite	-	23.6	1.1	-	-	-	-	-	24.7
Dimethoate	-	-	1.5	-	-	-	-	6.6	8.1
Disulfoton	-	-	-	-	-	170.4	418.5	-	588.9
Endosulfan	120.3	49.9	6.3	5.4	13.5	-	-	25.2	220.5
Esfenvalerate	179.4	28.6	6.5	43.1	97.0	-	-	30.2	384.8
Methamidophos	201.3	-	14.0	-	6.4	436.0	2,420.8	37.0	3,115.4
Methyl parathion	-	-	3.3	-	-	-	-	-	3.3
Oxamyl	-	-	-	-	-	199.9	-	845.0	1,044.9
Permethrin	-	8.7	6.7	97.2	-	11.2	216.9	57.1	397.9
Phorate	1,961.3	-	1.4	-	4.2	148.5	763.0	2.1	2,880.6
Phosmet	-	15.8	11.6	-	-	-	-	-	27.4
Piperonyl butoxide	-	4.5	30.7	-	-	-	-	-	35.2
Propargite	-	-	-	-	-	86.6	410.3	-	496.9
Total	2,982.5	197.9	103.2	181.3	154.3	1,243.6	4,492.4	1,011.0	10,366.0

[1]Includes application cost estimated at $5.00 per acre

Table 6. Break-even price for CPB-and PLRV-resistant seed potatoes (in addition to the market price for conventional seed)

	ID	ME	MI	MN	ND	OR	WA	WI	Total
Insecticide costs avoided ($1,000)	$2,982	$198	$103	$181	$154	$1,244	$5,337	$166	$10,365
Assumed acres of Newleaf (1,000)	153.9	5.8	0.6	1.7	1.5	14.5	45.0	1.8	224.7
Insecticide savings per acre ($/a)	$19	$34	$186	$105	$100	$86	$119	$94	$46
Assumed seeding rate (cwt/a)	20	13	20	19	20	23	23	18	20.6
Break-even price of seed ($/cwt)	$0.97	$2.61	$9.31	$5.55	$5.02	$3.73	$5.16	$5.21	$2.24

*Assumes no yield or quality benefits

Table 7. Economic benefits of enhanced yield and quality

Category	Amount	Amount	Amount
Assumed yield and quality impacts	2%	4%	6%
Yield increase	$8,157,538	$16,315,076	$24,472,614
Quality increase	$8,157,538	$16,315,076	$24,472,614
Market adjustment	$(9,789,046)	$(19,578,092)	$(29,367,137)
Total revenue change (8 states)	$6,526,031	$13,052,061	$19,578,092
Benefits to all US growers	$8,586,882	$17,173,765	$25,760,647
Benefits to US consumers	$44,495,663	$88,991,325	$133,486,988

Appendix 2. Certification of NewLeaf Plus Russet Burbank Potatoes in the U.S. and Canadian Seed Certification Programs

Summary

NewLeaf Plus Russet Burbank potato lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 were evaluated in the U.S. and Canadian Seed Certification programs in 1995 and 1996. The certification data demonstrates that the transgenic potato lines are equivalent to non-transgenic Russet Burbank potato with regard to agronomic performance and susceptibility to potato pathogens lines, except for the transgenic resistant to the Potato leafroll virus and the Colorado potato beetle.

Background

Description of the Seed Potato Certification Program

Production of seed potatoes, including transgenic potatoes, is under the oversight of seed potato certification agencies in the U.S. and Canada. Certification of seed potatoes is a regulatory activity conducted by government agencies or universities and is focused on ensuring that seed potatoes are suitable (free from disease, varietally pure) for replanting to produce seed and commercial crops. This approach to seed production is necessary because commercial potatoes are produced through vegetative propagation. Because the cleansing effect with regard to disease that occurs with true seed crops is not generally available in potato, it is imperative that disease is not introduced or is maintained at very low levels to avoid affecting the productivity of a potato crop. This requirement is the basis for the development of seed potato certification programs.

The certification process occurs as follows:

- Growers apply to agencies for certification evaluation of individual seed lots of potatoes

    1. Growers provide history on seed lot, including source, and previous production location.
    2. The grower source of seed potatoes provides a seed health certificate from a certification agency documenting disease incidence, varietal purity, generation, certification number (ID) from the previous season, and plant characteristics if unique.

- Certification agency monitors location (maps, description of location) of seed lots (field, storage), acres and quantity produced, lot segregation, disease incidence, varietal purity through:

    1. Field inspections to evaluate crop for disease type and incidence, varietal uniformity or purity, cultural conditions, making note of off-type plants, unsatisfactory cultural or crop conditions,
    2. Harvest/storage inspections for determining tuber condition, lot identification and segregation,
    3. Post-harvest tests (field, greenhouse or laboratory assay) to document that disease incidence did not increase during the growing season to levels unacceptable for reproduction of another generation; serological assay is used routinely to confirm disease diagnosis based on visible symptoms. At this point the potatoes are designated as CERTIFIED SEED if all requirements have been met. **For transgenic potatoes this designation also indicates that the potatoes are "true-to-type" for the specific variety transformed.**
    4. A shipping point inspection is conducted prior to seed delivery to ensure that the customer is receiving the correct seed lot and that the quality meets the requirements of certified seed for that jurisdiction.

Unsuitable seed potatoes (disease, varietal mixture, chemical/physical damage) are disqualified as certified seed and those potatoes are utilized for processing, consumption or destroyed (non-registered product).

Seed potato certification has been in place for more than 80 years in the U.S. and Canada. The processes for initiating seed potatoes and inspecting/testing the crop at various stages of production have advanced greatly in

recent years, utilizing tissue culture, sensitive pathogen testing techniques, sophisticated facilities. Many of seed potato inspectors have been examining potatoes in the field for 20-40 years and attest to the constancy of the crop and the pathogens which impact the certification system.

Another consideration in the containment of transgenic potato production is the multiplication rate with potatoes. For seed potatoes this is generally in the range of 10-12X increase each year. This is meager compared to true seed crops (50-150X) and necessitates a period of 4 or more years before quantities adequate for commercial production are available. Therefore, seed potato multiplication is a relatively slow process, with 4 years required to expand production from one acre to seed quantities adequate for 1% of the potato acres in North America (Table 1).

Table 1. Seed Potato Production - 4 years of increase (starting with plants or minitubers)

Years of Field Production	1	(X)	2	(X)	3	(X)	4
Generation	FG1		FG2		FG3		FG4
cwt[1]	200	12	2,400	12	28,800	12	345,600
Acres(from cwt)	10		120		1,440		17,280

[1]cwt; centiweight or 100 pounds

History of Multiple Viral Infections Potato

The history, multiplicity and stability of viral infections in potatoes, as documented by seed potato certification programs, suggests that the potential for virus modification in transgenic plants is not large. It is not uncommon to detect multiple viruses infecting the same potato plant. Several of the identified viruses infecting potatoes have more than one mod of transmission. A listing of the most common potato viruses in North America is included (Table 2).

Table 2. Common potato viruses in North America: symptoms and transmission..

VIRUS	YEAR DESCRIBED	SYMPTOMS	TRANSMISSION
Potato Leaf Roll Virus (PLRV)	1907	Foliar, tuber	Aphids only
Potato Virus A (PVA)	1932	Foliar	Aphids, mechanical
Potato Virus S (PVS)	1951	None	Mechanical, aphids
Potato Virus X (PVX)	1931	None - foliar	Mechanical
Potato Virus Y (PVY)	1932	Foliar, tuber (strain)	Aphids, mechanical
Potato virus M (PVM)	1930	Foliar - transient	Mechanical, aphids
Tobacco Rattle Virus (TRV)	1947	Tuber	Nematode, mechanical
Alfalfa Mosaic Virus (AlMV)	1957	Foliar	Aphids, mechanical

During the 40 to 80 years that the viruses in the table have been recognized as pathogens of potatoes, frequently as multiple infections, there has been little change in the viruses or attributes by which they are identified. With the opportunities that multiple infections provide for recombination of viral genomes it seems that there could have been many successful variants produced. Experience has demonstrated otherwise. The viruses have been noteworthy by their stability and consistency in epidemiology, including two multipartite viruses (AlMV, TRV) which are limited in impact by vector relationships and/or ability to move and replicate systemically.

The characteristics of potatoes viruses and disease symptoms they produce have remained stable since seed certification began. This can be attributed to the following.

1. **Host range** Individual potato varieties have been consistent in susceptibility to specific viruses
2. **Symptom expression** Experienced inspectors have observed consistent symptoms with individual variety/virus interactions for up to 50 years of examining seed potatoes.
3. **Limited strains** Few potato viruses have multiple strains. When those strains have developed they have been stable.
4. **Vector relationships** Vector-virus relationships have not changed since first recognized, both in terms of specificity and in efficiency of acquisition and transmission.
5. **Impact on potato productivity and quality** The PLRV/net necrosis/variety interaction has been consistent since identified, across both varieties and geographic areas.
6. **Failure to overcome resistance** Virus resistance developed in breeding programs, including resistance to PVX (Atlantic, Targhee); PVY (Norwis); PVA (Shepody); PLRV (Belrus, Abnaki, Penobscot) has been very durable.

NewLeaf Plus Russet Burbank and Seed Certification

In 1995 and 1996, seed potato certification programs in the U.S. and Canada evaluated seed lots of NewLeaf Plus Russet Burbank produced by seed growers. The seed potatoes were entered into the programs and evaluated as any other variety grown for seed. The process included field inspections, harvest/storage inspections, post-harvest seed trials, as well as additional testing by ELISA of plants with viral disease symptoms. These ELISA test assays were conducted by Idaho Crop Improvement Association on samples from the post-harvest test site in California (1995, Dr. Richard Clarke, Area Manager; 1996, Dr. Jonathon Whitworth, Area Manager).

If during inspections or the post-harvest test, a certification agency determines that plants and/or tubers in a seed lot are not typical or the response to virus and bacterial disease is atypical for the variety transformed, then the lot is disqualified from certification and required to be destroyed. Designation of seed lots as certified seed of NewLeaf Plus by the certification programs signifies that each program found the plants, tubers and disease response to be typical of Russet Burbank with the added advantage that the NewLeaf Plus plants are resistant to the Colorado potato beetle and to the Potato leafroll virus . At NatureMark's propagation and production facility/farm in Maine where screening of new transformed lines occurs, all potato production in the field is subjected to the seed certification process. When off-type transformed plants in a line (lot) are detected or a question arises about disease susceptibility, the line is destroyed at the direction of the certification agency. Each year since 1993, the Maine Seed Potato Certification program (lines were inspected by Reginald Brown, Supervisor and field inspectors, inspected under certification numbers 639 and/or 645) has evaluated and certified several thousand lines (Table 3) of transformed potatoes derived from up to eight different parental varieties at NatureMark's Maine site. During those inspections only a small percentage (< 1 %) of lines were designated as unsatisfactory on the basis of plant type. These plants were destroyed to comply with the certification process.

Table 3. Number of NewLeaf Plus lines evaluated for certified seed.

Year	Number of Lines
1993	1749
1994	2124
1995	2714
1996	2348

During 1995 and 1996, there were 183 seed lots (45 lines) of NewLeaf Plus Russet Burbank grown by 20 seed growers and evaluated by 9 different certification systems in the U.S. and Canada. Data obtained demonstrated that:

1. NewLeaf Plus Russet Burbank is equivalent to unimproved Russet Burbank in terms of plant habit, tuber type, phenotypic stability as shown by acceptance of these lines into certification programs;

2. NewLeaf Plus Russet Burbank resistance to infection by PLRV is substantial, illustrated by total freedom from infection in NLPlus seed lots, as determined by observations in field inspections and post-harvest tests;

3. The susceptibility and reaction of NewLeaf Plus Russet Burbank to other potato viruses (symptoms and incidence of infection) are comparable to the parent line of Russet Burbank, as demonstrated in-season visual inspection, visual inspection of post-season winter planting, and by the ELISA testing conducted by Idaho Crop Improvement Association.

Table 4 summarizes post-harvest test data for sites where comparisons can be made between NewLeaf Plus Russet Burbank (resistant to Colorado potato beetle and PLRV) and, Russet Burbank lines without the PLRV resistance phenotype. NewLeaf Plus Russet Burbank potato lines are completely resistant to infection by PLRV. The susceptibility to other potato viruses, particularly PVY and PVA, has been comparable to the Russet Burbank and Russet Burbank NewLeaf, both in incidence as well as symptom expression. The incidence of infection and the lack of altered symptoms in NewLeaf Plus Russet Burbank with viruses which produce mosaic disease symptoms, when compared to other Russet Burbank lines, indicates that expression of the PLRV replicase transgene does not result in a negative synergistic effect on NewLeaf Plus Russet Burbank potatoes.

Table 4. Summary of Post-Harvest Test results for NLPlus Russet Burbank Lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 and Russet Burbank/ NL Russet Burbank from same sites[1]

State/ Province	Crop Year	# Sites	NLPlus RB[2]			RB/NL RB[3]		
			# samples	# samples with PLRV	# samples with Mosaic[4]	# samples	# samples with PLRV	# samples with Mosaic
ND	1996	1	7	0	0	2	1	0
ME	1995	1	47	0	0	3	0	0
	1996	1	13	0	0	6	0	2
WI	1995	1	2	0	0	6	5	4
	1996	1	4	0	0	1	1	0
ID	1995	3	3	0	0	13	5	4
	1996	3	9	0	6	24	7	7
MT	1995	2	4	0	0	4	2	0
	1996	6	7	0	1	5	1	0
BC	1996	2	16	0	9	2	0	1
MB	1995	1	2	0	2	25	6	20
	1996	1	5	0	4	9	8	8
AB	1996	3	22	0	0	3	0	0
TOTAL			**141**	**0**	**22**	**101**	**36**	**46**

[1] Seed growers collect random samples of tubers (200-400) from each lot as it is harvested and submit the samples to the certification agencies for planting in a post-harvest field trial in either Florida or California. In plots managed by Idaho Crop Improvement Association at Oceanside, CA each plant with symptoms is flagged when inspected and a sample from that plant is tested by ELISA for the presence of the potato viruses PVX, PVY, PVA and PLRV. Other agencies will test plants with questionable symptoms.

[2]NL Plus RB, Russet Burbank NewLeaf Plus (CPB and PLRV resistant)

[3]RB/NL RB, Russet Burbank and/or NewLeaf® Russet Burbank(CPB resistant)

[4]Mosaic, mosaic disease incited by either PVY or PVA

Appendix 3. Blast Search Results of Nucleotide Sequences with Homology to the 5-prime and 3-prime 150 nucleotides of Potato leafroll virus

PLRV (Polish isolate) 3-prime 150 nucleotides versus GenBank

```
!!SEQUENCE_LIST 1.0

BLASTN 1.4.7 [1-Feb-95] [Build 12:02:41 Nov 12 1996]

Reference:  Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers,
and David J. Lipman (1990).  Basic local alignment search tool.  J. Mol. Biol.
215:403-10.

Notice:  this program and its default parameter settings are optimized to find
nearly identical sequences rapidly.  To identify weak similarities encoded in
nucleic acid, use BLASTX, TBLASTN or TBLASTX.

Query=  /usr2/people/hkredi/PLRVlast150.seq
        (151 letters)

Database:  genbank
           353,245 sequences; 547,840,210 total letters.

                                                                     Smallest
                                                                       Sum
                                                              High  Probability
Sequences producing High-scoring Segment Pairs:              Score  P(N)      N
..
GB_VI:PLVSQRN ! X74789 Potato leaf roll virus RNA sequenc...   755  5.6e-55   1
GB_VI:PLRVXX ! Y07496 Potato leaf roll virus genomic RNA....   728  9.9e-53   1
GB_VI:PLVRC ! D13954 Potato leafroll luteovirus (Canadian...   728  9.9e-53   1
GB_VI:PLLGRNA ! X14600 Potato leafroll luteovirus genomic...   728  9.9e-53   1
GB_VI:PLVGR ! D00530 Potato leafroll luteovirus genomic R...   728  9.9e-53   1
GB_VI:PLV3E ! D13746 Potato leaf roll virus, 3'end of vir...   687  1.9e-51   1
GB_VI:PLVRA ! D13953 Potato leafroll luteovirus (Australi...   710  3.1e-51   1
\\End of List

>GB_VI:PLVSQRN X74789 Potato leaf roll virus RNA sequence. 7/95
  Length = 5882

  Plus Strand HSPs:

 Score = 755 (208.6 bits), Expect = 5.6e-55, P = 5.6e-55
 Identities = 151/151 (100%), Positives = 151/151 (100%), Strand = Plus / Plus
```

[CBI DELETED]

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>GB_VI:PLRVXX Y07496 Potato leaf roll virus genomic RNA. 9/93
  Length = 5882

  Plus Strand HSPs:

 Score = 728 (201.2 bits), Expect = 9.9e-53, P = 9.9e-53
 Identities = 148/151 (98%), Positives = 148/151 (98%), Strand = Plus / Plus
```

[CBI DELETED]

[CBI DELETED

]

```
>GB_VI:PLVRC D13954 Potato leafroll luteovirus (Canadian isolate) genomic RNA,
  complete sequence. 12/92
  Length = 5883

  Plus Strand HSPs:

 Score = 728 (201.2 bits), Expect = 9.9e-53, P = 9.9e-53
 Identities = 148/151 (98%), Positives = 148/151 (98%), Strand = Plus / Plus
```

[CBI DELETED

]

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>GB_VI:PLLGRNA X14600 Potato leafroll luteovirus genomic RNA. 9/93
  Length = 5987

  Plus Strand HSPs:

 Score = 728 (201.2 bits), Expect = 9.9e-53, P = 9.9e-53
 Identities = 148/151 (98%), Positives = 148/151 (98%), Strand = Plus / Plus
```

[CBI DELETED

]

```
>GB_VI:PLVGR D00530 Potato leafroll luteovirus genomic RNA. 7/90
  Length = 5987

  Plus Strand HSPs:

 Score = 728 (201.2 bits), Expect = 9.9e-53, P = 9.9e-53
 Identities = 148/151 (98%), Positives = 148/151 (98%), Strand = Plus / Plus
```

[CBI DELETED

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```
>GB_VI:PLV3E D13746 Potato leaf roll virus, 3'end of virus genome. 11/92
  Length = 141

  Plus Strand HSPs:

 Score = 687 (189.8 bits), Expect = 1.9e-51, P = 1.9e-51
 Identities = 139/141 (98%), Positives = 139/141 (98%), Strand = Plus / Plus
```

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```
>GB_VI:PLVRA D13953 Potato leafroll luteovirus (Australian isolate) genomic
  RNA, complete sequence. 12/92
  Length = 5882

  Plus Strand HSPs:

 Score = 710 (196.2 bits), Expect = 3.1e-51, P = 3.1e-51
 Identities = 146/151 (96%), Positives = 146/151 (96%), Strand = Plus / Plus
```

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```
Parameters:
  V=250
  B=100

  -ctxfactor=2.00
  E=10
```

Query Strand	MatID	Matrix name	As Used Lambda	As Used K	As Used H	Computed Lambda	Computed K	Computed H
+1	0	+5,-4	0.192	0.173	0.357	same	same	same
-1	0	+5,-4	0.192	0.173	0.357	same	same	same

Query Strand	MatID	Length	Eff.Length	E	S	W	T	X	E2	S2
+1	0	151	151	10.	112	11	N/A	73	0.023	71
-1	0	151	151	10.	112	11	N/A	73	0.023	71

Statistics:

Query Strand	MatID	Expected High Score	Observed High Score	HSPs Reportable	HSPs Reported
+1	0	119 (32.9 bits)	755 (208.6 bits)	7	7
-1	0	119 (32.9 bits)	112 (30.9 bits)	0	0

Query Strand	MatID	Neighborhd Words	Word Hits	Excluded Hits	Failed Extensions	Successful Extensions	Overlaps Excluded
+1	0	144	10475	937	8923	626	0
-1	0	144	9525	682	8277	581	0

```
Database:  genbank
  Release date:  unknown
  Posted date:  2:08 AM CDT Jun 14, 1997
# of letters in database:  547,840,210
# of sequences in database:  353,245
# of database sequences satisfying E:  7
No. of states in DFA:  169 (338 KB)
Total size of DFA:  345 KB (384 KB)
Time to generate neighborhood:  0.01u 0.01s 0.03t  Real: 00:00:00
Time to search database:  13.68u 4.55s 18.23t  Real: 00:00:42
Total cpu time:  14.15u 4.78s 18.93t  Real: 00:00:42
```

PLRV (Polish isolate) 5-prime 150 nucleotides versus GenBank

```
!!SEQUENCE_LIST 1.0

BLASTN 1.4.7 [1-Feb-95] [Build 12:02:41 Nov 12 1996]

Reference:  Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers,
and David J. Lipman (1990).  Basic local alignment search tool.  J. Mol. Biol.
215:403-10.

Notice:  this program and its default parameter settings are optimized to find
nearly identical sequences rapidly.  To identify weak similarities encoded in
nucleic acid, use BLASTX, TBLASTN or TBLASTX.

Query=  /usr2/people/hkredi/PLRV-5prime-150nt.seq
        (150 letters)

Database:  genbank
           353,245 sequences; 547,840,210 total letters.

                                                                    Smallest
                                                                      Sum
                                                             High  Probability
Sequences producing High-scoring Segment Pairs:              Score  P(N)      N
..
GB_VI:PLVSQRN ! X74789 Potato leaf roll virus RNA sequenc...   750  1.5e-54   1
GB_VI:PLRVXX ! Y07496 Potato leaf roll virus genomic RNA....   732  4.6e-53   1
GB_VI:PLVRA ! D13953 Potato leafroll luteovirus (Australi...   714  1.4e-51   1
GB_VI:PLVRC ! D13954 Potato leafroll luteovirus (Canadian...   714  1.4e-51   1
GB_VI:PLLGRNA ! X14600 Potato leafroll luteovirus genomic...   671  5.5e-48   1
GB_VI:PLVGR ! D00530 Potato leafroll luteovirus genomic R...   671  5.5e-48   1
GB_IN:CELC44E4 ! AF003140 Caenorhabditis elegans cosmid C...   118  0.993     1
GB_IN:CEM199 ! Z81104 Caenorhabditis elegans cosmid M199....   118  0.993     1
\\End of List

>GB_VI:PLVSQRN X74789 Potato leaf roll virus RNA sequence. 7/95
  Length = 5882

  Plus Strand HSPs:

 Score = 750 (207.2 bits), Expect = 1.5e-54, P = 1.5e-54
 Identities = 150/150 (100%), Positives = 150/150 (100%), Strand = Plus / Plus
```

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```
>GB_VI:PLRVXX Y07496 Potato leaf roll virus genomic RNA. 9/93
  Length = 5882

  Plus Strand HSPs:

 Score = 732 (202.3 bits), Expect = 4.6e-53, P = 4.6e-53
 Identities = 148/150 (98%), Positives = 148/150 (98%), Strand = Plus / Plus
```

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```
>GB_VI:PLVRA D13953 Potato leafroll luteovirus (Australian isolate) genomic
  RNA, complete sequence. 12/92
  Length = 5882

  Plus Strand HSPs:

 Score = 714 (197.3 bits), Expect = 1.4e-51, P = 1.4e-51
 Identities = 146/150 (97%), Positives = 146/150 (97%), Strand = Plus / Plus
```

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```
>GB_VI:PLVRC D13954 Potato leafroll luteovirus (Canadian isolate) genomic RNA,
  complete sequence. 12/92
  Length = 5883

  Plus Strand HSPs:

 Score = 714 (197.3 bits), Expect = 1.4e-51, P = 1.4e-51
 Identities = 146/150 (97%), Positives = 146/150 (97%), Strand = Plus / Plus
```

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```
>GB_VI:PLLGRNA X14600 Potato leafroll luteovirus genomic RNA. 9/93
  Length = 5987

  Plus Strand HSPs:

 Score = 671 (185.4 bits), Expect = 5.5e-48, P = 5.5e-48
 Identities = 135/136 (99%), Positives = 135/136 (99%), Strand = Plus / Plus
```

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```
>GB_VI:PLVGR D00530 Potato leafroll luteovirus genomic RNA. 7/90
  Length = 5987

  Plus Strand HSPs:

 Score = 671 (185.4 bits), Expect = 5.5e-48, P = 5.5e-48
 Identities = 135/136 (99%), Positives = 135/136 (99%), Strand = Plus / Plus
```

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```
>GB_IN:CELC44E4 AF003140 Caenorhabditis elegans cosmid C44E4. 5/97
  Length = 35,169

  Minus Strand HSPs:

 Score = 118 (32.6 bits), Expect = 5.0, P = 0.99
 Identities = 38/56 (67%), Positives = 38/56 (67%), Strand = Minus / Plus
```

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```
>GB_IN:CEM199 Z81104 Caenorhabditis elegans cosmid M199. 3/97
  Length = 39,183

  Minus Strand HSPs:

 Score = 118 (32.6 bits), Expect = 5.0, P = 0.99
 Identities = 42/65 (64%), Positives = 42/65 (64%), Strand = Minus / Plus
```

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```
Parameters:
  V=250
  B=100

  -ctxfactor=2.00
  E=10

  Query                               ----- As Used -----      ----- Computed ----
  Strand MatID Matrix name           Lambda    K      H       Lambda    K      H
   +1      0   +5,-4                 0.192   0.173  0.357     same     same   same
   -1      0   +5,-4                 0.192   0.173  0.357     same     same   same

  Query
  Strand MatID  Length  Eff.Length   E     S  W   T  X     E2   S2
   +1      0     150       150      10.  112 11 N/A 73   0.023 71
   -1      0     150       150      10.  112 11 N/A 73   0.023 71

Statistics:
```

```
Query          Expected           Observed          HSPs       HSPs
Strand MatID  High Score         High Score      Reportable  Reported
 +1     0    119 (32.9 bits)  750 (207.2 bits)       6          6
 -1     0    119 (32.9 bits)  118 (32.6 bits)        2          2

Query        Neighborhd  Word       Excluded     Failed   Successful  Overlaps
Strand MatID   Words     Hits         Hits    Extensions Extensions  Excluded
 +1     0       143      21613        1875       17640      2115         1
 -1     0       143      21350        1578       18154      1630         0

Database:  genbank
  Release date:  unknown
  Posted date:  2:08 AM CDT Jun 14, 1997
# of letters in database:  547,840,210
# of sequences in database:  353,245
# of database sequences satisfying E:  8
No. of states in DFA:  157 (314 KB)
Total size of DFA:  321 KB (384 KB)
Time to generate neighborhood:  0.00u 0.01s 0.01t  Real: 00:00:00
Time to search database:  15.16u 3.06s 18.23t  Real: 00:00:34
Total cpu time:  15.75u 3.41s 19.16t  Real: 00:00:34
```

Appendix 4. Presence of Polyadenylation signal sequences in the reverse complement strands of the transgene expression cassettes in NewLeaf Plus

PV-STMT22

In PV-STMT22, the *PLRVrep*, *cry3A*, and CP4 genes are all synthesized in the same direction from the same DNA strand (Fig. 1). The reverse complement strand (Fig. 1) (downstream from the replicase expression cassette) was probed for the polyadenylation signal sequence termination sequence, AATAAT, which would terminate mRNA expression by the addition of poly-A.

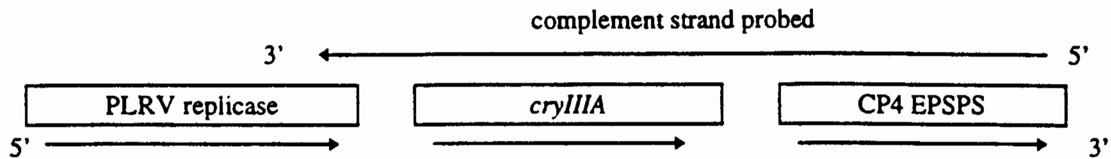

Figure 1. Direction of transcription for transgenes genes in PV-STMT22 and area of the complement strand probed for polyadenylation signal sequence.

Results: The polyA termination signal was found at four locations within the probed sequence (see data below). Therefore, a plant promoter present on the complement DNA strand downstream of the CP4 EPSPS expression cassette could not drive the synthesis of an RNA strand which could possibly reach the PLRV subgenomic promoter..

```
PMON18844E9-R_BORDER.RC  ck: 9876  len: 7,423 !   REVERSE-COMPLEMENT of:
PMON18844E9-R_BORDER.seQ  check: 7432  from: 1  t

                AATAAT
      4,920: GCTGG AATAAT GCCAC
      6,176: GAAAA AATAAT ATATA
      6,354: TAAAG AATAAT ACACA
      6,952: AAACA AATAAT ATTAA
```

PV-STMT21

In PV-STMT21, *cry3A* mRNA is synthesized on the opposite strand compared to the PLRVrep mRNA (Fig. 2). The expression of the *cry3A* mRNA is driven by the arabidopsis SSU promoter. The cry3A gene is terminated by a NOS terminator. Additionally, the the reverse complement strand (see Fig. 2) was probed for the polyadenylation signal sequence termination sequence, AATAAT, which would also terminate mRNA expression by the addition of poly-A.

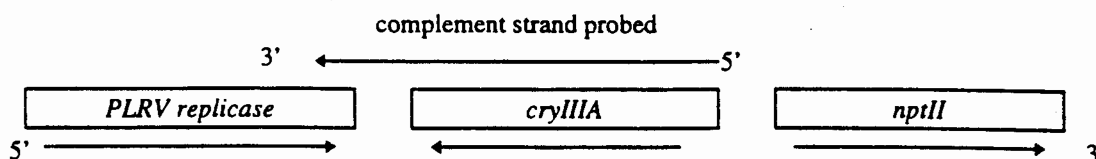

Figure 2. Direction of transcription for transgenes genes in PV-STMT21 and area of the complement strand probed for polyadenylation signal sequence.

Results: The poly-A signal sequence signal was found in the reverse complement of the E9 terminator. Therefore, this message contains two sequences (nos and the polyadenylation siginal) which will terminate the message. The minus-strand RNA containing the intergenic region and subgenomic binding site for PLRV replicase will not be produced.

```
! FINDPATTERNS on @/usr2/people/hkredi/.seqlab-
mendel/findpatterns_ss1_55.list allowing 0 mismatches

pat_0                AATAAT
           392: AAACA AATAAT ATTAA
```

Appendix 5. GenBank Comparison of Transgenic PLRV Replicase mRNA and Known Potato Viruses

Potato Virus A

Potato Virus A

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Potato virus A complete genome check: 5646 from: 1 to: 9585

```
LOCUS       PVCGA        9585 bp    RNA             VRL       13-FEB-1995
DEFINITION  Potato virus A complete genome.
ACCESSION   Z21670
NID         g671613
KEYWORDS    CI (cytoplasmic inclusion); coat protein; complete genome; HC;
            Nuclear inclusion protein a (NIa); Nuclear inclusion protein b . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:     50      Average Match: 10.000
      Length Weight:      3   Average Mismatch:  0.000

            Quality:  12298             Length:   9617
              Ratio:  3.241               Gaps:     31
 Percent Similarity: 37.709   Percent Identity: 37.709

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =    5
                    . =    1

 Transgenic PLRV replicase mRNA x Potato Virus A June 10, 1997 18:04  ..
```

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```
to: Potato Virus A  check: 5646  from: 1  to: 9585

LOCUS       PVCGA         9585 bp    RNA              VRL        13-FEB-1995
DEFINITION  Potato virus A complete genome.
ACCESSION   Z21670
NID         g671613
KEYWORDS    CI (cytoplasmic inclusion); coat protein; complete genome; HC;
            Nuclear inclusion protein a (NIa); Nuclear inclusion protein b . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch: -9.000

            Quality:     163             Length:      34
              Ratio:   4.939               Gaps:       1
 Percent Similarity:  81.818   Percent Identity:  81.818

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =    5
                    . =    1

 Transgenic PLRV replicase mRNA x Potato Virus A June 10, 1997 18:04 ...
```

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Potato Virus M

Potato Virus M

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Potato virus M complete genome check: 4922 from: 1 to: 8535

```
LOCUS       POPVMCG      8535 bp    RNA           VRL       12-SEP-1993
DEFINITION  Potato virus M complete genome.
ACCESSION   X53062
NID         g61291
KEYWORDS    coat protein; unidentified reading frame.
SOURCE      Potato virus M. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch:  0.000

            Quality:  12204             Length:   8567
              Ratio:  3.216               Gaps:     30
 Percent Similarity: 37.151   Percent Identity: 37.151

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1

 Transgenic PLRV replicase mRNA x Potato Virus M June 10, 1997 18:13 ..
```

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Potato Virus M

BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795 to: Potato Virus M check: 4922 from: 1 to: 8535

```
LOCUS       POPVMCG      8535 bp    RNA            VRL       12-SEP-1993
DEFINITION  Potato virus M complete genome.
ACCESSION   X53062
NID         g61291
KEYWORDS    coat protein; unidentified reading frame.
SOURCE      Potato virus M. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

        Gap Weight:     50      Average Match: 10.000
     Length Weight:      3   Average Mismatch: -9.000

           Quality:    143             Length:     20
             Ratio:  7.150               Gaps:      0
 Percent Similarity: 85.000   Percent Identity: 85.000

       Match display thresholds for the alignment(s):
                   | = IDENTITY
                   : =   5
                   . =   1

 Transgenic PLRV replicase mRNA x Potato Virus M June 10, 1997 18:14  ..
```

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Potato Virus X

Potato Virus X

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Potato Virus X complete genomic RNA check: 4296 from: 1 to: 6432

```
LOCUS       POPOVX       6432 bp    RNA             VRL        21-APR-1992
DEFINITION  Potato Virus X complete genomic RNA.
ACCESSION   X55802
NID         g61285
KEYWORDS    coat protein.
SOURCE      Potato virus X. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

        Gap Weight:      50      Average Match: 10.000
     Length Weight:       3   Average Mismatch:  0.000

           Quality:  12402              Length:   6483
             Ratio:  3.268                Gaps:     35
 Percent Similarity: 38.889   Percent Identity: 38.889

       Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =    5
                    . =    1

 Transgenic PLRV replicase mRNA x Potato Virus X June 10, 1997 18:15  ..
```

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Potato Virus X

BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Potato Virus X complete genomic RNA check: 4296 from: 1 to: 6432

```
LOCUS       POPOVX      6432 bp    RNA        VRL       21-APR-1992
DEFINITION  Potato Virus X complete genomic RNA.
ACCESSION   X55802
NID         g61285
KEYWORDS    coat protein.
SOURCE      Potato virus X. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

        Gap Weight:      50      Average Match: 10.000
     Length Weight:       3   Average Mismatch: -9.000

           Quality:     161             Length:     68
             Ratio:   2.403               Gaps:      1
 Percent Similarity: 64.179   Percent Identity: 64.179

       Match display thresholds for the alignment(s):
                   | = IDENTITY
                   : =   5
                   . =   1

 Transgenic PLRV replicase mRNA x Potato Virus X complete genomic RNA June 11, 1997
15:15  ..
```

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Potato Virus Y

Potato Virus Y

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Potato virus Y complete genome RNA check: 3704 from: 1 to: 9705

```
LOCUS       A08776       9705 bp    RNA             PAT       27-NOV-1993
DEFINITION  Potato virus Y complete genome RNA.
ACCESSION   A08776
NID         g492885
KEYWORDS    .
SOURCE      Potato virus Y. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch:  0.000

            Quality:  12244             Length:   9720
              Ratio:  3.226               Gaps:     40
 Percent Similarity: 39.841   Percent Identity: 39.841

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =    5
                    . =    1

 Transgenic PLRV replicase mRNA x Potato virus Y complete genome RNA June 10, 1997
17:46  ..
```

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Potato Virus Y

BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Potato virus Y complete genome RNA check: 3704 from: 1 to: 9705

```
LOCUS       A08776       9705 bp    RNA             PAT       27-NOV-1993
DEFINITION  Potato virus Y complete genome RNA.
ACCESSION   A08776
NID         g492885
KEYWORDS    .
SOURCE      Potato virus Y. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

        Gap Weight:      50      Average Match: 10.000
     Length Weight:       3   Average Mismatch: -9.000

           Quality:     172             Length:     40
             Ratio:   4.300               Gaps:      0
 Percent Similarity: 70.000   Percent Identity: 70.000

        Match display thresholds for the alignment(s):
                      | = IDENTITY
                      : =   5
                      . =   1

 Transgenic PLRV replicase mRNA x Potato virus Y complete genome RNA June 10, 1997
17:49  ..
```

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Tobacco Rattle Virus

Tobacco rattle virus RNA1

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tobacco rattle virus check: 5733 from: 1 to: 6791

```
LOCUS       MTRRNA1       6791 bp ss-RNA            VRL       04-DEC-1996
DEFINITION  Tobacco rattle virus (TRV), RNA-1, complete cds.
ACCESSION   D00155
NID         g222133
KEYWORDS    RNA-1.
SOURCE      Tobacco rattle virus (strain SYM), cDNA to viral genomic RNA, . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch:  0.000

            Quality:  12213             Length:   6813
              Ratio:  3.218               Gaps:     29
 Percent Similarity: 37.318   Percent Identity: 37.318

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =    5
                    . =    1

 Transgenic PLRV replicase mRNA x Tobacco rattle virus June 10, 1997 18:34  ..
```

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Tobacco rattle virus RNA1

BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tobacco rattle virus check: 5733 from: 1 to: 6791

```
LOCUS       MTRRNA1     6791 bp ss-RNA          VRL       04-DEC-1996
DEFINITION  Tobacco rattle virus (TRV), RNA-1, complete cds.
ACCESSION   D00155
NID         g222133
KEYWORDS    RNA-1.
SOURCE      Tobacco rattle virus (strain SYM), cDNA to viral genomic RNA, . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch: -9.000

            Quality:     169             Length:     34
              Ratio:   4.971               Gaps:      0
 Percent Similarity: 73.529   Percent Identity: 73.529

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =     5
                    . =     1

 Transgenic PLRV replicase mRNA x Tobacco rattle virus June 10, 1997 18:34  ..
```

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Tobacco rattle virus RNA2

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tobacco Rattle Virus RNA2 check: 8903 from: 1 to: 3389

```
LOCUS       TOTRVRN2     3389 bp    RNA             VRL       12-SEP-1993
DEFINITION  Tobacco rattle virus (TRV) strain TCM RNA2 sequence.
ACCESSION   X03955
NID         g62141
KEYWORDS    coat protein.
SOURCE      Pepper ringspot virus. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch:  0.000

            Quality:  10741             Length:   3833
              Ratio:  3.169               Gaps:     29
 Percent Similarity: 37.302   Percent Identity: 37.302

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1

 Transgenic PLRV replicase mRNA x Tobacco rattle virus June 10, 1997 18:34  ..
```

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Tobacco rattle virus RNA2

BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tobacco Rattle Virus RNA2 check: 8903 from: 1 to: 3389

```
LOCUS       TOTRVRN2     3389 bp    RNA             VRL       12-SEP-1993
DEFINITION  Tobacco rattle virus (TRV) strain TCM RNA2 sequence.
ACCESSION   X03955
NID         g62141
KEYWORDS    coat protein.
SOURCE      Pepper ringspot virus. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch: -9.000

            Quality:     169             Length:     34
              Ratio:   4.971               Gaps:      0
 Percent Similarity:  73.529   Percent Identity: 73.529

        Match display thresholds for the alignment(s):
                     | = IDENTITY
                     : =    5
                     . =    1

 Transgenic PLRV replicase mRNA x Tobacco rattle virus June 10, 1997 18:34  ..
```

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Tomato Spotted Wilt Virus

Tobacco rattle virus RNA2

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tobacco Rattle Virus RNA2 check: 8903 from: 1 to: 3389

```
LOCUS       TOTRVRN2     3389 bp    RNA             VRL       12-SEP-1993
DEFINITION  Tobacco rattle virus (TRV) strain TCM RNA2 sequence.
ACCESSION   X03955
NID         g62141
KEYWORDS    coat protein.
SOURCE      Pepper ringspot virus. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch:  0.000

            Quality:  10741             Length:   3833
              Ratio:  3.169               Gaps:     29
 Percent Similarity: 37.302   Percent Identity: 37.302

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1

 Transgenic PLRV replicase mRNA x Tobacco rattle virus June 10, 1997 18:34  ..
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Tomato Spotted Wilt Virus M RNA

BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tomato Spotted Wilt Virus check: 6302 from: 1 to: 4821

```
LOCUS       S48091       4821 bp    RNA             VRL       13-JAN-1993
DEFINITION  NSM, glycoprotein precursor {M segment} [tomato spotted wilt virus
            TSWV, Genomic RNA Complete, 4821 nt].
ACCESSION   S48091
NID         g259518
KEYWORDS    . . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch: -9.000

            Quality:     158             Length:     62
              Ratio:   2.590               Gaps:      1
 Percent Similarity: 65.574   Percent Identity: 65.574

        Match display thresholds for the alignment(s):
                     | = IDENTITY
                     : =    5
                     . =    1

 Transgenic PLRV replicase mRNA x Tomato Spotted Wilt Virus June 11, 1997 11:34  ..
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Tomato Spotted Wilt Virus S RNA

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tomato spotted wilt virus, genomic S RNA check: 5516 from: 1 to: 2837

```
LOCUS       TSWSNS       2837 bp ss-RNA          VRL        20-FEB-1993
DEFINITION  Tomato spotted wilt virus, genomic S RNA.
ACCESSION   D13926 D00821
NID         g222682
KEYWORDS    N protein; NSs protein; S RNA.
SOURCE      Tomato spotted wilt virus, strain L3, DSM# PV-0182, cDNA to genomic

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch:  0.000

            Quality:    8973             Length:   3810
              Ratio:   3.163               Gaps:     24
 Percent Similarity:  37.739   Percent Identity: 37.739

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1
```

Transgenic PLRV replicase mRNA x Tomato spotted wilt virus, genomic S RNA June 11, 1997 11:35 ..

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Tomato Spotted Wilt Virus S RNA

**BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tomato spotted wilt virus S RNA check: 5516 from: 1 to: 2837**

```
LOCUS       TSWSNS       2837 bp ss-RNA          VRL       20-FEB-1993
DEFINITION  Tomato spotted wilt virus, genomic S RNA.
ACCESSION   D13926 D00821
NID         g222682
KEYWORDS    N protein; NSs protein; S RNA.
SOURCE      Tomato spotted wilt virus, strain L3, DSM# PV-0182, cDNA to genomic

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

        Gap Weight:      50      Average Match: 10.000
     Length Weight:       3   Average Mismatch: -9.000

           Quality:     196             Length:     50
             Ratio:   4.083               Gaps:      1
 Percent Similarity: 75.000   Percent Identity: 75.000

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =    5
                    . =    1

 Transgenic PLRV replicase mRNA x Tomato spotted wilt virus, genomic S RNA June 11,
1997 11:35  ..
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Tomato spotted wilt virus L RNA

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tomato spotted wilt virus L RNA check: 4152 from: 1 to: 8897

```
LOCUS       TSWLRPOLM    8897 bp ss-RNA            VRL       17-APR-1992
DEFINITION  Tomato spotted wilt virus L RNA encoding RNA polymerase, complete
            cds.
ACCESSION   D10066 D01230
NID         g222680
KEYWORDS    RNA polymerase. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch:  0.000

            Quality:  12259             Length:   8925
              Ratio:  3.230               Gaps:     26
 Percent Similarity: 37.244   Percent Identity: 37.244

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1

 Transgenic PLRV replicase mRNA x Tomato spotted wilt virus L RNA June 11, 1997 11:35
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Tomato spotted wilt virus L RNA

BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tomato spotted wilt virus L RNA check: 4152 from: 1 to: 8897

```
LOCUS       TSWLRPOLM    8897 bp ss-RNA           VRL       17-APR-1992
DEFINITION  Tomato spotted wilt virus L RNA encoding RNA polymerase, complete
            cds.
ACCESSION   D10066 D01230
NID         g222680
KEYWORDS    RNA polymerase. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

        Gap Weight:      50      Average Match: 10.000
     Length Weight:       3   Average Mismatch: -9.000

           Quality:     173             Length:     23
             Ratio:   7.522               Gaps:      0
 Percent Similarity: 86.957   Percent Identity: 86.957

       Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =    5
                    . =    1

 Transgenic PLRV replicase mRNA x Tomato spotted wilt virus L RNA June 11, 1997 11:36
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Tomato spotted wilt virus S RNA

GAP of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tomato spotted wilt virus S RNA check: 6757 from: 1 to: 2916

```
LOCUS       TSWSRS       2916 bp ss-RNA            VRL       30-OCT-1990
DEFINITION  Tomato spotted wilt virus S RNA segment, complete sequence.
ACCESSION   D00645
NID         g222685
KEYWORDS    non-structural protein; nucleocapsid protein.
SOURCE      Tomato spotted wilt virus (isolate CPNH9), cDNA to viral RNA. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
 CompCheck: 8760

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch:  0.000

            Quality:    9253             Length:   3824
              Ratio:   3.173               Gaps:     23
 Percent Similarity: 37.271   Percent Identity: 37.271

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1

 Transgenic PLRV replicase mRNA x Tomato spotted wilt virus S RNA June 11, 1997 11:35
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Tomato spotted wilt virus S RNA

BESTFIT of: Transgenic PLRV replicase mRNA check: 5272 from: 1 to: 3795
to: Tomato spotted wilt virus S RNA check: 6757 from: 1 to: 2916

```
LOCUS       TSWSRS      2916 bp ss-RNA          VRL       30-OCT-1990
DEFINITION  Tomato spotted wilt virus S RNA segment, complete sequence.
ACCESSION   D00645
NID         g222685
KEYWORDS    non-structural protein; nucleocapsid protein.
SOURCE      Tomato spotted wilt virus (isolate CPNH9), cDNA to viral RNA. . . .

 Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

         Gap Weight:      50      Average Match: 10.000
      Length Weight:       3   Average Mismatch: -9.000

            Quality:     175             Length:     99
              Ratio:   2.083               Gaps:      1
 Percent Similarity:  64.286  Percent Identity: 64.286

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1

 Transgenic PLRV replicase mRNA x Tomato spotted wilt virus S RNA June 11, 1997 11:35
..
```

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Transgenic PLRV replicase mRNA Randomized

Transgenic PLRV replicase mRNA Randomized

GAP of: Transgenic PLRV replicase mRNA Randomized check: 3856 from: 1 to: 3795
to: Transgenic PLRV replicase mRNA: 5272 from: 1 to: 3795

```
Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/nwsgapdna.cmp
CompCheck: 8760

       Gap Weight:     50      Average Match: 10.000
    Length Weight:      3   Average Mismatch:  0.000

          Quality:  11865             Length:   3865
            Ratio:  3.126               Gaps:     26
Percent Similarity: 36.268   Percent Identity: 36.268

       Match display thresholds for the alignment(s):
                   | = IDENTITY
                   : =   5
                   . =   1

Transgenic PLRV replicase mRNA Randomized x Transgenic PLRV replicase mRNA
June 11, 1997 16:13  ..
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Transgenic PLRV replicase mRNA Randomized

```
 BESTFIT of: Transgenic PLRV replicase mRNA Randomized check: 3856  from: 1
to: 3795
to: Transgenic PLRV replicase mRNA check: 5272  from: 1  to: 3795

Symbol comparison table: /usr2/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

        Gap Weight:      50      Average Match: 10.000
     Length Weight:       3   Average Mismatch: -9.000

           Quality:     160             Length:     35
             Ratio:   4.571               Gaps:      0
 Percent Similarity: 71.429   Percent Identity: 71.429

       Match display thresholds for the alignment(s):
                   | = IDENTITY
                   : =    5
                   . =    1

 FMV-to-E9expressed_5.shuffle x FMV-to-E9expressed.seq June 11, 1997 16:13  ..
```

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Appendix 6. Efficacy of Phloem Enhanced and Constitutive Promoters for Transgenic Resistance to PLRV

Various promoters were used to drive the transcription of the PLRV coat protein (CP) gene in transgenic Russet Burbank potato. These tested for the ability to express CP in the plant tissues at levels necessary to provide effective resistance to PLRV infection. These included: the phloem enhanced promoter from the pea glutamine synthetase (GS) gene, the enhanced 35S promoter (E35S) from CaMV and the 35S promoter from FMV (FMV). The GS promoter was considered because of it's reported expression pattern being enhanced in the phloem tissue where PLRV is believed to be primarily localized. The E35S and FMV promoters are constitutively expressed in all plant tissues and highly expressed in phloem tissue. Plant expression vectors containing single and mixed promoter CP gene combinations were transformed into Russet Burbank potato. The resulting transgenic lines were assayed for resistance to PLRV infection in growth chamber tests. At least 20 lines from each expression vector were assayed. Constructs that had lines with reduced incidence of infection by PLRV in growth chamber tests were further tested in the field at multiple locations.

Russet Burbank potato lines transformed with GS/PLRV CP expression cassette (pMON8454; Fig. 1) did not demonstrate resistance to PLRV infection in growth chamber tests and as a result was determined to be an ineffective construct. pMON8515 (GS/PLRV-CP::E35S/PLRV-CP) (Fig. 2) and pMON8517 (FMV/PLRV-CP::E35S/PLRV-CP) (Fig. 3) contain two CP genes. In pMON8515, one gene was driven by E35S, the other gene driven by the GS promoter and in pMON8517, FMV and E35S promoters drove expression of the CP genes. Sixty-five lines were produced from pMON8515, five lines demonstrated resistance to PLRV infection (8 %). Fifty-three lines transformed with the pMON8517, produced nine lines with resistance to PLRV infection (17 %). One line from pMON8517 (FMV and E35S) was completely resistant to infection from the homologous PLRV isolate LR7. No lines transformed with the pMON8515 (GS and E35S) construct were highly resistant. Based on extensive field testing, the best pMON8517 lines always outperformed the best pMON8515 lines (Fig. 4). We concluded that the FMV promoter was more effective than the GS promoter by providing to transgenic potato plants a high level of resistance to PLRV infection. The use of the GS promoter was discontinued in favor of the FMV promoter to drive expression of all subsequent viral transgenes in potato.

pMON18685 (Fig. 5) which used the FMV promoter to drive expression of the replicase gene produced a high frequency (~15%) of transgenic plants which were extremely resistant to infection by PLRV. This provided us with a foundation to design a construct which would produce a sufficient number of resistant lines that could be evaluated for agronomic traits. Only then could a commercially acceptable line be selected.

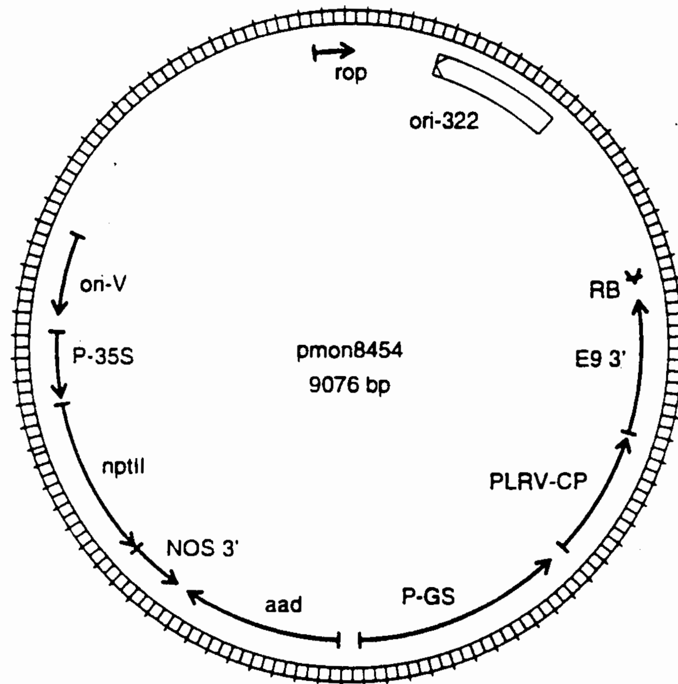


Figure 1. Plasmid of pMON8454.

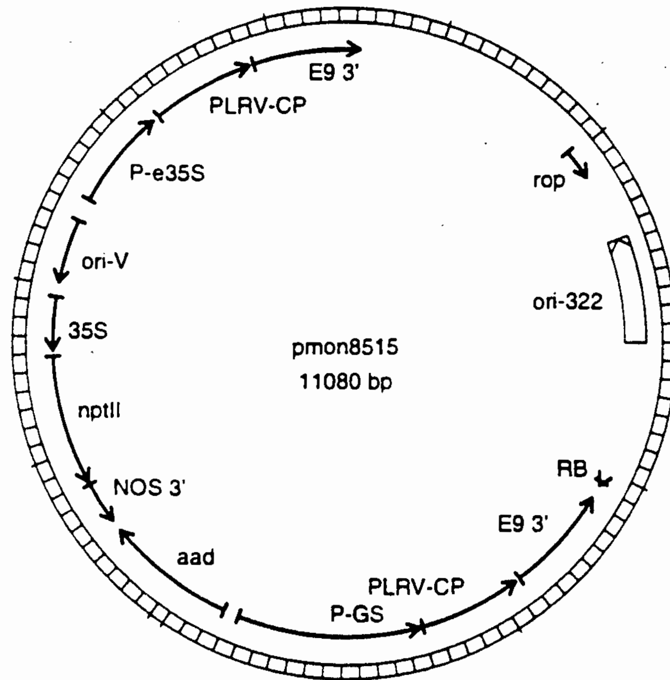


Figure 2. Plasmid map of pMON8515

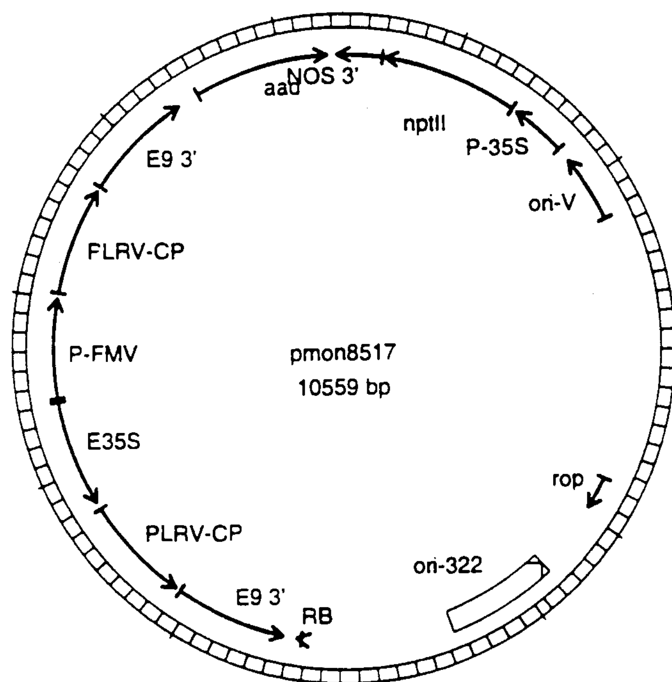
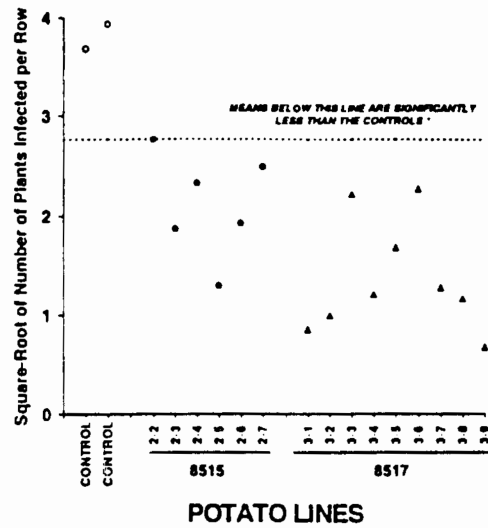


Figure 3. Plasmid map of pMON8517.

PLRV INFECTION OF NON-INOCULATED PLANTS RESULTING FROM CENTER SPREADER PLANT. (BASED ON VISUAL SYMPTOMS.)



* A 5% level 1-tailed t-comparison using a pooled variance-based standard error. The square-root transformation was used in all statistical analyses to equalize within-line variances. Block (replication) differences were non-significant and were ignored in the analyses.

Figure 4. Evaluation of NewLeaf Plus lines transformed with pMON8515 and pMON8517 for resistance to PLRV.

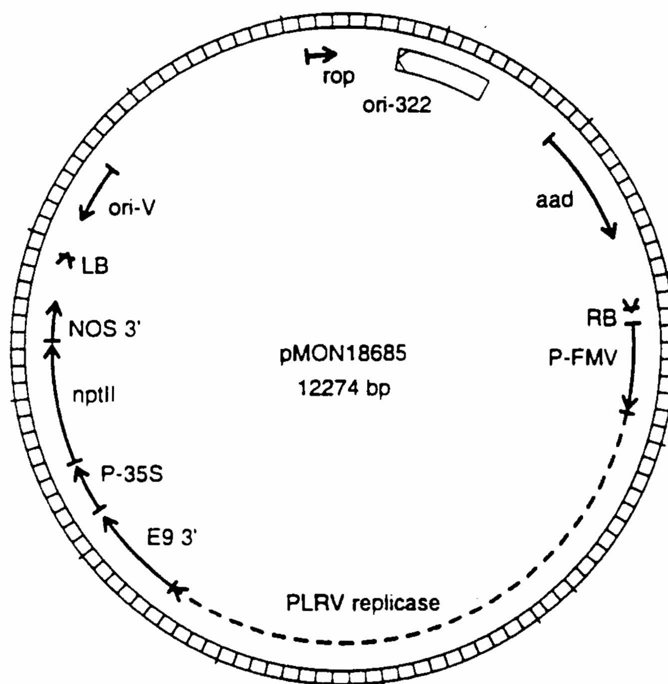


Figure 5. Plasmid map of pMON18685.

Appendix 7. USDA Field Data Reports

USDA PERMIT # 93-362-01R (MON # 93-187R)

FINAL REPORT

Tom Salaiz, NatureMark

USDA permit number 93-362-01 allowed the field evaluation of genetically modified potatoes resistant to the feeding of Colorado potato beetle (CPB) and/or infection by potato leafroll virus (PLRV). The trials were conducted by Monsanto and NatureMark, Inc. in collaboration with academic and/or private cooperators at the following locations:

Parma, Idaho
Wilder, Idaho
Island Falls, Maine
Prosser, Washington

Lines:
21-001, 21-003, 21-005, 21-006, 21-007, 21-008, 21-009, 21-010, 21-011, 21-012, 21-015, 21-016, 21-018, 21-019, 21-020, 21-021, 21-022, 21-023, 21-024, 21-025, 21-026, 21-027, 21-028, 21-030, 21-031, 21-033, 21-034, 21-035, 21-036, 21-037, 21-039, 21-040, 21-041, 21-042, 21-043, 21-045, 21-047, 21-050, 21-051, 21-052, 21-053, 21-054, 21-055, 21-056, 21-057, 21-059, 21-060, 21-062, 21-063, 21-064, 21-065, 21-066, 21-067, 21-069, 21-070, 21-072, 21-073, 21-074, 21-076, 21-077, 21-078, 21-079, 21-080, 21-082, 21-083, 21-084, 21-085, 21-086, 21-087, 21-088, 21-089, 21-090, 21-091, 21-093, 21-094, 21-095, 21-096, 21-097, 21-097, 21-098, 21-099, 21-100, 21-101, 21-102, 21-105, 21-107, 21-108, 21-110, 21-111, 21-112, 21-113, 21-114, 21-115, 21-116, 21-117, 21-118, 21-119, 21-120, 21-121, 21-121, 21-123, 21-124, 21-125, 21-126, 21-127, 21-128, 21-129, 21-130, 21-131, 21-132, 21-133, 21-134, 21-135, 21-136, 21-137, 21-138, 21-139, 21-140, 21-142, 21-143, 21-144, 21-145, 21-145, 21-146, 21-147, 21-148, 21-149, 21-150, 21-151, 21-152, 21-153, 21-154, 21-155, 21-157, 21-159, 21-160, 21-162, 21-163, 21-164, 21-166, 21-167, 21-170, 21-170, 21-171, 21-172, 21-173, 21-174, 21-176, 21-177, 21-178, 21-179, 21-180, 21-181, 21-182, 21-183, 21-184, 21-186, 21-187, 21-188, 21-189, 21-190, 21-191, 21-192, 21-193, 21-195, 21-196, 21-197, 21-198, 21-200, 21-201, 21-203, 21-204, 21-205, 21-206, 21-208, 21-209, 21-211, 21-212, 21-213, 21-214, 21-215, 21-216, 21-220, 21-222, 21-223, 21-224, 21-225, 21-226, 21-227, 21-228, 21-229, 21-231, 21-232, 21-233, 21-234, 21-235, 21-236, 21-237, 21-238, 21-240, 21-240, 21-242, 21-244, 21-246, 21-247, 21-249, 21-251, 21-252, 21-253, 21-256, 21-256, 21-258, 21-259, 21-260, 21-262, 21-263, 21-265, 21-266, 21-267, 21-268, 21-269, 21-270, 21-271, 21-273, 21-274, 21-275, 21-276, 21-277, 21-278, 21-279, 21-280, 21-280, 21-281, 21-282, 21-283, 21-284, 21-285, 21-286, 21-287, 21-289, 21-290, 21-291, 21-292, 21-293, 21-294, 21-297, 21-298, 21-299, 21-300, 21-301, 21-302, 21-303, 21-304, 21-305, 21-306, 21-307, 21-309, 21-310, 21-312, 21-313, 21-314, 21-315, 21-316, 21-318, 21-319, 21-320, 21-321, 21-324, 21-325, 21-326, 21-328, 21-330, 21-331, 21-332, 21-337, 21-340, 21-341, 21-343, 21-344, 21-346, 21-347, 21-348, 21-349, 21-350, 21-352, 21-354, 21-359, 21-360, 21-361, 21-362, 21-363, 21-365, 21-367, 21-369, 21-372, 21-373, 21-375, 21-378, 21-382, 21-383, 22-001, 22-002, 22-004, 22-005, 22-007, 22-008, 22-009, 22-010, 22-011, 22-012, 22-013, 22-014, 22-015, 22-016, 22-018, 22-020, 22-021, 22-022, 22-023, 22-024, 22-025, 22-026, 22-027, 22-028, 22-029, 22-030, 22-031, 22-032, 22-033, 22-034, 22-036, 22-037, 22-038, 22-038, 22-039, 22-040, 22-041, 22-042, 22-043, 22-044, 22-045, 22-046, 22-046, 22-047, 22-048, 22-049, 22-050, 22-051, 22-052, 22-053, 22-054, 22-055, 22-056, 22-057, 22-058, 22-059, 22-060, 22-061, 22-062, 22-063, 22-064, 22-065, 22-066, 22-067, 22-070, 22-071, 22-072, 22-073, 22-074, 22-075, 22-076, 22-077, 22-078, 22-079, 22-080, 22-081, 22-082, 22-083, 22-084, 22-085, 22-087, 22-088, 22-089, 22-090, 22-091, 22-092, 22-093, 22-094, 22-096, 22-097, 22-098, 22-099, 22-099, 22-100, 22-102, 22-103, 22-104, 22-105, 22-106, 22-107, 22-108, 22-109, 22-110, 22-111, 22-112, 22-113, 22-114, 22-115, 22-118, 22-120, 22-121, 22-122, 22-123, 22-124, 22-125, 22-127, 22-128, 22-129, 22-130, 22-131, 22-132, 22-133, 22-134, 22-135, 22-136,

22-137, 22-138, 22-139, 22-140, 22-141, 22-142, 22-144, 22-147, 22-148, 22-148, 22-152, 22-153, 22-154, 22-155, 22-156, 22-157, 22-158, 22-159, 22-160, 22-161, 22-162, 22-163, 22-164, 22-166, 22-167, 22-168, 22-169, 22-170, 22-171, 22-172, 22-174, 22-174, 22-175, 22-176, 22-177, 22-179, 22-180, 22-181, 22-182, 22-183, 22-185, 22-186, 22-187, 22-188, 22-189, 22-190, 22-191, 22-192, 22-193, 22-194, 22-195, 22-196, 22-197, 22-198, 22-199, 22-200, 22-201, 22-202, 22-203, 22-204, 22-205, 22-206, 22-207, 22-208, 22-209, 22-210, 22-211, 22-212, 22-213, 22-214, 22-215, 22-216, 22-217, 22-218, 22-221, 22-223, 22-224, 22-225, 22-226, 22-228, 22-229, 22-230, 22-231, 22-235, 22-236, 22-238, 22-239, 22-240, 22-243, 22-244, 22-245, 22-248, 22-249, 22-250, 22-251, 22-252, 22-254, 22-255, 22-256, 22-257, 22-260, 22-261, 22-262, 22-263, 22-264, 22-265, 22-268, 22-269, 22-270, 22-271, 22-273, 22-274, 22-275, 22-276, 22-277, 22-278, 22-280, 22-281, 22-282, 22-283, 22-284, 22-285, 22-287, 22-288, 22-289, 22-290, 22-291, 22-292, 22-294, 22-295, 22-296, 22-297, 22-298, 22-299, 22-301, 22-302, 22-303, 22-304, 22-305, 22-306, 22-307, 22-308, 22-309, 22-310, 22-311, 22-312, 22-313, 22-314, 22-315, 22-316, 22-317, 22-318, 22-320, 22-321, 22-322, 22-324, 22-327, 22-328, 22-330, 22-331, 22-332, 22-333, 22-376, 22-387,

EXPERIMENTAL PROGRAM

Trial: 94-14-01: PLRV Field Screening of New Constructs

Purpose:

To evaluate resistance to potato leafroll virus (PLRV) infection under field conditions, in Russet Burbank lines from new constructs.

Summary:

Plots were arranged in a completely random design with two replications per treatment at both Prosser, WA and Parma, ID. There were 624 transgenic lines and three Russet Burbank clones planted at each location.

The entire trial area was surrounded by at least 10' of unplanted ground at the Parma, ID location and by two rows of unplanted buffer followed by winter wheat at the Prosser, WA location. At each location, the area planted to transgenic potatoes was less than 1 acre and the entire plot area was less than 2 acres.

All plants were inoculated with 10-15 viruliferous aphids as described in permit protocol. One of the controls was left uninoculated in order to evaluate plant type on resistant lines. An insecticide was sprayed on the entire plot to kill aphids at seven days post-inoculation. All control lines and the controls in which less than 10% of the plants showed PLRV symptoms were harvested, stored for observation of net necrosis in tubers and plant back PLRV indexing. All other lines were lifted and left on the soil surface to freeze. The plot areas will not be planted to potatoes in 1995 and will be observed for volunteers in spring of 1995. Any volunteers will be mechanically or chemically destroyed.

No differences in susceptibility to diseases other than to PLRV or insects other than CPB were observed between transgenic and non-transgenic potatoes at either location. Some infestation by aphids was noted. Some lines showed atypical phenotype which is normal when screening large numbers of transformants. These lines did not meet the selection criteria and were destroyed by being left to freeze after harvest lifting.

Harvested tubers were held in a locked storage prior to examination of tuber net necrosis and plant back for PLRV indexing. All tuber and plant material was disposed of according to conditions of the field release permit.

Trial: 94-04-02: Field exposure to natural PLRV strains

Purpose:

To evaluate under field conditions, the resistance of transgenic potato clones to naturally occurring strains of PLRV and to net necrosis.

Summary:

The trial was conducted at Hermiston, OR and Prosser, WA. The experimental design was a randomized complete block with four replications per treatment. The area planted to transgenic potatoes was 0.13 acres and the entire trial area including buffer rows did not exceed 1 acre. The border area was planted to BelRus, a PLRV resistant variety, in order to facilitate the objectives of the trial.

No differences in susceptibility to diseases other than to PLRV were observed between transgenic and non-transgenic potatoes at either location. No differences were observed between the transgenic potatoes and non-transgenic controls with respect to morphological type, vigor and uniformity. The trial area will not be planted to potatoes in 1995 and will be observed for volunteers in spring of 1995.

Harvested tubers were held in a locked storage prior to examination of tuber net necrosis and plant back for PLRV indexing. All tuber and plant material was disposed of according to conditions of the field release permit.

Trial: 94-14-03: Yield Efficiency

Purpose:

To determine the distribution of yields from a large number of transformed potato lines.

Summary:

The trial was conducted at Wilder, ID. The experimental design was a randomized complete block with one to four replications per treatment depending on platelet availability and condition. The area planted to transgenic potatoes did not exceed 1.6 acres and the entire trial area including buffer rows did not exceed 2 acres. The border area was a two row unplanted buffer.

No differences in susceptibility to diseases were observed between transgenic and non-transgenic potatoes at either location. Some lines showed atypical phenotype which is normal when screening large numbers of transformants. These lines did not meet the selection criteria and were destroyed by being left to freeze after harvest lifting. The trial area will not be planted to potatoes in 1995 and will be observed for volunteers in spring of 1995.

Harvested tubers were held in a locked storage prior to examination of tuber net necrosis and plant back for PLRV indexing. All tuber and plant material was disposed of according to conditions of the field release permit.

Trial: 94-14-04: Seed Production

Purpose:

To increase the quantity of selected potato lines, while evaluating yield potential and phenotypic uniformity.

Summary:

Tubers, plantlets and/or minitubers were planted in multi-row, non-replicated blocks and grown following the production practices for seed potatoes that are standard for each site location. All pests, including Colorado potato beetle, and fungal and viral diseases were controlled with conventional methods. There was a six foot barrier separating the transgenic potatoes from adjacent potatoes. All production at the site was submitted for seed certification with the agency responsible for certification in Maine. Potato lines of vectors PV-STMT21 and PV-STMT22 and PV-STLR9, PV-STLR11, and PV-STLR12 evaluated, respectively.

The transgenic plants grew identically to the non-transformed controls. Observations by the cooperator detected no differences in phenotypic traits of the foliage or tubers. Yields were comparable. In addition, no differences in susceptibility to diseases other than PLRV (PVY, early or late blight, verticillium, rusts, or leaf spot) or insects (aphids, leafhoppers, mites or cutworms) were observed between transgenic and non-transgenic potatoes. There were no unusual or unexpected results which would pose a threat to the environment.

All tubers that met the selection criteria for certified seed were lifted, harvested and stored for use in 1994. Tubers not meeting the selection criteria were spread back on the surface of the plot area to freeze over the winter. The plot areas will not be planted to potatoes in 1995 and will be monitored for volunteers. Volunteers will be chemically or mechanically destroyed.

Overall, these potatoes do not appear to be any more susceptible to other potato diseases than the nontransgenic parental variety. No deleterious effects were noted on plants, nontarget organisms or the environment.

USDA Final Report 94-217-02R

USDA Number: **94-217-02R**

Purpose of trial Evaluate the seed potatoes during winter plant back which is part of the seed certification process in the U.S.A. The evaluation determines amount of viral infection that occurred during in-season seed production.

Methods of observation: Visual inspection by trained potato seed certification personnel.

Resulting data. Virus infection in the seed samples did not exceed t Tolerances allowed by certification agencies for certified seed potatoes. No symptoms related to infection by PLRV. No weedy tendencies were noted for tubers remaining in the field post-harvest.

Analysis. These potatoes are resistant to PLRV and do not appear to be any more susceptible to other potato diseases than the nontransgenic parental variety. No deleterious effects were noted on plants, nontarget organisms or the environment.

USDA Final Report 94-342-01R

USDA Number: **94-342-01R**

Purpose of trial

1. To confirm the resistance of transgenic lines to potato leafroll virus
2. Investigate the effect of transgenic CPB and/or potato leafroll virus resistant Russet Burbank plants (with and without systemic insecticide) on populations of predacious insects, pest insects and vectored viruses.
3. To increase the seed volume of selected transgenic potato lines.
4. To evaluate resistance of various transgenic leafroll resistant potato clones to several different isolates of PLRV
5. To study the development of disease symptoms, virus populations and virus distribution in transgenic versus standard Russet Burbank clones.
6. To note an difference of disease susceptibility (see Table 1) of transgenic plants compared to control non-transgenic plants

Lines:
21-005, 21-016, 21-020, 21-023, 21-030, 21-033, 21-052, 21-063, 21-066, 21-072, 21-101, 21-111, 21-129, 21-134, 21-152, 21-203, 21-204, 21-212, 21-228, 21-240, 21-257, 21-281, 21-291, 21-350, 21-365, 21-377, 22-015, 22-037, 22-045, 22-062, 22-078, 22-082, 22-084, 22-141, 22-161, 22-180, 22-186, 22-238, 22-250, 22-262, 22-270, 22-294, 22-297, 22-298, 22-333

Methods of observation. Visual inspection at least monthly by trained potato researchers/growers and by ELISA.

Resulting data. The amount of leafroll and CPB resistance varied depending on the line. Lines that provided good control of CPB and PLRV were selected and saved for future seed increase. No differences were observed for disease susceptibility between experimental plants and control plants. No weedy tendencies were noted for tubers remaining in the field post-harvest.

Analysis. These potatoes are resistant to PLRV and do not appear to be any more susceptible to other potato diseases than the nontransgenic parental variety. No deleterious effects from the transgenic potatoes were noted on plants, nontarget organisms or the environment.

Table 1. Diseases and disease symptoms which were looked for during monthly scouting of transgenic potato field trials.

Organism or Pathogen	Disease or Symptoms
Insect	
Emposasca fabae (Potto leafhopper)	Leaf feeding damage
Epitrix species (Flea Beetle)	Shotholes in leaves
Leptinotarsa decemlineata (Colorado Potato Beetle)	Defoliation
Limonius californicus (Wireworm)	Bored holes in tubers and shoots
Ostrinia nubilalis (European Corn Borer)	Sever vine wilting above point of injury
Paratrioza cockerelli (Potato Psyllid)	Yellows
Phthorimaea operculella (Tuberworm)	Foliar and tuber damage
Various aphid species	Leaf suckling damage
Virus and Virus-Like	
Aster Yellows MLO	Purple top disease
Potato Leafroll Virus	rolling of leaves and net necrosis
Potato Spindle Tuber Viroid	Potato spindle tuber disease
Potato Virus A.M.X.Y	Mosaic symptoms
Tobacco Rattle Virus	Stem mottling
Bacteria and Fungi	
Erwinia carotovora	Blackleg, Aerial stem rot and Tuber soft rot
Corynebactium sepedonicum	Bacterial ring rot
Phytophthora infectans	Late blight
Verticillium spp.	Early dying
Sclerotinia sclerotiorum	Sclerotinia stalk rot
Rhizoctonia solani	Canker
Streptomyces scabies	Scab
Fusarium sp.	Dry rot
Phytophthora and Pythium	Water rot
Nematodes	
Globodera rostochiensis	Cysts
Meloidogyne ep.	Root knot
Paratrichodorus sp.	Stubby root
Pratylenchus sp	Root lesions

USDA Final Report 96-277-01R

USDA Number: **96-277-01R**
Lines: RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262

Purpose of trial. Evaluate the seed potatoes during winter plant back which is part of the seed certification process in the U.S.A. The evaluation determines amount of viral infection that occurred during in-season seed production.

Methods of observation. Visual inspection by trained potato seed certification personnel.

Resulting data. Virus infection in the seed samples did not exceed the Tolerances allowed by certification agencies for certified seed potatoes. No symptoms related to infection by PLRV. No weedy tendencies were noted for tubers remaining in the field post-harvest.

Analysis. These potatoes are resistant to PLRV and do not appear to be any more susceptible to other potato diseases than the nontransgenic parental variety. No deleterious effects were noted on plants, nontarget organisms or the environment.

97-204-01p

Appendix 8

Agronomic Performance (vigor, emergence, stem count, flowering, yield, tuber size and tubers per plant) of NewLeaf Plus Lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262

October 30, 1997

Submitted by

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NewLeaf Plus Potato Agronomic Field Trials

NewLeaf Plus potatoes were evaluated during the 1996 growing season in three sites in Canada and two sites in USA The potatoes were compared to two different control Russet Burbank potatoes, one produced in Maine, USA (CTR-ME) and one produced in Idaho, U.S.A (CTR-ID). Initially, many lines were evaluated to determine the line which performed the best agronomically in the field. Data from all of these lines is included below. The lines have been reduced to the following lines which have been submitted for commercial approval: lines RBMT21-129, RBMT21-RBMT21-129, RBMT21-152, RBMT21-350-82, RBMT22-186, RBMT22-238 and RBMT22-262. The data demonstrates that the transgenic NewLeaf® Plus Russet Burbank potato lines are substantially equivalent to non-transgenic Russet Burbank potatoes.

Field Trial 1: [CBI DELETED] Alberta, Canada

Method

Potatoes were planted May 21, 1996 in a randomized complete block design. Each treatment consisted of 1 row containing 15 seed pieces. The plot was replicated 4 times.

Vigor measurement taken July 23, 1996. Values within plot: 1. below average; 2. average; 3. above average.

Results

[CBI DELETED] Analysis of plant vigor[1].

Row	Range	Plot #	Line	TRT#	Rep	Vigor[2]
1	A	1A	CTRL-ME	8	1	2
2	A	2A	RBMT21-129	1	1	2
3	A	3A	RBMT21-152	2	1	2
4	A	4A	RBMT21-350	3	1	2
5	A	5A	RBMT22-262	7	1	3
6	A	6A	RBMT22-082	4	1	3
7	A	7A	RBMT22-238	6	1	3
8	A	8A	RBMT22-186	5	1	2
1	B	1B	RBMT22-186	5	2	1
2	B	2B	RBMT22-082	4	2	3
3	B	3B	RBMT22-262	7	2	2
4	B	4B	RBMT21-152	2	2	2
5	B	5B	RBMT21-350	3	2	3
6	B	6B	RBMT21-129	1	2	3
7	B	7B	RBMT22-238	6	2	3
8	B	8B	CTRL-ME	8	2	2
1	C	1C	RBMT21-129	1	3	2
2	C	2C	RBMT21-152	2	3	2
3	C	3C	RBMT22-238	6	3	2
4	C	4C	RBMT22-186	5	3	1
5	C	5C	RBMT22-082	4	3	3
6	C	6C	RBMT21-350	3	3	3
7	C	7C	CTRL-ME	8	3	2
8	C	8C	RBMT22-262	7	3	2
1	D	1D	RBMT22-082	4	4	2
2	D	2D	CTRL-ME	8	4	2
3	D	3D	RBMT22-186	5	4	2
4	D	4D	RBMT21-350	3	4	2
5	D	5D	RBMT22-238	6	4	3
6	D	6D	RBMT21-129	1	4	2
7	D	7D	RBMT21-152	2	4	2
8	D	8D	RBMT22-262	7	4	2

[CBI DELETED] ANOVA

Screening Fit

Vigor

Summary of Fit

Analysis of Variance

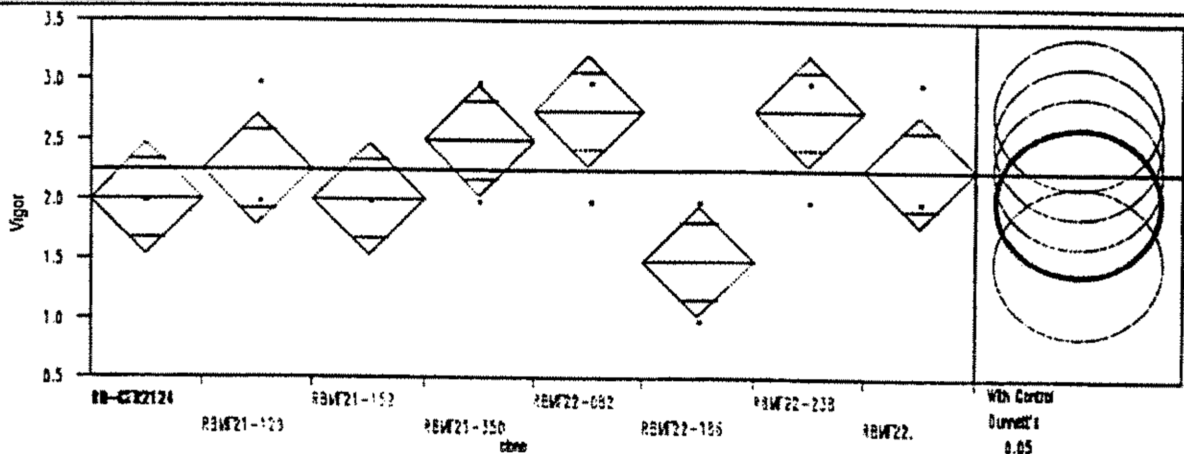
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	5.400000	0.675000	3.3750
Error	23	4.600000	0.200000	Prob>F
C Total	31	10.000000		0.0105

Parameter Estimates

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
clone	7	7	5.0000000	3.5714	0.0096
Rep	1	1	0.4000000	2.0000	0.1707

Prediction Profile



Oneway Anova

Means Comparisons

[CBI DELETED] ANOVA

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Line	7	7	5	3.3333	0.0151
Rep	3	3	0.5	0.7778	0.5195
Error	21	4.5	0.214286		

Dependent variable = Vigor

[CBI DELETED] **LSD of Means**

Line	Vigor (LS Means)
21-129	2.3
21-152	2.0
21-350	2.5
RB Control	2.0
22-082	2.8 *
22-186	1.5
22-238	2.8 *
22-262	2.3

*, Significantly different than the control

Field Trial 2: [CBI DELETED] Manitoba, Canada

Method

Potatoes were planted June 3, 1996 in a randomized complete block design. Each treatment consisted of 1 row containing 15 seed pieces. The plot was replicated 4 times.

Vigor measurement was taken July 24, 1996. Values within plot: 1. below average; 2. average; 3. above average

Results

[CBI DELETED] Analysis of Plant Vigor

Row	Range	Plot	Line	TRT#	Rep	Vigor
1	A	A1	RBMT22-238	6	1	3
2	A	A2	RBMT21-152	2	1	2
3	A	A3	RBMT22-262	7	1	2
4	A	A4	CTRL-ME	8	1	3
5	A	A5	RBMT21-129	1	1	2
6	A	A6	RBMT21-350	3	1	2
1	B	B1	RBMT22-186	5	1	2
2	B	B2	RBMT22-082	4	1	2
3	B	B3	RBMT22-082	4	2	2
4	B	B4	RBMT21-350	3	2	3
5	B	B5	RBMT22-262	7	2	1
6	B	B6	RBMT21-129	1	2	2
1	C	C1	CTRL-ME	8	2	3
2	C	C2	RBMT22-238	6	2	2
3	C	C3	RBMT21-152	2	2	2
4	C	C4	RBMT22-186	5	2	3
5	C	C5	CTRL-ME	8	3	2
6	C	C6	RBMT21-129	1	3	2
1	D	D1	RBMT22-082	4	3	3
2	D	D2	RBMT22-262	7	3	3
3	D	D3	RBMT21-350	3	3	2
4	D	D4	RBMT21-152	2	3	2
5	D	D5	RBMT22-186	5	3	2
6	D	D6	RBMT22-238	6	3	2
1	E	E1	RBMT22-082	4	4	3
2	E	E2	RBMT22-238	6	4	3
3	E	E3	RBMT21-350	3	4	2
4	E	E4	CTRL-ME	8	4	3
5	E	E5	RBMT21-152	2	4	2
6	E	E6	RBMT22-262	7	4	2
1	F	F1	RBMT22-186	5	4	3
2	F	F2	RBMT21-129	1	4	2

[CBI DELETED] ANOVA

Screening Fit

Vigor

Summary of Fit

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	2.6000000	0.325000	1.1912
Error	23	6.2750000	0.272826	Prob>F
C Total	31	8.8750000		0.3465

Parameter Estimates

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
Clone	7	7	2.3750000	1.2436	0.3204
Rep	1	1	0.2250000	0.8247	0.3732

Prediction Profile

[CBI DELETED] LSD of Means

Average of Vigor	
Line	Vigor
CTRL-ME	2.8
21-129	2.0
21-152	2.0
21-350	2.3
22-082	2.5
22-186	2.5
22-238	2.5
22-262	2.0

Field Trial 3: [CBI DELETED] New Brunswick, Canada

Method

Potatoes were planted May 31, 1996 in a randomized complete block design. Each treatment consisted of 2 rows containing 15 seed pieces per row. The plot was replicated 4 times.

Emergence data was collected July 11, 1996. These data indicate the percentage of plant which had emerged by this date.

Flowering data was taken July 24, 1996. Values within plot: 1, below average; 2, average; 3, above average

Tubers were harvested and the total yield was taken on October 1, 1996.

Results

[CBI DELETED] Analysis of Plant Emergence, Flowering, and Total Yield

Plot	Range/rep	Line	trt#	Emergence	Flowering	POSITION	Total Yield (cwt/acre)
8	A	CTRL-ME	8	96.7	1	A8	360.35
1	B	CTRL-ME	8	100	1	B1	342.89
5	C	CTRL-ME	8	100	1	C5	358.26
3	D	CTRL-ME	8	100	1	D3	353.41
9	A	CTRL-ID	9	100	2	A9	399.32
7	B	CTRL-ID	9	100	2	B7	367.20
8	C	CTRL-ID	9	100	2	C8	384.30
6	D	CTRL-ID	9	100	3	D6	391.62
4	A	RBMT21-129	4	100	1	A4	398.01
4	B	RBMT21-129	4	100	1	B4	402.87
2	C	RBMT21-129	4	100	1	C2	340.26
9	D	RBMT21-129	4	100	1	D9	395.35
1	A	RBMT21-152	1	100	1	A1	317.95
2	B	RBMT21-152	1	100	1	B2	283.17
1	C	RBMT21-152	1	100	1	C1	345.76
5	D	RBMT21-152	1	100	1	D5	332.02
2	A	RBMT21-350	2	100	1	A2	318.00
5	B	RBMT21-350	2	100	1	B5	375.57
7	C	RBMT21-350	2	100	1	C7	358.49
2	D	RBMT21-350	2	100	1	D2	370.09
6	A	RBMT22-082	6	100	1	A6	413.04
3	B	RBMT22-082	6	100	1	B3	317.02
6	C	RBMT22-082	6	100	1	C6	411.12
7	D	RBMT22-082	6	100	1	D7	437.41
3	A	RBMT22-186	3	100	1	A3	278.39
8	B	RBMT22-186	3	100	1	B8	368.35

4	C	RBMT22-186	3	100	1	C4	361.01
8	D	RBMT22-186	3	100	1	D8	355.36
5	A	RBMT22-238	5	100	1	A5	378.25
9	B	RBMT22-238	5	100	1	B9	398.32
9	C	RBMT22-238	5	100	1	C9	409.09
1	D	RBMT22-238	5	100	1	D1	344.69
7	A	RBMT22-262	7	100	1	A7	381.73
6	B	RBMT22-262	7	100	1	B6	407.05
3	C	RBMT22-262	7	100	1	C3	337.99
4	D	RBMT22-262	7	100	1	D4	351.84

[CBI DELETED] **ANOVA Type III Sums of Squares**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
range/rep	3	526.704	175.568	0.162	0.9209
Line	8	19413.056	2426.632	2.238	0.0607
Residual	24	26022.65	1084.277		

Dependent: Total Yield cwt/acre

Screening Fit

% emergence

Summary of Fit

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	3.327500	0.302500	1.0000
Error	24	7.260000	0.302500	Prob > F
C. Total	35	10.587500		0.4744

Parameter Estimates

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Clone	8	8	2.4200000	1.0000	0.4613
range/rep	3	3	0.9075000	1.0000	0.4098

flowering

Summary of Fit

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	5.6388889	0.512626	18.4545
Error	24	0.6666667	0.027778	Prob > F
C. Total	35	6.3055556		< .0001

Parameter Estimates

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Clone	8	8	5.5555556	25.0000	< .0001
range/rep	3	3	0.0833333	1.0000	0.4098

Total Yield

Summary of Fit

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	19940.015	1812.73	1.6719
Error	24	26021.465	1084.23	Prob > F
C. Total	35	45961.480		0.1413

Parameter Estimates

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Clone	8	8	19413.327	2.2382	0.0607
range/rep	3	3	526.689	0.1619	0.9209

Prediction Profile

[CBI DELETED] **LSD of Means**

Line	Emergence	Flowering	Total Yield cwt/acre	
CTRL-ME	99.175	1	353.7275	RB Control 1
CTRL-ID	100	2.25 *	385.61	RB Control 2
RBMT21-129	100	1	384.1225	
RBMT21-152	100	1	319.725	
RBMT21-350	100	1	355.5375	
RBMT22-082	100	1	394.6475	
RBMT22-186	100	1	340.7775	
RBMT22-238	100	1	382.5875	
RBMT22-262	100	1	369.6525	

*= significantly different from CTRL-ME (RB control 1)

Field Trial 4: [CBI DELETED] Island Falls, Maine, USA

Method

Potatoes were planted May 218, 1996 in a randomized complete block design. Each treatment consisted of 1 row containing 15 seed pieces. The plot was replicated 4 times.

Tubers were harvested October 2, 1996.

Results

[CBI DELETED] Analysis of total yield, tuber size, culls, and tubers per plant.

REP POS	REP	Line	Total yield	cwt <4 oz	cwt 4-6 oz	cwt 6-10 oz	cwt 10-14 oz	cw >14
A14	REP 1	RB 2124	521.7278	142.4654	245.5816	133.6808	0	0
B07	REP 2	RB 2124	435.1402	113.7158	136.851	150.8628	33.7106	0
C04	REP 3	RB 2124	417.813	120.8064	174.361	110.1584	12.4872	0
D22	REP 4	RB 2124	399.3	88.2574	166.617	132.8822	11.5434	0
A17	REP 1	RB ID	478.9422	146.8214	182.2018	108.5854	41.3336	0
B22	REP 2	RB ID	492.6152	121.6534	150.3788	137.8432	82.7398	0
C03	REP 3	RB ID	499.3186	126.0094	169.037	115.8454	88.4268	0
D05	REP 4	RB ID	497.4068	127.05	231.11	77.7788	61.468	0
A01	REP 1	RBMT21-005	451.8382	189.7522	161.5592	77.7062	22.8206	0
B20	REP 2	RBMT21-005	408.2056	164.8746	148.5396	73.6406	21.1508	0
C14	REP 3	RBMT21-005	405.8582	203.6188	157.905	31.1454	13.189	0
D13	REP 4	RBMT21-005	338.8	155.6302	114.103	69.0668	0	0
A08	REP 1	RBMT21-111	334.0326	269.5396	64.493	0	0	0
B02	REP 2	RBMT21-111	395.428	192.8256	158.6068	43.9956	0	0
C06	REP 3	RBMT21-111	454.6696	144.232	232.6588	51.4492	26.3296	0
D08	REP 4	RBMT21-111	373.9142	157.3968	136.9236	50.094	29.4998	0
A13	REP 1	RBMT21-129	410.8192	210.7578	168.5772	31.4842	0	0
B03	REP 2	RBMT21-129	485.9602	248.9696	173.6834	55.5148	7.7924	0
C11	REP 3	RBMT21-129	460.3082	152.7262	178.2572	87.8218	41.503	0
D15	REP 4	RBMT21-129	430.9536	228.3996	168.8676	33.6864	0	0
A09	REP 1	RBMT21-152	352.2068	202.554	112.5542	37.0986	0	0
B13	REP 2	RBMT21-152	341.6072	164.0518	116.7892	37.026	23.7402	0
C15	REP 3	RBMT21-152	463.5026	197.6656	218.9616	20.3522	26.5232	0
D01	REP 4	RBMT21-152	418.5148	168.7466	201.8038	47.9644	0	0
A20	REP 1	RBMT21-212	405.6162	220.9702	142.8768	41.7692	0	0
B17	REP 2	RBMT21-212	469.3348	196.6734	202.1668	52.6108	17.8838	0
C05	REP 3	RBMT21-212	461.0826	175.087	186.4368	84.2886	15.2702	0
D18	REP 4	RBMT21-212	411.9566	172.062	176.4422	45.0604	18.392	0
A11	REP 1	RBMT21-291	356.829	253.7612	103.0678	0	0	0
B01	REP 2	RBMT21-291	442.013	208.3136	233.6994	0	0	0
C12	REP 3	RBMT21-291	401.2118	178.5476	198.198	24.4662	0	0
D06	REP 4	RBMT21-291	405.108	156.7676	191.7608	56.5796	0	0
A05	REP 1	RBMT21-350	425.4602	212.9842	182.6858	15.246	14.5442	0
B15	REP 2	RBMT21-350	463.9624	194.2776	178.6928	76.835	14.157	0
C13	REP 3	RBMT21-350	445.4252	189.7038	179.2736	59.2658	17.182	0
D12	REP 4	RBMT21-350	454.7422	197.6656	177.2408	68.486	11.3498	0
A18	REP 1	RBMT21-377	415.3446	155.848	176.902	57.7896	24.805	0
B05	REP 2	RBMT21-377	473.5456	192.4384	190.091	78.4322	12.584	0
C22	REP 3	RBMT21-377	436.9794	207.7086	177.3134	51.9574	0	0

D19	REP 4	RBMT21-377	432.7928	156.1626	180.6046	80.2472	15.7784	0
A15	REP 1	RBMT22-045	415.4172	254.3662	147.6442	13.4068	0	0
B09	REP 2	RBMT22-045	408.5444	183.0972	151.0806	69.3088	5.0578	0
C17	REP 3	RBMT22-045	448.4986	211.7984	178.7896	57.9106	0	0
D14	REP 4	RBMT22-045	438.0926	255.2132	173.03	9.8494	0	0
A21	REP 1	RBMT22-082	458.8804	193.358	193.0434	46.8512	25.6278	0
B19	REP 2	RBMT22-082	419.8216	123.2264	183.436	113.1592	0	0
C08	REP 3	RBMT22-082	434.027	142.8526	243.2342	41.7934	6.1468	0
D09	REP 4	RBMT22-082	443.707	190.2846	189.4134	64.009	0	0
A19	REP 1	RBMT22-141	434.6078	163.8582	190.0668	58.685	21.9978	0
B10	REP 2	RBMT22-141	445.1106	164.3906	182.5164	77.0528	21.1508	0
C07	REP 3	RBMT22-141	491.5988	152.2664	171.4086	167.9238	0	0
D17	REP 4	RBMT22-141	420.9348	142.2476	199.3112	79.376	0	0
A16	REP 1	RBMT22-161	448.7406	230.142	182.3228	24.6598	11.616	0
B11	REP 2	RBMT22-161	423.1612	210.8062	159.5022	44.891	7.9618	0
C16	REP 3	RBMT22-161	455.565	164.0518	250.712	19.8924	20.9088	0
D03	REP 4	RBMT22-161	389.741	163.9308	189.2198	36.5904	0	0
A03	REP 1	RBMT22-180	420.354	230.747	157.663	31.944	0	0
B16	REP 2	RBMT22-180	467.544	191.6882	201.9732	62.7022	11.1804	0
C19	REP 3	RBMT22-180	458.3964	151.4436	169.8114	112.0702	25.0712	0
D10	REP 4	RBMT22-180	440.2706	224.697	163.0112	52.5624	0	0
A12	REP 1	RBMT22-186	431.607	129.8814	176.7084	109.142	15.8752	0
B08	REP 2	RBMT22-186	405.4468	102.8258	132.9064	80.1746	89.54	0
C01	REP 3	RBMT22-186	436.447	133.1242	170.2228	115.918	17.182	0
D21	REP 4	RBMT22-186	376.2374	100.3332	160.3734	91.0404	24.4904	0
A04	REP 1	RBMT22-238	419.6038	180.169	171.3602	58.3462	9.7284	0
B04	REP 2	RBMT22-238	462.341	182.2018	178.4024	79.1098	22.627	0
C09	REP 3	RBMT22-238	468.9718	176.0792	225.302	44.2134	23.3772	0
D02	REP 4	RBMT22-238	431.849	165.6006	194.205	50.5054	21.538	0
A06	REP 1	RBMT22-250	462.3168	247.4934	189.5586	25.2648	0	0
B06	REP 2	RBMT22-250	419.0956	161.535	188.5422	57.4266	11.5918	0
C18	REP 3	RBMT22-250	403.9464	128.2842	140.0938	116.3778	19.1906	0
D04	REP 4	RBMT22-250	399.6146	124.3638	193.7936	81.4572	0	0
A22	REP 1	RBMT22-262	421.9996	218.3566	156.5014	42.229	4.9126	0
B21	REP 2	RBMT22-262	422.411	179.5156	168.2142	64.6382	10.043	0
C20	REP 3	RBMT22-262	422.895	191.3978	180.1448	40.2446	11.1078	0
D20	REP 4	RBMT22-262	445.6188	189.5344	218.6712	37.4132	0	0
A07	REP 1	RBMT22-294	433.5188	130.68	193.9146	92.3714	16.5528	0
B18	REP 2	RBMT22-294	427.251	125.6948	191.8334	70.8818	38.841	0
C10	REP 3	RBMT22-294	368.6386	129.0586	166.3266	73.2534	0	0
D16	REP 4	RBMT22-294	398.0658	161.1962	153.0892	77.5852	6.1952	0
A10	REP 1	RBMT22-298	334.7344	195.8506	103.1888	25.0954	10.5996	0
B14	REP 2	RBMT22-298	439.9318	177.1198	161.4866	84.6274	16.698	0
C02	REP 3	RBMT22-298	363.363	127.0984	160.1798	70.543	5.5418	0
D11	REP 4	RBMT22-298	410.6498	107.811	191.422	111.4168	0	0
A02	REP 1	RBMT22-333	415.5382	181.4274	174.24	23.1594	36.7114	0
B12	REP 2	RBMT22-333	505.5864	174.119	250.6878	71.995	8.7846	0
C21	REP 3	RBMT22-333	405.8824	114.3692	141.8604	63.3072	86.3456	0
D07	REP 4	RBMT22-333	445.5704	136.1734	204.7804	81.2636	23.353	0

[CBI DELETED] **ANOVA**

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
REP	3	7458.421	2486.14	2.26	0.0901
LINE	21	52744.062	2511.622	2.283	0.0062
Residual	63	69300.577	1100.009		

Dependent: Total yield

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
REP	3	20401.083	6800.361	9.817	0.0001
LINE	21	75379.399	3589.495	5.182	0.0001
Residual	63	43639.883	692.697		

Dependent: cwt <4 oz

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
REP	3	6283.163	2094.388	1.942	0.1318
LINE	21	18843.311	897.301	0.832	0.6716
Residual	63	67928.092	1078.224		

Dependent: cwt 4-6 oz

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
REP	3	8152.948	2717.649	4.77	0.0046
LINE	21	60813.44	2895.878	5.083	0.0001
Residual	63	35889.749	569.679		

Dependent: cwt 6-10 oz

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
REP	3	2149.102	716.367	3.333	0.025
LINE	21	18558.771	883.751	4.111	0.0001
Residual	63	13542.726	214.964		

Dependent: cwt 10-14 oz

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
REP	3	42.536	14.179	6.383	0.0008
LINE	21	160.071	7.622	3.431	0.0001
Residual	63	139.947	2.221		

Dependent: tubers/ plant

[CBI DELETED] **LSD of Means**

Line	Total Yield	< 4 oz	4-6 oz	6-10 oz	> 10 oz	Culls	Tubers / Plant
			(100 lb/Acre)				
RB 2124	443 cd	116 a	181 NS	132 i	14 a	0	12.5 ab
RB ID	492 e	130 ab	183	110 hi	68 c	0	14.1 bcd
RBMT21-005	401 abc	178 cdefg	146	63 bcdef	14 a	0	14.6 bcdef
RBMT21-111	390 a	191 defgh	148	36 abc	14 a	0	14.7 cdef
RBMT21-129	447 cde	210 gh	172	52 abcde	12 a	0	17 g
RBMT21-152	394 ab	183 cdefg	163	36 abc	13 a	0	14.6 bcde
RBMT21-212	437 bcd	191 defgh	177	56 bcde	13 a	0	15.4 defg
RBMT21-291	401 abcd	199 efgh	182	20 a	0 a	0	14.2 bcd
RBMT21-350	447 cde	199 efgh	179	55 bcde	14 a	0	16.3 efg
RBMT21-377	440 bcd	178 cdefg	181	67 cdefg	13 a	0	14.4 bcde
RBMT22-045	428 abcd	226 h	163	38 abcd	1.3 a	0	16.8 g
RBMT22-082	439 bcd	162 bcde	202	66 cdefg	7.9 a	0	15.1 cdefg
RBMT22-141	448 de	156 bcd	186	96 fgh	11 a	0	13.7 bcd
RBMT22-161	429 abcd	192 defgh	195	32 ab	10 a	0	14.9 cdefg
RBMT22-180	447 cde	200 fgh	173	65 bcdef	9.1 a	0	16.7 fg
RBMT22-186	412 abcd	117 a	160	99 ghi	37 b	0	11.5 a
RBMT22-238	446 cde	176 cdefg	192	58 bcde	19 ab	0	15 cdefg
RBMT22-250	421 abcd	165 bcdef	178	70 defg	7.7 a	0	15.1 cdefg
RBMT22-262	428 abcd	195 efgh	181	46 abcde	6.5 a	0	15.3 defg
RBMT22-294	407 abcd	137 ab	176	79 efgh	15 a	0	13 abc
RBMT22-298	387 a	152 abc	154	73 efg	8.2 a	0	13.1 abc
RBMT22-333	443 cd	152 abc	193	60 bcde	39 b	0	14.4 bcde
P-Value	0.006	0.0001	0.67	0.0001	0.0001		0.0001

Field Trial 5: [CBI DELETED] Parma, Idaho, USA

Method

Potatoes were planted April 9, 1996 in a randomized complete block design. Each treatment consisted of 4 row containing 20 seed pieces. The plot was replicated 7 times.

Measurements were taken on the following data: Emergence, May 10, 1996; Stem count, May 10, 1996; Vigor, May 30, 1996; Senescence: August 29, 1996; Harvest; September 9, 1996. Values within plot: 1, below average; 2, average; 3, above average.

Vigor measurement taken July 23, 1996. Values within plot: 1, below average; 2, average; 3, above average.

[CBI DELETED] **Analysis of emergence, stem count, vigor, senescence, tuber size, culls, yield and specific gravity.**

Rep	Line	Emergence (%)	Stem count Avg./10 plants	Vigor	Senescence (%)	Tuber size 100 lb./acre <4 oz	4-6 oz	6-12 oz	>12 oz	CULLS	Total Yield	Specific gravity
1	CTR-ID	76	3	3	75	107	85	340	125	9	668	1.08274
2	CTRL-ID	66	4	3	70	118	152	198	115	15	598	1.07980
3	CTRL-ID	94	3	3	95	106	94	325	136	13	674	1.07303
4	CTRL-ID	80	2	2.5	85	94	107	281	40	44	566	1.07694
5	CTRL-ID	99	2	3	80 •	•	•	•	•	•		1.07656
6	CTRL-ID	99	3	3	95	129	122	269	60	15	595	1.07125
7	CTRL-ID	86	3	3	90	84	79	373	97	23	657	1.07533
1	CTR-ME	51	2	3	50	96	120	293	142	35	686	1.07764
2	CTR-ME	83	3	3	90	90	108	390	94	16	699	1.07786
3	CTR-ME	74	3	3	70	85	116	379	98	16	693	1.07688
4	CTR-ME	63	4	3	90	113	167	288	37	15	620	1.07563
5	CTR-ME	73	3	3	85	84	145	319	31	20	599	1.07926
6	CTR-ME	96	3	3	95	119	124	268	14	5	530	1.07913
7	CTR-ME	81	3	2.5	95	104	91	302	52	19	568	1.07339
1	RBMT21-023	85	3	3	65	92	86	342	141	53	714	1.07569
2	RBMT21-023	71	3	3	60	92	99	340	182	0	713	1.07742
3	RBMT21-023	61	3	2.5	75	95	116	257	87	17	572	1.07921
4	RBMT21-023	95	3	3	95	107	79	377	86	19	668	1.07987
5	RBMT21-023	94	3	3	95 •	•	•	•	•	•		1.07708
6	RBMT21-023	60	4	2.5	100	105	134	249	31	18	538	1.07691
7	RBMT21-023 •	•	•	•	•	•	•	•	•	•		•
1	RBMT21-129	73	4	3	60	111	160	238	88	42	638	1.07485
2	RBMT21-129	81	3	3	55	117	137	302	105	40	700	1.07769
3	RBMT21-129	83	2	3	90	91	109	290	150	15	654	1.08120

4	RBMT21-129	85	3	3	85	112	125	325	87	7	655	1.07434
5	RBMT21-129	98	3	3	90	101	109	335	66	13	626	1.07874
6	RBMT21-129	89	3	3	95	91	112	316	88	19	626	1.07729
7	RBMT21-129	84	3	3	90	136	79	385	76	6	681	1.07679
1	RBMT21-152	78	3	3	40	114	124	318	88	65	709	1.07671
2	RBMT21-152	70	3	3	35	111	131	277	151	16	687	1.07728
3	RBMT21-152	92	2	3	60	91	119	255	125	65	655	1.07548
4	RBMT21-152	91	3	3	75	75	149	297	103	9	632	1.07558
5	RBMT21-152	91	2	2.5	95	73	50	329	99	49	600	1.07817
6	RBMT21-152	88	3	2.5	95	107	88	254	76	10	534	1.07540
7	RBMT21-152	75	3	2	75	92	107	255	147	23	625	1.07455
1	RBMT21-350	58	3	3	95	114	108	229	32	15	498	1.07441
2	RBMT21-350	74	3	3	80	108	158	244	94	10	613	1.07954
3	RBMT21-350	69	3	2.5	80	79	109	294	227	12	722	1.08132
4	RBMT21-350	98	3	3	70	105	138	351	82	5	682	1.08263
5	RBMT21-350	99	3	3	90	102	93	311	56	18	581	1.08007
6	RBMT21-350	79	3	2.5	90	73	88	344	112	10	627	1.07977
7	RBMT21-350	85	3	3	90	96	117	325	48	21	608	1.08003
1	RBMT22-082	56	3	3	90	90	143	276	92	15	615	1.08058
2	RBMT22-082	76	3	3	40	96	131	300	45	23	594	1.07973
3	RBMT22-082	81	3	3	85	76	106	349	166	15	711	1.07569
4	RBMT22-082	63	4	2.5	55	142	126	273	30	8	579	1.07680
5	RBMT22-082	85	3	2.5	90	115	133	250	37	47	582	1.07857
6	RBMT22-082	95	3	3	85	120	107	376	44	44	691	1.07623
7	RBMT22-082	84	3	3	70	89	90	316	121	53	670	1.07516
1	RBMT22-186	54	2	3	100	113	98	145	67	3	426	1.07534
2	RBMT22-186	69	3	2.5	60	90	82	340	192	10	714	1.08330
3	RBMT22-186	44	3	2.5	85	94	110	275	74	6	559	1.07834
4	RBMT22-186	73	2	2.5	85	69	114	279	128	2	593	1.07773
5	RBMT22-186	91	3	2.5	95	134	143	249	18	10	554	1.08017
6	RBMT22-186	78	3	2.5	95	91	106	276	57	19	549	1.07981

7	RBMT22-186	59	3	2	85	111	110	254	51	47	572	1.08012
1	RBMT22-238	71	3	3	35	95	139	305	91	30	659	1.07670
2	RBMT22-238	83	3	3	90	88	95	306	168	22	679	1.07988
3	RBMT22-238	94	3	3	95	107	112	292	79	18	608	1.10905
4	RBMT22-238	96	2	3	75	126	105	329	89	17	667	1.07665
5	RBMT22-238	81	3	3	90	178	60	254	3	68	562	1.07895
6	RBMT22-238	75	3	2.5	95	125	107	205	32	42	511	1.07958
7	RBMT22-238	94	3	3	95	132	130	236	34	11	543	1.07999
1	RBMT22-262	39	5	2.5	50	105	110	299	55	37	606	1.07485
2	RBMT22-262	78	3	3	70	96	115	322	99	18	649	1.07772
3	RBMT22-262	88	3	2.5	80	106	115	273	85	3	582	1.08273
4	RBMT22-262	73	3	2.5	70	107	126	225	27	25	509	1.08598
5	RBMT22-262	71	3	3	90	150	105	182	21	10	468	1.08298
6	RBMT22-262	83	4	2.5	90	87	118	297	27	5	535	1.07427
7	RBMT22-262	54	2	2	90	87	86	270	70	21	533	1.07825

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ANOVA

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Rep	6	3562.991	593.832	4.462	0.001
Line	9	2778.757	308.751	2.32	0.0277
Residual	53	7054.343	133.101		

Dependent: Emergence

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Rep	6	0.436	0.073	0.347	0.9087
Line	9	1.786	0.198	0.948	0.4929
Residual	53	11.099	0.209		

Dependent: Avg. stems/plant

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Rep	6	0.884	0.147	2.648	0.0254
Line	9	1.715	0.191	3.424	0.0022
Residual	53	2.949	0.056		

Dependent: Vigor

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
rep	6	7504.927	1250.821	7.156	0.0001
Line	9	2083.38	231.487	1.324	0.2469
Residual	53	9264.12	174.795		

Dependent: % Senescence

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Rep	6	2654.595	442.433	1.219	0.3118
Line	9	3729.579	414.398	1.142	0.3515
Residual	51	18502.77	362.799		

Dependent: <4 oz

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Rep	6	5071.524	845.254	1.577	0.1729
Line	9	3329.606	369.956	0.69	0.7142
Residual	51	27331.91	535.92		

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LSD of Means

Line	Emergence	Avg. Stems/Plant	Vigor	Senescence	< 4 oz	4-6 oz	6-12 oz	Culls	Total Yield	Specific gravity
CTR-ID	85.7 c	2.8 NS	2.9 cd	84.3 NS	106 NS	107 NS	298 NS	20 NS	626 bc	1.077 NS
CTR-ME	74.4 abc	3	2.9 cd	82.1	99	124	320	18	628 c	1.077
MT21-023	77.7 abc	3	2.8 bcd	81.7	98	103	313	21	641 c	1.078
MT21-129	84.7 c	2.8	3 d	80.7	108	119	313	20	654 c	1.077
MT21-152	83.6 c	2.6	2.7 abc	67.9	95	110	284	34	635 c	1.076
MT21-350	80.3 bc	2.9	2.9 cd	85	97	116	300	13	619 bc	1.080
MT22-082	77.1 abc	2.9	2.9 cd	73.6	104	119	306	29	635 c	1.078
MT22-186	66.9 a	2.7	2.5 a	86.4	100	109	260	14	567 ab	1.079
MT22-238	84.9 c	2.9	2.9 cd	82.1	122	107	275	30	604 ab	1.083
MT22-262	69.4 ab	3.2	2.6 ab	77.1	105	111	267	17	555 a	1.080
P value	0.03	0.49	0.002	0.25	0.35	0.71	0.33	0.2	0.05	0.18

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Pages 1 through 43]

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LSD of Means

Line	Emergence	Avg. Stems/Plant	Vigor	Senescence	< 4 oz	4-6 oz	6-12 oz	Culls	Total Yield	Specific gravity
CTR-ID	85.7 c	2.8 NS	2.9 cd	84.3 NS	106 NS	107 NS	298 NS	20 NS	626 bc	1.077 NS
CTR-ME	74.4 abc	3	2.9 cd	82.1	99	124	320	18	628 c	1.077
MT21-023	77.7 abc	3	2.8 bcd	81.7	98	103	313	21	641 c	1.078
MT21-129	84.7 c	2.8	3 d	80.7	108	119	313	20	654 c	1.077
MT21-152	83.6 c	2.6	2.7 abc	67.9	95	110	284	34	635 c	1.076
MT21-350	80.3 bc	2.9	2.9 cd	85	97	116	300	13	619 bc	1.080
MT22-082	77.1 abc	2.9	2.9 cd	73.6	104	119	306	29	635 c	1.078
MT22-186	66.9 a	2.7	2.5 a	86.4	100	109	260	14	567 ab	1.079
MT22-238	84.9 c	2.9	2.9 cd	82.1	122	107	275	30	604 ab	1.083
MT22-262	69.4 ab	3.2	2.6 ab	77.1	105	111	267	17	555 a	1.080
P value	0.03	0.49	0.002	0.25	0.35	0.71	0.33	0.2	0.05	0.18

Addendum to USDA Petition 97-204-01P

On page 8, second full paragraph, the sentence "The parental potato line RB1 was transformed with either PV-STMT21 or PV-STMT22 to insert the DNA between the left and right border of the *Agrobacterium tumefaciens* Ti plasmid (Klee and Rogers, 1989)" should be inserted after the sentence "Information on potato plant lines derived from seven independent transformation events are ..."

On page 9 at the start of Section II, the sentence "The following section was written by Dr. Steve Love, University of Idaho, Aberdeen" should be changed to read "The following section, Section II. A - E, was written by Dr. Steve Love, University of Idaho, Aberdeen".

On page 24 at the end of the first partial paragraph, the sentences "Data from the PCR experiments, summarized on page 8, demonstrate that only the genetics elements between left and right borders of PV-STMT21 or PV-STMT22 was transferred; and according to Klee and Rogers (1989), the transformed lines also contain a portion of the left and right border. No elements outside the left and right borders are present in any of the seven transgenic lines."

On page 24, the text (in italics and bold) should be added to the following paragraph

> 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.13 and V.14, respectively. *PLRVrep* RNA transcript was not detected by dot hybridization analysis in either leaf or tuber tissue of the seven transgenic lines in this assay. Based on the amount of *PLRVrep* RNA standard spotted (Lane 1, Figure V.12 and V.13), 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 PRLV ORF1/2 mRNA*** (Figure V.12.).. Since the average amount of RNA isolated from leaf and tuber of the seven transgenic lines corresponded to 840 ng/mg leaf fresh weight and 190 ng/mg tuber fresh weight, the limits 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 seven transgenic lines.

The Monsanto technical report referenced as "Dean, 1993" on page 25 last paragraph is attached. Additional, the sentence "However, the purified protein standard was detected" should be changed to "The purified protein standard, which was purified from recombinant *E. coli* and used to produce anti-PLRV ORF1/ORF2 protein antiserum, was detected".

> **NOTE: The Dean, 1993 is marked a confidential; however, this document is no longer considered confidential and USDA/APHIS is free to distribute to the public.**

On page 41, Figure V.12, panel B, the sentence "Plasmid DNA containing PLRV ORF1/ORF2 served as a positive hybridization control for the probe (lane not shown)

On page 44, Section V.B., text should be added or altered (indicated by italics and bold) in the following paragraph:

> Potato lines RBMT21-129, RBMT21-152, RBMT21-350, RBMT22-82, RBMT22-186, RBMT22-238 and RBMT22-262 were evaluated in the field from 1994 - 1997 under USDA permits 93-362-01r, 94-217-02R, 94-342-01r, 96-277-01r, and 97-017-03r (in progress). ***The lines selected for commercialization are substantially equivalent agronomically, including weediness, to unmodified Russet Burbank potatoes and when analyzed for vigor, emergence, stem count, flowering, yield, tuber size and tubers per plant (Appendix 8). Additionally, the transgenic lines*** highly resistant to the Colorado potato beetle and the potato leafroll virus (see USDA Final Reports, Appendix 7; and Certification of NewLeaf Plus Russet Burbank Potatoes in the U.S. and Canadian Seed Certification Programs Appendix 2). During field trial evaluating of the transgenic potato lines, the potato plants were monitored for any unusual susceptibility to potato insect pests or pathogens (Table V.4.). 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 U.S. Leafhoppers were seen but mostly in the midwestern states (MN and WI). However, except for the intended PLRV and CPB resistance traits in the transgenic lines, no differences in susceptibility to insect pests or diseases between the transgenic and control lines were noted.

An Agronomic Performance report is included as Appendix 8.

97-204-01p

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July 13, 1998

Dr. James White
USDA, APHIS, PPQ, BSS
Unit 147
4700 River Road
Riverdale, MD 20737-1236

Dear Dr. White,

SUBJECT: USDA Petition 97-204-01P
Petition for Determination of Regulatory Status
for NewLeaf® Plus Potatoes

In response to your letter of December 17, 1997, attached is the research report of a study designed to address your specific questions.

In your letter, two questions were asked concerning the small percentage of NewLeaf® Plus plants that developed leafroll symptoms when challenged with potato leafroll virus (PLRV):

1. *Has a resistance breaking virus strain arisen?*
    - If so, this strain must be characterized.
2. *Is the resistance transgene not expressed?*
    - If the resistance gene is being lost in a small percentage of the transformation events, describe how this could occur.

The two-part laboratory experiment conducted under the protocol, discussed with and approved by you, provided results that answer these two questions. The results show that:

1. The potato leafroll virus (PLRV) found in a low percentage of NewLeaf Plus potato plants was shown not to be resistance breaking in a subsequent passage through transgenic plants, as there were no significant changes in the presence of PLRV as detected by ELISA.

2. Analysis established the presence of the intact replicase gene in PLRV infected NewLeaf Plus potato plants.

These experiments confirm the results from extensive field and laboratory studies, previously submitted to the USDA, that show a very low incidence of PLRV viral infection in NewLeaf Plus potato plants. Furthermore, they confirm that the observed infection is not due to a new recombinant resistance breaking strain of PLRV. When taken together, our data clearly demonstrate that the modified potato plants do not pose a plant pest risk and are not otherwise deleterious to human health or the environment.

Monsanto considers USDA determination of NewLeaf Plus potatoes as a non-regulated article to be a top priority and respectfully request that you conduct a review of this submission and issue the final decision as soon as possible; potato growers desire access to this important technology for the 1999 growing season.

The enclosed addendum does not contain confidential business information.

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

Signature Elizabeth Owens GJR Signature Glen Rogan

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Attachment:

Report: Biological Characterization of Potato Leafroll Virus Found in NewLeaf Plus Russet Burbank and Russet Burbank Potato Plants

[CBI DELETED

Pages 1 through 43]