FOOD DERIVED FROM INSECT AND POTATO LEAFROLL VIRUS PROTECTED (NEW LEAF® PLUS) POTATO LINES RBMT21-129, RBMT21-350, AND RBMT22-82

A Safety Assessment

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ANZFA Australia
PO Box 7186
Canberra BC ACT 2610
Australia
Tel +61 2 6271 2241
Fax +61 2 6271 2278
Email info@anzfa.gov.au

ANZFA New Zealand
PO Box 10599
Wellington
New Zealand
Tel + 64 4 473 9942
Fax +64 4 473 9855
Mail nz.reception@anzfa.gov.au

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SUMMARY

Food derived from GM potato lines BT RBMT21-129, RBMT21-350 and RBMT22 has been evaluated to determine its suitability for human consumption. The evaluation criteria included characterisation of the transferred genes, analysis of changes at the DNA, protein and whole food levels, and assessment of the potential allergenicity and toxicity of any newly expressed proteins. Examination of these criteria has enabled both the intended and unintended changes to be identified, characterised and evaluated for safety.

Nature of the genetic modification

Three lines of Russet Burbank potatoes (RBMT21-129, RBMT21-350 and RBMT22-82) were protected against Colorado potato beetle (CPB) and potato leafroll virus (PLRV) through the *Agrobacterium tumefaciens* mediated transfer of two genes — the *cry3Aa* gene from the soil bacterium *Bacillus thuringiensis* subspecies *tenebrionis* (*B.t.t.*) and the *PLRVrep* gene from PLRV. The insect and virus-protected potatoes are known commercially as New Leaf® Plus potatoes.

The *cry3Aa* gene is responsible for the production of the Cry3Aa protein, which is toxic to a narrow range of beetles, including the Colorado potato beetle. When ingested by a susceptible beetle, Cry3Aa causes lysis of midgut epithelial cells in the insect gut, leading to gut paralysis, cessation of feeding and the eventual death of the insect. Cry3Aa produces this toxic effect by binding to specific receptors in the target insects. As there are no receptors for Cry3Aa on the surface of mammalian intestinal cells, humans are not susceptible to Cry3Aa. A number of microbial pesticide products based on Cry3Aa are commercially available in the United States, with some being in use since 1989.

The *PLRVrep* gene is responsible for the production of the PLRV replicase protein. The replicase is an enzyme whose normal function is to copy (i.e., replicate) the genome of the virus within the plant cell. It has been found that plants can be protected from viral infection through the expression of one of a number of viral genes in the plant. The exact mechanism by which the viral protection occurs is unknown.

Other genes transferred to the New Leaf® Plus potatoes were the *nptII* gene (in lines RBMT21-129 and RBMT21-350), and the *CP4 EPSPS* and *aad* genes (in line RBMT22-82 only). The *nptII* and *CP4 EPSPS* genes are marker genes used for selection of transformed plant lines during the potato transformation procedure. The *nptII* gene codes for the enzyme neomycin phosphotransferase II (NPTII) and confers resistance to the antibiotics neomycin, kanamycin, and geneticin (G418). The *CP4 EPSPS* gene codes for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and confers tolerance to the herbicide, glyphosate. The glyphosate tolerant trait of the New Leaf® Plus potatoes was included for selection purposes only. New Leaf® Plus potato line RBMT22-82 is not marketed as a herbicide-tolerant potato and the trait is not utilised in the field. The *aad* gene is a marker used to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells. It codes for the enzyme streptomycin adenyltransferase, which confers resistance to the antibiotics spectinomycin and streptomycin.

The transferred genes appear to be stably integrated and both protection traits are stably maintained over multiple generations.

History of use

The potato (*Solanum tuberosum* L.) is a major food crop throughout the world and has a long history of safe use as human food. The main food products to be derived from the New Leaf® Plus potatoes will be processed food commodities such as processed potato crisps, pre-cooked French fries, potato flour and potato starch.

Antibiotic resistance genes

One of the important issues to consider in relation to genetically modified foods is the impact on human health from potential transfer of novel genetic material to cells in the human digestive tract. Much of the concern in this regard is with antibiotic resistance genes. In the case of the New Leaf® Plus potatoes, it was concluded that the *nptII* and *aad* genes would be extremely unlikely to transfer to bacteria in the human digestive tract because of the number and complexity of the steps that would need to take place consecutively. More importantly, however, in the highly unlikely event that transfer did occur, the human health impacts would be negligible because bacteria harbouring resistance to kanamycin and streptomycin are already widespread in nature or are found to naturally inhabit the human digestive tract. Furthermore, kanamycin/neomycin and streptomycin are rarely used clinically because of unwanted side effects.

Characterisation of novel protein

The New Leaf® Plus potatoes each express three of four novel proteins — Cry3Aa, the PLRV replicase, and either NPTII or CP4 EPSPS. The Cry3Aa protein is expressed in tubers at levels ranging from 0.11 - 0.9 μg protein/g fresh weight (equivalent to 0.0006 to 0.005% of the total tuber protein). The PLRV replicase protein was unable to be detected in the plants, although other evidence suggests that it is expressed. NPTII is expressed in the tuber but at levels below the limit of detection (0.3 ng protein/g fresh weight) and CP4 EPSPS is expressed in the tuber at levels ranging from 0.21 - 0.78 μg protein/g fresh weight (equivalent to 0.001 to 0.004% of the total tuber protein).

Acute oral toxicity testing in mice has been done previously for the Cry3Aa, NPTII and CP4 EPSPS proteins where it was concluded that all three proteins are non-toxic to humans. No additional evidence has come to light in this evaluation that would alter this conclusion. Dietary intake assessments indicate that exposure to all three proteins from the consumption of New Leaf® Plus potatoes will be low.

The potential toxicity of the PLRV replicase has not previously been considered. Human beings have a long history of exposure to the PLRV replicase through the consumption of PLRV-infected plants. In addition, the expression levels of the PLRV replicase are likely to be lower in New Leaf® Plus potatoes than in PLRV-infected potatoes. Therefore, human populations consuming New Leaf® Plus potatoes will most likely have lower exposure levels to the PLRV replicase than they would through the consumption of PLRV-infected potatoes. There is also no significant similarity between the PLRV replicase and any known toxins. On the basis of this evidence, it was concluded that the PLRV replicase protein, as expressed in the New Leaf® Plus potatoes, is non-toxic to humans.

In terms of the potential allergenicity of the four novel proteins, it has previously been concluded that Cry3Aa, NPTII and CP4 EPSPS are unlikely to be allergenic to humans. No additional data or evidence has emerged which would necessitate revising this conclusion. The PLRV replicase protein in the New Leaf® Plus potatoes has no significant similarity to any known allergens, nor is it present in large quantities in potato tubers. On the basis of this information and the fact that humans have a long history of exposure to low levels of this protein, through the consumption of PLRV-infected potatoes, with no recorded instances of allergenicity, it can be concluded that the PLRV replicase protein is unlikely to be allergenic to humans.

Comparative analyses

Detailed compositional analyses were done to establish the nutritional adequacy of the New Leaf® Plus potatoes, and to compare them to non-modified control lines. Analyses were done of total solids, dextrose, sucrose, soluble protein, proximate (total protein, fat, crude fibre, ash, total carbohydrates and calories), amino acid, and vitamin and mineral content. Some minor differences were observed

for some constituents however these were not biologically significant and the values reported were all within the literature reported ranges for the Russet Burbank cultivar. On the basis of this information it was concluded that the New Leaf® Plus potatoes are similar to other commercial varieties in terms of these key constituents.

The levels of naturally occurring toxins in New Leaf® Plus potatoes were also assessed. The only naturally occurring toxins in potatoes are the glycoalkaloids. The glycoalkaloid levels in the New Leaf® Plus potatoes were slightly elevated compared to the control but were at the lower end of the range reported for commercial varieties of Russet Burbank potatoes. The slightly elevated levels of glycoalkaloids are not statistically significant and do not represent a safety concern.

Conclusion

Based on the currently available data, food from New Leaf® Plus potato lines RBMT21-129, RBMT21-350 and RBMT22-82 is as safe and nutritious as food from other commercially available potato cultivars.

FOOD DERIVED FROM INSECT AND POTATO LEAFROLL VIRUS PROTECTED (NEW LEAF® PLUS) POTATO LINES RBMT21-129, RBMT21-350, AND RBMT22-82:

A SAFETY ASSESSMENT

INTRODUCTION

A safety assessment has been conducted on food derived from potatoes that have been genetically modified to be protected against Colorado potato beetle (*Leptinotarsa decemlineata* Say.), one of the major pests of potatoes in North America, and potato leafroll virus (PLRV), a major viral disease of potatoes worldwide. The potatoes are known commercially as New Leaf® Plus potatoes.

Protection against Colorado potato beetle is achieved through expression in the plant of the insecticidal protein, Cry3Aa. Cry3Aa is produced naturally by the *tenebrionis* subspecies of the spore-forming soil bacterium *Bacillus thuringiensis* (*B.t.t.*). The majority of described *B. thuringiensis* strains produce insecticidal proteins active against lepidopteran insects (larvae of moths and butterflies) and a few are reported to have activity against dipteran insects (mosquitos and flies). The Cry3Aa protein, however, is toxic to a narrow spectrum of coleopteran insects (beetles) and shows no activity against other groups of insects such as the lepidopterans or dipterans (Herrnstadt *et al* 1986).

Two commercially available microbial pesticide products based on *B.t.t.* (M-One® and Foil®) have been in use in the United States since 1989. In addition, a bio-insecticide known commercially as MYX 1806 comprising Cry3Aa genetically engineered into the bacterium *Pseudomonas fluorescens,* which has been rendered non-viable, has been commercially available in the United States since 1991.

PLRV is a spherical RNA virus belonging to the luteovirus group of plant viruses and is transmitted primarily by the green peach aphid (*Myzus persicae*). Protection against PLRV is achieved through the expression, in the plant, of a gene derived from the viral genome, which codes for the viral replicase protein. The replicase is an enzyme that copies (i.e., replicates) the RNA genome of the virus within the plant cell. The expression of plant viral genes in plants has been shown to confer varying degrees of protection against subsequent infection by the plant virus from which the gene was derived (reviewed in Lomonossoff 1995). The exact mechanism by which the protection is conferred is unknown.

New Leaf® Plus potatoes are not grown in Australia or New Zealand and are currently not permitted to be imported into Australia and New Zealand as fresh produce. Rather, they currently enter into the market in imported processed food commodities such as processed potato crisps, pre-cooked French fries, potato flour and potato starch.

HISTORY OF USE

The potato (*Solanum tuberosum* L.) is a major food crop throughout the world (Simmonds 1976). It was introduced into Europe from South America in the 16th century and is cultivated for the production of underground tubers.

Potatoes are generally consumed either cooked (as a fresh vegetable) or processed into crisps, potato flour or potato starch. They are rarely consumed raw because of the indigestibility of ungelatinised potato starch and the presence of protease inhibitors (Burton 1989).

DESCRIPTION OF THE GENETIC MODIFICATION

Methods used in the genetic modification

Russet Burbank potatoes were transformed with one of two plasmids, PV-STMT21 and PV-STMT22, using *Agrobacterium*-mediated transformation of stem sections.

Function and regulation of the novel genes

Agrobacterium-mediated transformation of potatoes with PV-STMT21 and PV-STMT22 resulted in the transfer of three of four gene expression cassettes — *cry3Aa*, *PLRVrep*, and either *nptII* or *CP4 EPSPS*. Each of these expression cassettes is described in Table 1 below.

Table 1: Description of the gene expression cassettes in PV-STMT21 and PV-STMT22

Cassette	Genetic element	Source	Function
cry3Aa	ArabSSU1A promoter	*Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit ats 1A promoter (Almeida *et al* 1989, Wong *et al* 1992).	Constitutive plant promoter.
	cry3Aa	Coding region of the *B.t.t.* Band 3 protein (Perlak *et al* 1993).	Confers protection against a narrow spectrum of Coleopterans, including Colorado potato beetle.
	NOS 3' terminator	The 3' terminator region of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens* (Depicker *et al* 1982, Bevan *et al* 1983).	Contains signals for termination of transcription and directs polyadenylation.
PLRVrep	35S promoter	A promoter derived from Figwort mosaic virus (FMV) (Richins *et al* 1987) containing the soybean heatshock protein 17.9 kDa 5' 77-nucleotide leader sequence (Raschke *et al* 1988).	A promoter of high-level constitutive gene expression in plant tissues.
	PLRVrep	Coding region for the putative PLRV replicase protein (Van der Wilk *et al* 1989).	Replicates the RNA genome of the virus.
	E9 3'	The 3' non-translated region of the pea ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) E9 gene (Coruzzi *et al* 1984).	Contains signals for termination of transcription and directs polyadenylation.
***nptII* (PV-STMT21)**	P-NOS	The promoter region of the nopaline synthase gene from the Ti plasmid of Agrobacterium tumefaciens (Fraley *et al* 1983).	A promoter of low-level constitutive gene expression in plant tissues.
	nptII	The gene coding for neomycin phosphotransferase II from Tn5 in *Escherichia coli* (Beck *et al* 1982).	Confers resistance to the antibiotics kanamycin and neomycin. Used as a selectable marker for plant transformation (Horsch *et al* 1984, DeBlock *et al* 1984).

	NOS 3'	The 3' terminator region of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens* (Depicker *et al* 1982, Bevan *et al* 1983).	Contains signals for termination of transcription and directs polyadenylation.
***CP4 EPSPS* (PV-STMT22)**	35S promoter	A promoter derived from Figwort mosaic virus (FMV) (Richins *et al* 1987) containing the soybean heatshock protein 17.9 kDa 5' 77-nucleotide leader sequence (Raschke *et al* 1988).	A promoter of high-level constitutive gene expression in plant tissues.
	CTP2	The chloroplast transit peptide from the EPSPS gene of *Arabidopsis thaliana* (Della-Cioppa *et al* 1986)	Targets proteins to the chloroplasts of higher plants.
	CP4 EPSPS	The gene from *Agrobacterium* sp. strain CP4 (Barry *et al* 1992) coding for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).	Confers tolerance to the herbicide, glyphosate. Used as a dominant selectable marker in tissue culture following transformation (Howe *et al* 1992).
	E9 3'	The 3' non-translated region of the pea ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) E9 gene (Coruzzi *et al* 1984).	Contains signals for termination of transcription and directs polyadenylation.

The cry3Aa gene

The *cry3Aa* gene was isolated from the DNA of *B.t.t* strain BI 256-82 (Krieg *et al* 1983). A full length clone and complete nucleotide sequence of the *cry3Aa* gene has been published (McPherson *et al* 1988, Perlak *et al* 1993). The gene is one of several that have been isolated from *B. thuringiensis* and which encode a group of toxins known as the δ-endotoxins or the crystal proteins. These toxins are selectively active against several Orders of insects such as the Lepidoptera, Coleoptera, and Diptera. The crystal proteins are produced by the bacterium during sporulation. The protein product of the *cry3Aa* gene, Cry3Aa, is selectively active against a narrow spectrum of Coleoptera (MacIntosh *et al* 1990). When ingested by susceptible insect species, the crystal proteins cause lysis of midgut epithelial cells in the insect gut, which leads to gut paralysis, cessation of feeding and the eventual death of the insect (Höfte and Whiteley 1989). Cytolytic effects on the midgut cells are mediated by binding of the activated toxin to specialised receptors on the cell surface. This binding of the toxin to specialised receptors has been shown to be essential for the onset of toxicity (Wolfersberger 1990, Ferré *et al* 1991). Following binding of activated toxin to the receptors, a rapid change in permeability of midgut cells is observed where there is an influx of ions and water in the cell, resulting in its eventual lysis (Knowles and Ellar 1987).

The PLRVrep gene

The *PLRVrep* gene was isolated from PLRV isolate LR-7 (Kaniewski *et al* 1995). The genome of PLRV has been fully sequenced (Mayo *et al* 1989). The *PLRVrep* gene consists of two overlapping open reading frames, which together are thought to encode the PLRV replicase (Miller *et al* 1995, Murphy *et al* 1995, Van der Wilk *et al* 1989).

The strategy of expressing viral genes in plants to confer protection against an infecting virus is known as pathogen-derived resistance. Sanford and Johnson (1985) first developed pathogen-derived resistance as a theoretical concept, when they proposed that resistance genes against a pathogen could be derived from the genome of the pathogen itself. This approach was first successfully applied against tobacco mosaic virus (TMV) where disease development was found to be delayed in TMV

inoculated plants expressing the TMV coat protein gene (Powell *et al* 1986). The exact mechanism by which the protection occurs is unknown.

The nptII gene

The *nptII* gene is widely used as a selectable marker in the transformation of plants (Kärenlampi 1996). The gene 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). It codes for the enzyme neomycin phosphotransferase II (NPTII) and confers resistance to the aminoglycoside antibiotics, neomycin, kanamycin, and geneticin (G418). The *nptII* gene is transferred along with the *cry3Aa* and *PLRVrep* genes, enabling those plant cells successfully transformed with the *cry3Aa* and *PLRVrep* genes to grow in the presence of kanamycin. Those cells that lack the *nptII* gene, and hence the *cry3Aa* and *PLRVrep* genes, will not grow and divide in the presence of kanamycin.

The CP4 EPSPS gene

The *CP4 EPSPS* gene was isolated from *Agrobacterium* sp. strain CP4 (Barry *et al* 1992). The CP4 EPSPS gene codes for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and confers resistance to the herbicide, glyphosate. Plant EPSPS is inhibited by glyphosate and plant cells treated with glyphosate are unable to synthesize the aromatic amino acids essential for protein synthesis resulting in the death of the plant cells (Barry *et al* 1992). In contrast, the CP4 EPSPS is not inhibited by glyphosate. Thus when the *CP4 EPSPS* gene is transferred along with the *cry3Aa* and *PLRVrep* genes it enables those plant cells successfully transformed with the *cry3Aa* and *PLRVrep* genes to grow in the presence of glyphosate. Those cells that lack the *CP4 EPSPS* gene, and hence the *cry3Aa* and *PLRVrep* genes, will not grow and divide in the presence of glyphosate.

Other genetic elements

The plasmid vectors, PV-STMT21 and PV-STMT22, are double border binary plant transformation vectors, which differ only in the plant cell selectable marker region. Both plasmid vectors contain well characterised DNA segments required for selection and replication of the plasmids in bacteria as well as the right and left borders delineating the region of DNA (T-DNA) which is transferred into the plant genomic DNA. This is the region into which the gene of interest, and the plant cell selectable marker, is inserted. DNA residing outside the T-DNA region does not normally get transferred into plant genomic DNA (Zambryski 1992).

The genetic elements are described in Table 2 below.

Table 2: Description of other genetic elements contained within PV-STMT21 and PV-STMT22

Genetic element	Source	Function
***aad* (resides outside the T-DNA)**	Gene coding for streptomycin adenyltransferase from transposon Tn7 in *Escherichia coli* (Fling *et al* 1985).	Confers resistance to the antibiotics spectinomycin and streptomycin.
LB	A 0.45 kb fragment of the octopine Ti plasmid pTi5955, which contains the 24 bp T-DNA left border (LB) region (Barker *et al* 1983).	Terminates the transfer of the T-DNA from *A. tumefaciens* to the plant genome.
***oriV* (resides outside the T-DNA region)**	A 1.3 kb origin of replication region derived from the broad-host range RK2 plasmid of *Agrobacterium* (Stalker *et al* 1981).	Allows plasmids to replicate in *A. tumefaciens*.
***ori-322/rop* region (resides outside the T-DNA region)**	A 1.8 kb segment of the plasmid pBR322 which contains the origin of replication region and the *bom* site for the conjugational transfer.	Allows for maintenance of plasmids in *E. coli* and their conjugal transfer into *A. tumefaciens* cells (Bolivar *et al* 1977, Sutcliffe 1978).
RB	A 0.36 kb fragment from the pTiT37 plasmid	The RB region is used to initiate T-DNA

containing the 24 bp nopaline-type T-DNA right border (RB) region. (Depicker *et al* 1982).	transfer from *A. tumefaciens* to the plant genome.

The *aad* gene is used as a marker to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells. Only those bacterial cells that have been transformed with the plasmid containing the *aad* gene, and hence the genes of interest (in this case the *cry3Aa* and *PLRVrep* genes) will grow. The *aad* gene is under the control of a bacterial promoter and would therefore not be expressed in transformed plant cells.

The host for all DNA cloning and vector construction was *E. coli* strain MV1190, a derivative of the common laboratory *E. coli* K-12 strain (Bachmann 1987).

Characterisation of the genes in the plant

Studies evaluated:

Rochester, D.E. and Lavrik, P.B. (1996). Molecular characterisation of potato leafroll virus and Colorado potato beetle resistant Russet Burbank potato lines transformed with plant vector PV-STMT21. Monsanto Study No. 96-01-37-20.

Rochester, D.E. and Lavrik, P.B. (1996). Molecular characterisation of potato leafroll virus and Colorado potato beetle resistant Russet Burbank potato lines transformed with plant vector PV-STMT22. Monsanto Study No. 96-01-37-22.

Reiser, S.E. *et al* (1999). Characterisation of T-DNA inserts present in New Leaf® Plus potato line number RBMT21-350 by Southern blot analysis. Monsanto Study No. 98-01-37-31.

Krohn, B.M. *et al* (1999). Characterisation of T-DNA inserts present in New Leaf® Plus potato line number RBMT22-082 by Southern blot analysis. Monsanto Study No. 98-01-37-32.

Rogan, G.J. *et al* (1999). Characterisation of T-DNA inserts present in New Leaf® Plus potato line number RBMT21-129 by Southern blot analysis. Monsanto Study No. 98-01-37-30.

Seven lines of transformed Russet Burbank potatoes were produced but only three lines have been commercialised as New Leaf® Plus potatoes. Two of the potato lines, RBMT21-129 and RBMT21-350 were transformed with plasmid PV-STMT21, which contains the *nptII* gene as a selectable marker and the third potato line, RBMT22-82, was transformed with plasmid PV-STMT22 which contains the *CP4 EPSPS* gene as the selectable marker.

The transferred genes in the New Leaf® potatoes were characterised using the polymerase chain reaction (PCR) technique (Saiki *et al* 1985). PCR is a method whereby a particular segment of DNA between two known sequences can be specifically amplified using a pair of complementary single stranded DNA primers. The PCR product can be visualised and analysed using agarose gel electrophoresis by comparison against appropriate molecular size markers and positive controls.

Genomic DNA was isolated from young leaf tissue from potato lines RBMT21-129, RBMT21-350 and RBMT22-82 and a control Russet Burbank line that were collected from a 1995 field trial in the United States. PCR was used to obtain the following information: (1) the identity of the genetic elements inserted into the genomic DNA of each of the potato lines; (2) the integrity of the inserted T-DNA; and (3) whether there had been any transfer of sequences residing outside of the T-DNA region of the plant transformation vectors (e.g. the *aad* gene and the *oriV* region).

PCR primer pairs were designed to detect the presence of each of the transferred genes by specifically amplifying their coding regions. Different sets of primers were also designed to ascertain whether the integrity of the linkage between the three transferred genetic elements had been maintained during the T-DNA transfer. Primers were also designed to detect the presence of the *aad* gene and the *oriV* region, both of which reside outside the T-DNA region. PCR products obtained from these reactions

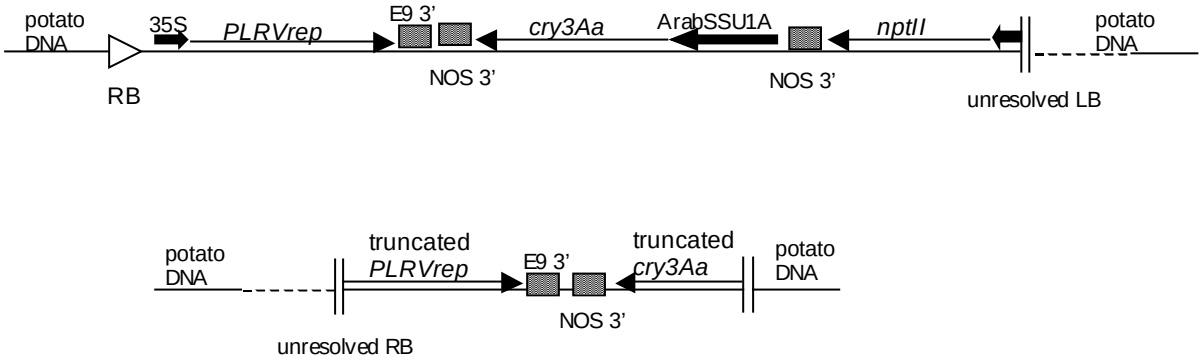
using genomic DNA from the transformed potatoes were compared to PCR products obtained from purified plasmid DNA.

The results of the PCR analysis confirm that potato lines RBMT21-129 and RBMT21-350 contain intact *PLRVrep, cry3Aa* and *nptII* genes and that line RBMT22-82 contains intact *PLRVrep*, *cry3Aa* and *CP4 EPSPS* genes. The PCR analysis did not detect the transfer of either the *aad* gene or the *oriV* region in any of the lines.

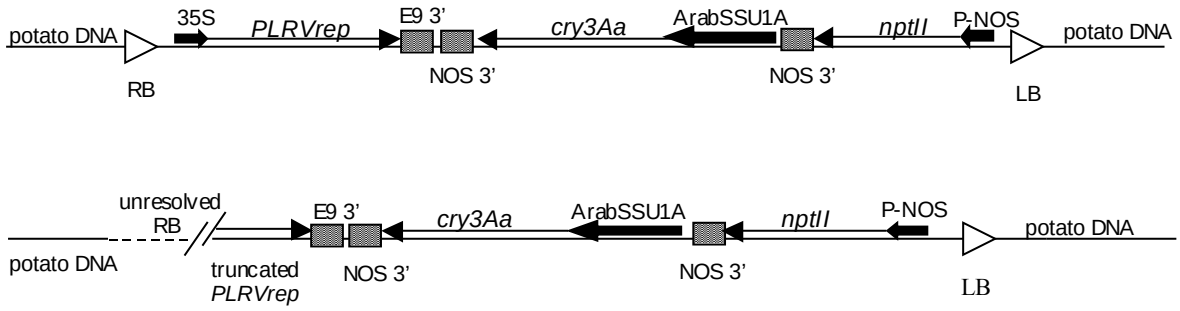
The applicant also used Southern blotting to characterise the T-DNA insertion into the potato lines and to confirm the results of the PCR. Southern blotting is a sensitive technique that enables the detection of specific sequences among DNA fragments separated using gel electrophoresis (Southern 1975). The overall pattern of the specific fragments detected can be used to characterise the nature of the T-DNA insertion into the genome (e.g. how many sites in the genome has the T-DNA have inserted into, whether the inserted copies are intact, etc).

The results of the Southern blotting indicate the following for each of the New Leaf® potato lines:

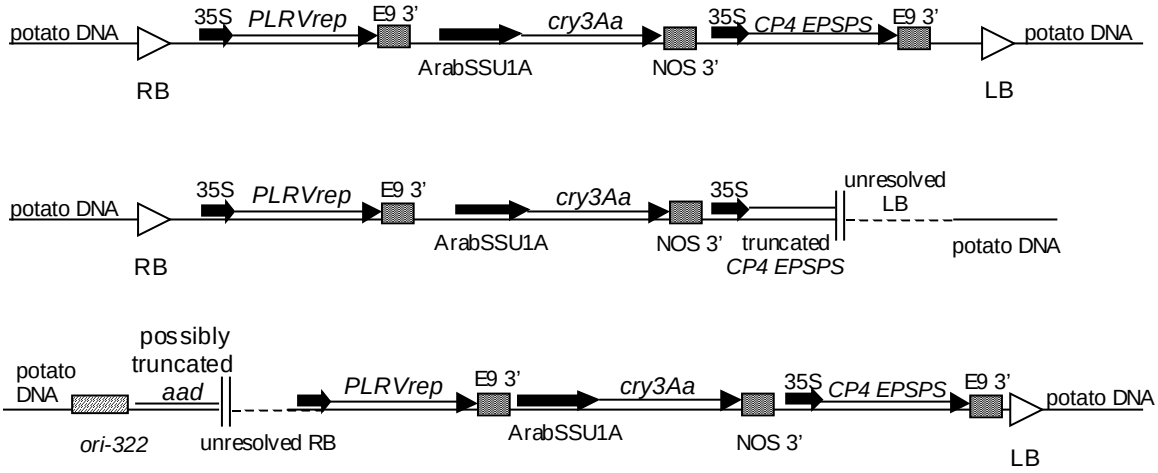
(i) line RBMT21-129 — insertion of the T-DNA occurred at two sites. One of the insertion events begins at the right border of the T-DNA and continues through the *PLRVrep* gene cassette, the *cry3Aa* gene cassette and the *nptII* coding region, terminating within the NOS promoter. This T-DNA insertion was not completely resolved at the left border resulting in the partial deletion of the 5' end of the NOS promoter used to express the *nptII* gene. The second insert consists of the *PLRVrep* gene and a partially deleted *cry3Aa* gene cassette. The ArabSSU1A promoter of the *cry3Aa* gene, as well as a portion of the 5' coding region of the *cry3Aa* gene, were deleted upon integration into the plant genome. The partial *cry3Aa* gene is still associated with its NOS 3' terminator region. Northern and western blot analysis was unable to detect any protein or mRNA from the truncated *cry3Aa* gene. This T-DNA insertion was not completely resolved at the right border, resulting in the deletion of the 35S promoter as well as a portion of the 5' end of the *PLRVrep* gene. Northern blot analysis confirmed that the partial *PLRVrep* gene does not give rise to any detectable mRNA. A map of the two insertion sites is given below:



(ii) line RBMT21-350 — insertion of the T-DNA occurred at two sites. At one site, intact copies of all three genes had been inserted. At the second site, a less than full-length copy of the T-DNA had been inserted resulting in a truncated copy of the *PLRVrep* gene, lacking the 35S promoter region. Northern blot analysis demonstrated that no detectable RNA transcript was produced from the truncated *PLRVrep* gene. The remaining two genes were intact. A map of the two insertion sites is given below:



(iii) line RBMT22-82 — insertion of the T-DNA occurred at three sites. All three copies of the T-DNA contain intact coding regions for the *PLRVrep* gene and the *cry3Aa* gene. Two copies of the T-DNA contain an intact coding region of the *CP4 EPSPS* gene. At one site, however, a less than full-length copy of the *CP4 EPSPS* gene had been inserted. Northern analysis was not able to detect any mRNA being produced from the truncated *CP4 EPSPS* gene suggesting that, as the promoter region for the truncated CP4 EPSPS gene is intact, mRNA does not accumulate to any detectable levels. For another T-DNA, DNA sequence beyond the RB was also inserted. This DNA is adjoined to the RB of the T-DNA and contains the *aad* gene and the *ori-322* region. This result conflicts with that of the PCR analyses, which were unable to detect the *aad* gene. The failure to detect the *aad* gene by PCR suggests that the gene is probably not intact. A map of the three insertion sites is given below:



Conclusion

The genetic elements transferred to each of the New Leaf® Plus lines are summarised in Table 3 below.

Table 3: Intact genetic elements present in the New Leaf® Plus potatoes

Plant line	*PLRVrep*	*cry3Aa*	*nptII*	*CP4 EPSPS*	*ori-322*	*aad*
RBMT21-129	√	√	√	-	-	-
RBMT21-350	√	√	√	-	-	-

RBMT22-82	√	√	-	√	√	√ (probably not intact)

Stability of the genetic changes

The New Leaf® Plus potatoes have been planted in field trials since 1994. The applicant reports that the potato lines, which are the subject of this application, were selected on the basis that both the PLRV and Colorado potato beetle protection traits were stable throughout multiple generations of vegetative propagation of potato for seed production.

Conclusion

The *cry3Aa* gene and the *PLRVrep* gene in the New Leaf® Plus potato lines appear to be stably integrated with both traits being stably maintained over multiple generations.

Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes to select transformed cells. It is generally accepted that there are no safety concerns with regard to the presence in the food of antibiotic resistance gene DNA *per se* (WHO 1993). There have been concerns expressed, however, that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of antibiotics.

This section of the report will therefore concentrate on evaluating the human health impact of the potential transfer of antibiotic resistance genes from the New Leaf® Plus potatoes to microorganisms present in the human digestive tract.

Two antibiotic resistant genes have been transferred to the New Leaf® Plus potato lines — the *nptII* gene and the *aad* gene. The transferred *aad* gene may not be intact. The *nptII* gene confers resistance to the aminoglycoside antibiotics, neomycin, kanamycin, and geneticin (G418) and the *aad* gene confers resistance to the antibiotics spectinomycin and streptomycin. These antibiotics only have very limited clinical use. Neomycin is not used orally because of its toxicity but is still used topically in certain circumstances (Davis *et al* 1980). Streptomycin has mostly been replaced by newer aminoglycosides, although it is still used for special indications, such as in the treatment of tuberculosis and brucellosis (Kärenlampi 1996) and spectinomycin is rarely used clinically.

Lines RMBT21-129 and RMBT21-350 contain the *nptII* gene, under the control of the NOS promoter, meaning it will be expressed in plant cells but not bacterial cells, and line RMBT22-82 contains a copy or partial copy of the *aad* gene, under the control of a bacterial promoter.

The first issue that must be considered in relation to the presence of the *nptII* and *aad* genes in the New Leaf® Plus potatoes is the probability that these gene would be successfully transferred to and expressed in microorganisms present in the human digestive tract. The following steps are necessary for this to occur:

1. a fragment of DNA, containing the coding region of each gene, would have to be released, probably as a linear fragment, from the DNA in the GM food;

2. the DNA fragment would then have to survive exposure to various nucleases excreted by the salivary glands, the pancreas and the intestine;

3. the DNA fragment would have to compete for uptake with dietary DNA and would have to be available at a time and place in which competent bacteria develop or reside;

4. the recipient bacteria would have to be competent for transformation;

5. the DNA fragment would have to be stably integrated into the bacterium, either as a self-replicating plasmid or through a rare recombination event with the bacterial chromosome;

6. the *nptII* and *aad* genes would have to be expressed, that is, would have to be integrated into the bacterial chromosome in close association with a promoter or would need to already be associated with a promoter that will function in the recipient bacterium;

7. the antibiotic resistance gene would have to be stably maintained by the bacterial population.

The transfer of either the *nptII* or *aad* genes to microorganisms in the human digestive tract is considered to be highly unlikely because of the number and complexity of the steps that would need to take place consecutively.

In the case of line RBMT22-82, there may be a slightly higher probability of horizontal gene transfer of the *aad* gene because of the transfer to the plant genome of a linked *Escherichia coli* origin of replication (*ori322*). Depending on the integrity of these components, the presence of these elements on the same DNA fragment could lead to the reconstitution of a plasmid capable of autonomous replication in *E. coli*. A plasmid is more likely to be successfully taken up than an isolated fragment of DNA. This however, would still be an extremely unlikely event.

The second and most important issue that must be considered is the potential impact on human health in the unlikely event successful transfer of a functional antibiotic resistance gene to microorganisms in the human digestive tract did occur.

In the case of transfer of the *nptII* gene and the *aad* gene, the human health impacts are considered to be negligible. In the case of *nptII*, this gene occurs naturally in bacteria inhabiting the human digestive tract therefore the additive effect of an *nptII* gene entering the human gastrointestinal flora from a genetically modified plant would be insignificant compared to the population of kanamycin resistant microorganisms naturally present. In the case of the *aad* gene, this gene is common and can be found at high frequencies in natural populations of bacteria as well as clinical isolates (Shaw *et al* 1993). Natural populations of streptomycin resistant bacteria are far more likely to be sources of transferred antibiotic resistance than ingested plant material.

Conclusion

It is extremely unlikely that the *nptII* or *aad* genes would transfer from the New Leaf® Plus potatoes to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively. In the highly unlikely event that the genes were transferred the human health impacts would be negligible because both antibiotic resistance genes are already commonly found in bacteria in the environment as well as inhabiting the human digestive tract and both antibiotics have very little, if any, clinical use in Australia and New Zealand.

CHARACTERISATION OF NOVEL PROTEIN

Biochemical function and phenotypic effects

Cry3Aa

Cry3Aa is a protein of 644 amino acids (molecular mass 73 kDa), which is produced by *B. thuringiensis* during sporulation and is encoded by the *cry3Aa* gene. The *cry3Aa* gene was isolated from *B. thuringiensis* subsp. *tenebrionis* (*B.t.t*) strain BI 256-82. In addition to the full length

Cry3Aa protein, *B.t.t* also produces a smaller form of the protein known as *B.t.t* band 3 (McPherson *et al* 1988). *B.t.t* band 3 has a molecular weight of 68 kDa (597 amino acids) and results from an internal translation initiation event within the same gene starting at an internal methionine codon at amino acid position 48. This protein has been shown to possess the same insecticidal activity and selectivity to Colorado potato beetle larvae as the full-length Cry3Aa.

The gene encoding *B.t.t* band 3 protein was engineered for plant expression by being completely re-synthesised to substitute the existing bacteria-preferred codons with plant-preferred codons (Perlak *et al* 1993). The genetic code is degenerate meaning that a given amino acid may be specified by more than one codon. For example, four different codons can be used to specify alanine. It has been found that plants often prefer different codons to bacteria to specify the same amino acid, and this can affect the expression levels of bacterial genes when they are transferred to plant cells. It has been shown that the plant expression of bacterial genes can be improved if the bacteria-preferred codons are substituted with plant-preferred codons (Perlak *et al* 1990). The re-synthesis of the gene encoding the band 3 protein, to substitute plant-preferred codons for bacteria-preferred codons, changed 399 out of 1791 nucleotides without altering the amino acid sequence. The re-synthesised *cry3Aa* gene therefore expresses a protein that is identical to that produced by *B. thuringiensis* subsp. *tenebrionis*.

PLRV replicase protein

The PLRV replicase protein has a molecular mass of 130 kDa and is encoded by two overlapping open reading frames, ORF 1 and ORF 2 (Miller *et al* 1995, Murphy *et al* 1995, Van der Wilk *et al* 1989). ORF 1 encodes a genome-linked protein (VPg) (Van der Wilk *et al* 1997) and motifs characteristic of serine-like proteases (Gorbalenya *et al* 1989, Bazan and Fletterick 1989). The VPg is covalently bound to the 5'end of the PLRV genome and is thought to be important in the initiation of viral replication. The VPg and the serine protease are produced in equimolar amounts. The serine protease is an endoprotease that is thought to be responsible for cleaving the ORF1/ORF2 gene product into its functional components. This would be required to release the VPg and make it available to bind to the viral genome. The serine protease is not packaged into the PLRV virion. ORF 2, which overlaps ORF1 is expressed through a –1 translational frameshift at a ratio of approximately 99:1 to ORF1 (Prüfer *et al* 1992). ORF2 encodes the putative RNA dependent RNA polymerase (Van der Wilk *et al* 1989, Mayo *et al* 1989, Habili and Symons 1989). The role of the replicase is to replicate the PLRV RNA genome. The replicase is not packaged into the PLRV virion.

Neomycin phosphotransferase II

Neomycin phosphotransferase II (NPTII) is an enzyme with a molecular mass of 29 kDa that catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics, including neomycin, kanamycin and gentamicin A and B, thereby inactivating the antibiotics (Davies *et al* 1986). The enzyme is encoded by the *nptII* gene, which is derived from transposon Tn5 from the bacterium *E. coli* (Beck *et al* 1982).

CP4 5-enolpyruvylshikimate-3-phosphate synthase

CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) is an enzyme with a molecular weight of 48 kDa and is part of the shikimate metabolic pathway for the biosynthesis of aromatic amino acids in plants, bacteria and fungi (Levin and Springson 1964). The aromatic amino acid pathway is not present in mammalian cells (Cole 1985). The CP4 EPSPS enzyme is targeted to the chloroplast using a chloroplast transit peptide sequence derived from the *Arabidopsis thaliana* EPSPS (CTP 2). The aromatic amino acid biosynthetic pathway and endogenous EPSPS activity is located in the chloroplast. *In vitro* chloroplast uptake assays have shown that the *A. thaliana* EPSPS CTP delivers CP4 EPSPS to the chloroplast and is subsequently cleaved from the pre–protein, yielding mature CP4 EPSPS with no CTP amino acids retained (della Ciopa *et al* 1986).

Protein expression analyses

Studies evaluated:

Lavrik, P.B. (1997). Registration of the plant pesticide potato leafroll virus (PLRV) *orf1*/*orf2* gene: product chemistry. Monsanto Study No. 97-094E.

Lavrik, P.B. and Grace, A.M. (1996). Expression levels of *B.t.t.*, NPTII and CP4 EPSPS proteins in tissues derived from Russet Burbank potato plants resistant to Colorado potato beetle and potato leafroll virus. Monsanto Study No. 96-01-37-21.

Reding, H.K. (1998). Safety of PLRV replicase in NewLeaf® Plus potatoes. Monsanto Company. EPA application No. 198176.

Efforts by the performing laboratory to develop a method to quantify the concentration of the PLRV replicase in either the New Leaf® Plus or PLRV-infected potatoes were not successful. This is despite the fact that an mRNA of the correct size (3.8 kb) could be detected, demonstrating that the transferred *PLRVrep* gene is transcribed *in vivo*. Therefore, there is no direct evidence that any replicase protein is produced in the New Leaf® potatoes.

The applicant, however, was able to provide indirect evidence for the expression of the replicase protein from a separate set of studies where a frameshift mutation was introduced into the *PLRVrep* gene and subsequently transferred to potatoes (Kaniewski *et al* 1995). The frameshift mutation would result in the translation of a non-functional protein. Transgenic plants containing the mutated *PLRVrep* gene are no longer resistant to infection by PLRV suggesting that protection against PLRV is protein-mediated. These data taken together with the RNA expression data suggest that the PLRV replicase is most likely expressed in the New Leaf® Plus potatoes but at a level or in a form which is unable to be detected using standard immunological detection methods. Because of the difficulties in developing a detection method for the protein, no limit of detection has been established for the assay.

It should be noted that the PLRV replicase could also not be detected in PLRV-infected plants (Van der Wilk *et al* 1997). It is speculated that the failure to detect the PLRV replicase in both virus infected and New Leaf® Plus potatoes expressing the *PLRVrep* gene is due to self-proteolysis mediated by a putative serine endoprotease domain contained within the protein.

The expression levels of the other three proteins, Cry3Aa, NPTII and CP4 EPSPS, in leaf and tuber tissues of transformed potato lines RBMT21-129, RBMT21-350, and RBMT22-82 were determined using enzyme linked immunosorbent assay (ELISA). ELISA is a technique that uses highly specific antibodies to identify proteins. The assay system is capable of quantifying proteins in crude tissue extracts.

Tissues were obtained from potato plants grown in field trials during 1995 at three locations in the Northwest region of the USA. A non-transformed Russet Burbank line was used as the control. At all locations, the field trials were arranged in eight replicate randomised complete block design. Leaf samples were collected approximately 10 weeks post planting. The first fully expanded compound leaf from each of four or six plants within each plot (replicate) were collected. Tuber samples were collected at harvest from each of the field sites. Ten tubers per plot from four of the eight plots were collected. All extracts were analysed in triplicate. The results are summarised in Table 4 below.

Table 4: Protein expression levels in tissue of Russet Burbank lines transformed with either PV-STM21 or PV-STM22 – multi site data.

		Protein Expression level (µg protein/g fresh weight)		
Plant line	**Tissue**	**Cry3Aa**	**NPTII**	**CP4 EPSPS**
RBMT21-129	Leaf (n=18)	12.81 (9.12-16.53)	below LOD[1]	Not determined
	Tuber (n=12)	0.35 (0.11-0.90)	"	
RBMT21-350	Leaf (n=19)	20.54 (7.71-35.66)	"	Not determined
	Tuber (n=11)	0.28 (0.12-0.61)	"	

RBMT22-82	Leaf (n=18)	20.97 (14.97-29.44)	Not determined	28.34 (17.36-35.66)
	Tuber (n=12)	0.63 (0.49-0.79)		0.53 (0.21-0.78)

[1] limit of detection. The limit of detection was 0.3 ng/g tissue.

Using total protein levels of 1.6 and 2.0% for leaf and tuber fresh weight, respectively, the above protein expression levels correspond to the following % total protein.

Table 5: Protein expression levels in tissue of Russet Burbank lines as a percentage of total protein

	Protein expression levels (% total protein)		
Tissue	**Cry3Aa**	**NPTII**	**CP4 EPSPS**
Leaf	0.05-0.2	<0.0001	0.1-0.2
Tuber	0.0006-0.005	<0.0001	0.001-0.004

Conclusion

The expression levels of all three novel proteins in potato tubers are low. Protein expression levels in tubers are significantly less than those in the corresponding leaf tissue.

Potential toxicity of novel protein

All three New Leaf® Plus potato lines express the Cry3Aa protein and the data suggests, although there is no direct evidence, that they may also express the PLRV replicase. In addition to these two proteins, lines RBMT21-129 and RBMT21-350 also express NPTII and line RBMT22-82 expresses CP4 EPSPS. This section of the report will therefore assess the potential toxicity of all four proteins.

Studies evaluated[#]:

Lavrik, P.B. and Grace, A.M. (1997). Assessment of the equivalence of *B.t.t.*, NPTII and CP4 EPSPS proteins produced in *E. coli* and in Russet Burbank potato plant lines resistant to potato leafroll virus and Colorado potato beetle. Monsanto Study No. 96-01-37-26.

Berberich, S.A. *et al* (1993). Preparation and verification of dose for a mouse acute oral toxicity study with neomycin phosphotransferase II protein (NPTII). Monsanto Study ML-91-409.

Reding, H.K. (1998). Safety of PLRV replicase in NewLeaf® Plus potatoes. Monsanto Company. EPA application No. 198176.

Naumovich, L. (1994) The infection of the Solanum tuberosum, Russet Burbank potato, by PVX, PVY, and PLRV viruses during the cultivation of the tuber. A biology study presented to the Monsanto/St Louis Post-Dispatch Greater St Louis Science Fair.

[#]Refer also to the evaluations for Roundup Ready soybeans (CP4 EPSPS) and New Leaf® potatoes (Cry3Aa, NPTII)

Cry3Aa

Cry3Aa is insecticidal only to Coleopteran insects (MacIntosh *et al* 1990) and its specificity of action is directly attributable to the presence of specific receptors in the target insects (Wolfersberger 1990, Ferré *et al* 1991). There are no receptors for the δ-endotoxins of *B. thuringiensis*, including Cry3Aa, on the surface of mammalian intestinal cells (Hubert *et al* 1995), therefore, humans, as well as other mammals, are not susceptible to this protein.

The potential toxicity of Cry3Aa was assessed for New Leaf® potatoes under Application A382 where acute oral toxicity studies in mice were submitted for evaluation. These studies are also relevant to this application as the gene construct for the *cry3Aa* gene used in the New Leaf® Plus potatoes will give rise to an identical protein to that produced in the New Leaf® potatoes. Acute animal toxicity tests are used since – if toxic – proteins are known to act via acute mechanisms and laboratory animals have been shown to exhibit acute toxic effects from exposure to proteins known to be toxic to humans (Sjoblad *et al* 1992). For a detailed summary of the acute toxicity study, refer to the safety evaluation for New Leaf® potatoes. A brief summary of the findings is presented below.

The Cry3Aa protein used in the toxicity study was produced in *E. coli* because the plant lines did not express enough protein for purification of large quantities for toxicity testing. Data was presented to indicate that the bacterially produced Cry3Aa is equivalent to the plant produced Cry3Aa in terms of its molecular mass, N-terminal amino acid sequence, lack of glycosylation, and biological activity. Therefore, the *E. coli* produced Cry3Aa is considered to be a suitable substitute for plant produced Cry3Aa.

The Cry3Aa protein was administered by gavage to CD-1 mice at doses up to 5220 mg/kg body weight, the mice being observed for a period of seven days. No abnormal clinical signs were observed in the mice during the study that could be attributed to the treatment. No significant differences were observed in body weight, cumulative body weight or food consumption. Several minor pathologic changes were observed at necropsy but these were randomly distributed among all groups and could not be attributed to the treatment. On the basis of these findings the Cry3Aa protein was considered to be non-toxic.

PLRV replicase protein

The applicant was unable to successfully develop a method for the detection of the PLRV replicase in the New Leaf® Plus potatoes. Other evidence (discussed in Section 5.2 above), however, suggests that the replicase protein is expressed. In the light of only equivocal evidence for non-expression of the PLRV replicase, it is necessary to also consider the potential toxicity of the PLRV replicase.

The applicant did not submit any data from acute oral toxicity studies with the PLRV replicase protein. Instead, the applicant has provided the following arguments and evidence for the safety of the PLRV replicase protein.

History of safe consumption

PLRV must produce its replicase protein to be able to replicate in potatoes. Although it has not been possible to detect viral replicase in PLRV-infected potatoes, it logically follows that the replicase must be present in plants where the virus can be detected. PLRV infection of potatoes has been known to occur since the early twentieth century (Slack 1993). In some potato crops in the United States, the incidence of PLRV infection routinely approaches 100% if insecticides to control the aphid vector of the virus are not used (Thomas 1997). In a survey undertaken on behalf of the applicant, between 7-14% of fresh potatoes in supermarkets in the United States were found be infected with PLRV. Therefore, it can be said that there is a long history of human exposure to the PLRV replicase protein through the consumption of PLRV-infected potatoes, without any reported adverse effects.

PLRV replicase levels in New Leaf® Plus potatoes

The applicant provided evidence which suggests that the level of replicase protein expressed in the New Leaf® Plus potatoes is likely to be lower than what would be expressed in PLRV-infected potatoes. This comes from two sources of data. Firstly, for a number of plant viruses it appears that the level of certain viral proteins produced in a virus infected plant is significantly greater than if a 35S promoter in a transgenic plant expressed the same viral gene. Secondly, the New Leaf® Plus potatoes have been determined to produce <0.05ng *PLRVrep* mRNA/mg total RNA compared to the 3.4ng viral RNA/mg total RNA produced by PLRV-infected plants. In this instance the viral RNA can be regarded as equivalent to mRNA as the PLRV replicase is translated directly from viral RNA. Assuming that the same translation rates apply, it can be inferred from this data that the New Leaf® Plus potatoes will contain substantially lower amounts of PLRV replicase than that present in PLRV-infected potatoes.

Similarity with known toxins

The amino acid sequence of the PLRV replicase was compared to a database of 2625 known toxins. No significant amino acid similarity was observed.

Conclusion

There is a long history of safe human exposure to the PLRV replicase protein through the consumption of PLRV-infected potatoes. In addition, the evidence suggests that exposure to the PLRV replicase protein from the consumption of the New Leaf® Plus potatoes is likely to be lower than that from consumption of PLRV-infected potatoes. Furthermore, there is no significant similarity between the PLRV replicase and any known protein toxins. On the basis of this evidence, animal studies of the toxicity of the PLRV replicase protein are considered to be unnecessary and it is concluded that the PLRV replicase protein, as expressed in the New Leaf® Plus potatoes, is non-toxic to humans.

Neomycin phosphotransferase II

The potential toxicity of NPTII was assessed for New Leaf® potatoes where acute oral toxicity studies in mice were submitted for evaluation. These studies are also relevant to this evaluation as the gene construct for the *nptII* gene used in the New Leaf® Plus potatoes will give rise to an identical protein to that produced in the New Leaf® potatoes. For a detailed summary of the toxicity study, refer to the safety evaluation for the New Leaf® potatoes. A brief summary of the findings is presented below.

The NPTII protein used in the study was produced from *E. coli* because the plant lines did not express enough protein for purification of large quantities for toxicity testing. Data was presented to show that the *E. coli* produced NPTII is equivalent to the plant produced NPTII in terms of its molecular mass, N-terminal amino acid sequence, lack of glycosylation, and biological activity. The *E. coli* produced NPTII is therefore considered to represent a suitable substitute for plant produced NPTII.

The NPTII protein was administered by gavage to mice at doses up to 5000 mg/kg body weight for a period of 8-9 days. There were no statistically significant differences in mean body weights or cumulative body weight gain in any of the treated groups. No abnormal clinical signs were noted, there were no unscheduled deaths and there were no differences in mean terminal body weights. No gross lesions were observed at necropsy. On the basis of these findings NPTII was considered to have low oral toxicity.

CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)

The *CP4 EPSPS* gene construct used for the production of the New Leaf® Plus potato lines encodes a protein that is identical to the CP4 EPSPS protein produced in Roundup Ready soybeans. The potential toxicity of CP4 EPSPS was previously assessed for Roundup Ready soybeans where it was concluded to be of low toxic potential.

For Roundup Ready soybeans, the potential toxicity of CP4 EPSPS was assessed by three methods — firstly, through an acute oral toxicity study in mice, secondly through comparison of its amino acid sequence with the amino acid sequences of known toxins and lastly by its similarity with other EPSPS proteins which have a safe history of human consumption. The findings of these evaluations are summarised briefly below.

Acute oral toxicity study

The CP4 EPSPS protein, lacking the chloroplast transit peptide, was administered by gavage to mice at doses up to 572 mg/kg body weight. No adverse effects were observed at these dose levels.

Comparison with known toxins

CP4 EPSPS was compared to the amino acid sequence of 1935 known protein toxins. No meaningful similarity was found other than would be expected given that certain functional domains are generally conserved between proteins.

Similarity to other EPSPS proteins

All crop, fungal and microbial food sources such as Baker's yeast (*Saccharomyces cerevisiae*) or *Bacillus subtilis* contain EPSPS proteins (Kishore *et al* 1988) and, as such, EPSPS proteins have a long history of safe human consumption. The CP4 EPSPS protein shares significant amino acid sequence homology with other EPSPS proteins therefore it is unlikely to produce any toxic effects in humans.

Conclusion

As the CP4 EPSPS protein expressed in New Leaf® Plus potato line RBMT22-82 is identical to the EPSPS protein expressed in Roundup Ready soybeans, the conclusions of that safety evaluation are relevant here. No additional data or evidence has emerged which would necessitate revising the conclusion of the previous study. Furthermore, the CP4 EPSPS protein has itself now been consumed for at least five years in Roundup Ready soybeans without any reports of adverse effects. Therefore, it can be concluded that the CP4 EPSPS protein is non-toxic to humans.

Potential allergenicity of novel protein

The concerns regarding potential allergenicity of novel proteins are two fold. Firstly, there are concerns that the ability to express new or different proteins in food will result in the transfer of allergens from one food to another, thereby causing some individuals to develop allergic reactions to food they have not previously been allergic to. Secondly, there are concerns that the transfer of novel proteins to food will lead to the development of new allergies in certain individuals. The former is more easily addressed than the latter because if an allergen is already known it is possible, using human sera or human skin tests, to test if it has been transferred. There are no reliable tests or animal models, however, which enable the prediction of the allergenic potential of novel proteins. Instead, potential allergenicity can only be indicated by examination of a number of characteristics of the novel protein, such as whether it is derived from a known allergenic source, its physical/chemical characteristics (most allergens have a molecular mass of between 10 and 70 kDa, are glycosylated and are resistant to acid and protease degradation), whether it has any sequence similarity to any known allergens, and whether it is likely to be present in large amounts in the food as consumed and therefore have potential for allergic sensitisation.

There are four new proteins expressed in the New Leaf® Plus potatoes – Cry3Aa, PLRV replicase, NPTII, and CP4 EPSPS. The potential allergenicity of Cry3Aa and NPTII was considered for New Leaf® potatoes, and the potential allergenicity of the CP4 EPSPS protein was considered for Roundup Ready soybeans. The findings of those assessments are briefly summarised.

Cry3Aa, NPTII and CP4 EPSPS

For Cry3Aa, NPTII and CP4 EPSPS it was concluded that each protein is within the size range of known allergens, however, none are glycosylated and all are rapidly degraded in the proteolytic and acid conditions of simulated gastric fluid suggesting they would not survive mammalian digestion. None of the proteins have any significant similarity to known allergens, nor are they present in large amounts in potato tubers. On the basis of this data and on the basis that humans have a prior history of exposure to these proteins with no recorded instances of allergenicity, it was concluded that Cry3Aa, NPTII and CP4 EPSPS are unlikely to be allergenic to humans. No additional data or evidence has emerged which would necessitate revising this conclusion.

PLRV replicase

The potential allergenicity of the PLRV replicase protein has not previously been considered.

Source of the protein

The PLRV replicase is encoded by the *PLRVrep* gene, which was derived from a PLRV isolate obtained from a naturally infected potato in the United States (Kaniewski *et al* 1995). The PLRV replicase protein in the New Leaf® Plus potatoes is therefore identical to that which occurs in PLRV infected potatoes. PLRV infection of potatoes is a common occurrence (see Section 5.3). Infected potatoes are still suitable for human consumption. PLRV replicates in the vascular tissues of the tuber (Weidemann and Casper 1992) therefore both the virus and its expressed proteins, including the replicase, are routinely consumed. Despite the widespread consumption of PLRV-infected tubers, there have been no reports of allergic reactions to any of the viral proteins.

Physical and chemical characteristics

The PLRV replicase protein has a molecular weight of 130 kDa, which is outside the size range of known allergens (10-70 kDa). However, it is speculated that the replicase protein undergoes post-translation processing, therefore the processed products could well fall within the size range of known allergens. The PLRV replicase, however, is not glycosylated as it is expressed in the plant cell cytoplasm. For glycosylation of the PLRV replicase to occur, the protein would need to be transported through the endoplasmic reticulum and Golgi bodies (Taiz and Zeiger 1991). This requires the presence of specific targeting sequences on the protein and none of these were included in the *PLRVrep* gene construct.

Similarity to known allergens

The amino acid sequence of the PLRV replicase protein was compared to a database of 265 known allergen and gliadin sequences. In agreement with current understanding of allergen epitope structure (O'Hehir *et al* 1991), a significant sequence similarity was defined as a sequence identity of greater than seven contiguous amino acids. No significant sequence identity with any of the 265 allergens was found.

Presence of the protein in food as consumed

One of the factors contributing to the allergenicity of certain proteins is their high concentration in foods that elicit an allergenic response (Taylor *et al* 1987, Taylor 1992, Taylor *et al* 1992). This is true for milk (Baldo, 1984, Taylor *et al* 1987), soybean (Shibasaki *et al* 1980, Burks *et al* 1988, Pendersen and Djurtoft 1989) and peanuts (Barnett *et al* 1983, Sachs *et al* 1981, Barnett and Howden 1986, Kemp *et al* 1985).

The PLRV replicase protein, in contrast, cannot be detected in tubers of New Leaf® Plus potatoes. There is only equivocal evidence that the protein is expressed at all.

Conclusion

It has previously been concluded that Cry3Aa, NPTII and CP4 EPSPS are unlikely to be allergenic to humans. No additional data or evidence has emerged which would necessitate revising this conclusion.

The PLRV replicase protein in the New Leaf® Plus potatoes has no significant similarity to any known allergens, nor is it present in large quantities in potato tubers. On the basis of this evidence and the fact that humans have a long history of exposure to this protein, through the consumption of PLRV-infected potatoes, with no recorded instances of allergenicity, it can be concluded that the PLRV replicase protein is unlikely to be allergenic to humans.

COMPARATIVE ANALYSES

There are concerns that genetic modification will affect the overall nutritional composition of a food, or cause unintended changes that could adversely affect the safety of the product. Therefore a safety assessment of food produced from genetically modified plants must include analysis of the composition of the food, based on a comparison with other commercial varieties of the crop. Generally, comparisons are made not only with the parental line but also with other non-transformed lines. If the parameter for the transformed line is within the normal range for non-transformed lines, this is considered acceptable (Hammond and Fuchs 1998).

Key nutrients

Studies evaluated:

Lavrik, P.B. (1995). Compositional analysis of potato tubers derived from Colorado potato beetle and potato leafroll virus resistant (NewLeaf®) Russet Burbank potato plants grown under field conditions. Monsanto Study No. 96-01-37-23.

Rogan, G. J. *et al*. (1999). Composition analysis of potato tubers from NewLeaf® Y and NewLeaf® Plus potato plants grown under field conditions. Monsanto Study No. 98-01-37-27.

In undertaking a compositional analysis of potatoes there are a number of key defining nutrients and constituents that should be measured as part of that analysis. They are total tuber solids (measured as tuber dry matter), sugars, protein and vitamin C. Tuber solids are an important quality factor for processing and are also the single most important determinant of culinary appeal (Murphy *et al* 1967). Approximately 75% of the dry matter content of potatoes consists of starch. The remainder is composed of sugars, protein, and assorted cell and cell wall components (Storey and Davies, 1992). The major sugars in potatoes are sucrose as well as the reducing sugars fructose and glucose. They are present in small quantities and are inconsequential as a source of energy. However, like total solids, they are a very important factor in processed food quality. Potatoes also contain measurable amounts of proteins, fats, carbohydrates, and numerous vitamins and minerals. However, they are only a significant dietary source for two of these constituents – protein and vitamin C (Storey and Davies 1992, Pennington and Wilkening 1997). Potato proteins are highly digestible, have a fairly good balance of amino acids and are especially high in the essential amino acid lysine. Measurement of total protein is considered more informative than measurement of individual amino acids as nearly all of the proteins in potato tubers (albumin, globulin, glutelin, and prolamin) have a similar amino acid composition, therefore, changes in their respective ratios will have little impact on the amino acid profile (Storey and Davies, 1992).

The applicant undertook two separate field studies of the New Leaf® Plus potatoes. The first study was conducted in 1995 and 1996 at three locations in the United States and three locations in Canada. At each location, eight to fifteen replicated plots were grown per line. Tubers were collected at harvest from four replicated plots and analysed for total solids, dextrose, sucrose, vitamin C, soluble protein, and proximate composition (total protein, fat, crude fibre, ash, total carbohydrates and

calories). The controls were tubers isolated from non-transformed Russet Burbank potato lines grown at the same field location. The field trials were carried out in a randomised complete block design consisting of eight to fifteen replicated plots per line. This study was limited to tubers collected from four replicates per site.

The second study was conducted in 1998 at three locations in the United States. Four replicated plots were grown at one of the sites, whereas plants were grown in non-replicated plots at the other two sites. Compositional analyses were done of amino acid, vitamin B_6, niacin, copper, magnesium, and potassium content.

Major constituents

Summaries of the results of proximate and other major constituent analyses are presented in Table 6 and 7 below.

Table 6: Proximate and major constituent content[1] of New Leaf® Plus potatoes – Canadian data

	New Leaf® Plus lines				
Constituent	**RBMT21-129**	**RBMT21-350**	**RBMT22-82**	**RB Control**	**Lit. range**
Total solids (% fw)	21.1 (20.4-22.4)	21.6 (20.2-23.7)	21.0 (20.2-22.5)	21.7 (19.9-23.5)	16.8-24.5
Sugars (%fw):					
Dextrose	0.24 (0.05-0.35)	0.21 (0.05-0.38)	0.24 (0.08-0.37)	0.24 (0.05-0.40)	0.04-0.52
Sucrose	0.14 (0.10-0.20)	0.14 (0.11-0.18)	0.14 (0.11-0.16)	0.16 (0.12-0.22)	0.1-0.88
Soluble protein (% dw)	4.6 (4.1-5.4)	4.6 (4.0-5.2)	4.5 (3.9-5.1)	4.4 (3.9-4.7)	3.4-7.3
Proximate[2]:					
Moisture	1.39 (1.03-1.42)	1.43 (1.02-1.68)	1.20 (1.01-1.44)	1.48 (1.03-2.71)	-
Total protein	9.86 (7.16-12.75)	9.94 (7.6-13.4)	9.93 (8.20-11.98)	9.90 (7.81-12.59)	7.1-14.6
Fat	0.20 (0.13-0.32)	0.19 (0.10-0.30)	0.20 (0.13-0.27)	0.16 (0.11-0.23)	0.1-0.8
Ash	4.68 (4.21-4.85)	4.70 (4.08-5.72)	4.78 (3.95-5.69)	4.75 (3.93-5.43)	2.2-9.5
Crude fibre	1.64 (1.15-1.78)	1.55 (1.12-1.78)	1.61 (1.24-1.79)	1.68 (1.39-2.15)	0.2-3.5
Carbohydrates	85.2 (81.2-86.5)	85.2 (82.5-86.6)	85.1 (81.7-85.7)	85.2 (80.74-86.84)	84.5 (avg)
Calories	378 (376-379)	377 (372-380)	377 (374-381)	376 (370-380)	350 (avg)

[1] mean values, range in brackets (n=12 except for RBMT21-129 where n=8)
[2] except for moisture and calories, reported values are in g/100 g dry weight. Moisture is reported in g/100 g of lyophilised tuber powder. Calories are reported in calories/100 g dry weight.

Table 7: Major constituents[#] of New Leaf® Plus potatoes – USA data

	New Leaf® Plus lines				
Constituent	**129**	**350**	**82**	**RB Control**	**Lit. range**
Total solids (% FW)	21.6 (20.3-23.0)	21.9 (20.6-24.1)	21.0 (19.4-22.1)	21.5 (20.5-22.5)	16.8-24.5
Sugars (%FW):					
Dextrose	0.09 (0.04-0.12)	0.09 (0.06-0.16)	0.11 (0.05-0.16)	0.10 (0.07-0.13)	0.04-0.52
Sucrose	0.18 (0.14-0.23)	0.20 (0.16-0.25)	0.18 (0.13-0.23)	0.20 (0.14-0.26)	0.1-0.88
Soluble protein (% DW)	5.0 (4.4-5.8)	5.1 (4.6-5.5)	5.0 (4.6-5.8)	5.0 (4.5-5.6)	3.4-7.3

[#] mean values, range in brackets (n=12, except for RBMT21-350 where n=11)

There were no statistically significant differences between New Leaf® Plus potatoes and the control lines for any of the major constituents and the levels reported were all comparable to the normal ranges for Russet Burbank cultivars.

Amino acid content

The concentration of 18 out of a total of 20 amino acids was measured for the New Leaf® Plus potato lines. The two amino acids not analysed were asparagine and glutamine. The data obtained on the amino acid composition of the New Leaf® Plus potato lines is summarised in Table 8 below.

Table 8: Amino acid content[#] of New Leaf® Plus Russet Burbank potato lines

	New Leaf® Plus lines				
Amino acid	**RBMT21-129**	**RBMT21-350**	**RBMT22-82**	**Control**	**Lit. range**
	(mg/200 g tuber fresh weight)				
Aspartic acid	1280 (812-1822)	1096 (681-1419)	1296 (815-1982)	1250 (728-1630)	677-1476
Threonine	147 (116-193)	134 (106-162)	152 (115-211)	147 (119-173)	102-214
Serine	151 (109-197)	147 (121-175)	159 (121-215)	155 (124-185)	125-255
Glutamic acid	771 (550-1017)	701 (491-858)	779 (588-1115)	793 (516-1055)	583-1207
Proline	124 (92-160)	111 (78-141)	129 (84-186)	119 (88-160)	89-366
Glycine	122 (101-148)	112 (96-127)	126 (102-178)	121 (107-143)	92-195
Alanine	118 (96-147)	112 (92-133)	124 (101-171)	117 (99-135)	87-238
Cysteine	62 (56-67)	59 (55-63)	64 (56-78)	62 (57-70)	48-93
Valine	217 (171-248)	199 (162-245)	237 (192-374)	218 (175-284)	196-363
Methionine	59 (48-66)	52 (42-62)	62 (48-100)	56 (41-84)	57-100
Isoleucine	140 (109-162)	123 (100-151)	148 (117-230)	139 (117-178)	119-238
Leucine	224 (173-286)	200 (156-240)	229 (169-323)	220 (176-263)	171-346
Tyrosine	128 (97-162)	129 (111-150)	149 (122-209)	144 (117-178)	114-236
Phenylalanine	167 (134-203)	156 (130-184)	180 (141-268)	168 (133-208)	138-272
Histidine	78 (64-95)	72 (57-83)	84 (67-123)	82 (66-100)	33-117
Lysine	235 (194-284)	211 (175-241)	245 (198-360)	233 (193-291)	154-342
Arginine	196 (147-253)	180 (131-219)	215 (170-320)	200 (145-254)	175-362
Tryptophan	43 (38-46)	39 (36-42)	45 (37-64)	42 (34-54)	29-70

[#] mean values, range in brackets (n=6).

The levels reported for amino acids in the New Leaf® Plus potatoes are comparable to the control values as well as the literature reported ranges.

Vitamin and mineral content

Data obtained on the vitamin and mineral composition of the New Leaf® Plus potato lines is summarised in Table 9 below.

Table 9: Mean vitamin and mineral content[#] of New Leaf® Plus potato lines

	New Leaf® Plus lines				
Vitamin/ mineral[1]	**RBMT21-129**	**RBMT21-350**	**RBMT22-82**	**RB Control**	**Lit. range**
Vitamins:					
Vitamin C (Can)	16.7 (14.2-17.9)	16.8 (14.3-20.2)	16.8 (15.3-19.3)	16.4 (14.2-18.6)	10.3-22.0
Vitamin C (USA)	10.1 (8.2-13.0)	9.9 (8.2-11.3)	10.4 (7.6-12.8)	10.0 (8.8-12.6)	"
Vitamin B6	0.51 (0.32-0.58)	0.56 (0.31-0.57)	0.52 (0.30-0.78)	0.52 (0.45-0.56)	0.26-0.82
Niacin	4.03 (2.81-5.10)	3.99 (3.20-4.44)	4.28 (2.67-5.11)	4.06 (3.49-4.60)	0.18-6.2
Minerals:					
Copper	0.39 (0.14-0.61)	0.30 (.14-0.50)	0.33 (0.11-0.64)	0.32 (0.14-0.50)	0.03-1.4
Magnesium	52.2 (48.5-56.6)	45.7 (43.0-50.8)	46.9 (38.5-67.8)	61.5 (47.1-66.1)	22.5-110
Potassium	1072.6 (955.9-1185.6)	1026.6 (966.7-1120.2)	1038.2 (931.2-1512-6)	1080.7 (979.2-1202.7)	700-1250

[1] Except for vitamin C content, values are reported as mg/200 g fresh weight. Vitamin C content is reported as mg/100 g fresh weight.
mean values, range in brackets (n=6, except for vitamin C content where the number of samples corresponds to those for tables 5 and 6)

There were no statistically significant differences between New Leaf® Plus potatoes and the control line for any of vitamins or minerals analysed and the levels reported were comparable to the literature reported ranges for Russet Burbank cultivars.

Conclusion

Based on the data submitted in the present application, The New Leaf® Plus potato lines are compositionally equivalent to other commercial Russet Burbank potato varieties.

Key toxicants

Studies evaluated:

Lavrik, P.B. *et al* (1997). Compositional analysis of potato tubers derived from Colorado potato beetle and potato leafroll virus resistant (New Leaf® Plus) Russet Burbank potato plants grown under field conditions. Monsanto Study No. 96-01-37-23.

Wild tuberous *Solanum* species contain high concentrations of the toxic glycoalkaloids, which are very bitter in taste. The presence of glycoalkaloids in *Solanum* species is generally believed to be a natural plant defense mechanism against pests and diseases (Conner 1995). Modern potato cultivars accumulate high glycoalkaloid concentrations in green shoot tissue and in tubers upon exposure to light. In some cultivars, significant concentrations of glycoalkaloids can also accumulate in tubers not exposed to light. The variation in glycoalkaloid content of tubers can be attributed to both genetic effects and the environmental conditions under which the plants are grown and stored following harvest (van Gelder 1990). The concentration of glycoalkaloids in potato tubers in advanced lines of modern breeding programs is usually routinely monitored (Morris and Lee 1984).

The New Leaf® Plus potato lines were grown in six locations across the USA during 1995 and 1996. Tubers were collected at harvest from four replicated plots from each site and analysed for glycoalkaloid levels. The controls were tubers isolated from non-transformed Russet Burbank potato lines grown at the same field location. The field trials were carried out in a randomised complete block design consisting of eight to fifteen replicated plots per line. This study was limited to tubers collected from four replicates per site. A summary of the results is presented in Table 10 below.

Table 10: Total glycoalkaloid content[#] of tubers from New Leaf® Plus and control lines.

Potato line	Total glycoalkaloids (mg/100 g fresh weight)
RBMT21-129	5.4 (2.1-8.9)
RBMT21-350	4.8 (1.7-16.3)
RBMT22-82	5.1 (2.8-17.5)
Russet Burbank control	4.3 (2.6-25.0)
Literature range (Russet Burbank)	3.1-16.1

[#] mean values, range in brackets (n=24, except for RBMT21-129 where n=20)

The data presented in Table 6 demonstrates that the New Leaf® Plus potato lines have total glycoalkaloid levels which are slightly elevated compared to those of the non-transformed Russet Burbank control line. These differences, however, are not statistically significant. The range of levels for glycoalkaloids reported for both the New Leaf® Plus and control lines varied widely but are generally comparable to the literature reported range for Russet Burbank cultivars. There are no safety concerns regarding the slightly elevated glycoalkaloid levels in the New Leaf® Plus lines.

Key anti-nutrients

The only known anti-nutrient present in potato is trypsin inhibitor. Trypsin inhibitors are classed as anti-nutrients because they interfere with the digestion of proteins leading to decreased animal growth. Trypsin inhibitors are heat labile and are destroyed during the cooking process or during processing when heat treatment is applied.

As heating inactivates trypsin inhibitor, its presence is only an issue when raw potatoes are consumed. Humans rarely consume raw potatoes due to the indigestibility of the ungelatinised starch.

Naturally-occuring allergenic proteins

Potatoes are not generally regarded as major sources of food allergy, although patatin, the main storage protein of potatoes, has recently been reported to induce an allergic reaction in some individuals (Seppälä *et al*., 1999). The clinical importance of patatin as a food allergen has yet to be confirmed.

As potatoes are not classified as major sources of food allergy, and there have yet to be any confirmed potato allergens described, an assessment of the naturally-occurring allergenic proteins of New Leaf® Plus potatoes is unnecessary.

NUTRITIONAL IMPACT

Animal feeding studies

In assessing the safety of food produced using gene technology, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences together with an extensive compositional analysis of the food. Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further re-assurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

The compositional and other data presented in the application are considered adequate for establishing the ability of New Leaf® Plus potatoes to support typical growth and well-being. Additional studies, including animal feeding studies, are therefore not required.

Estimation of dietary intake of novel proteins

If the concentration of a substance in a food is known and data is available on the human consumption of that food then it is possible to estimate the dietary intake of that substance for the population. In safety assessments, dietary intakes are usually only estimated in circumstances where a substance is considered to be hazardous. In this way it is possible to determine the likely human exposure to the hazard and thus ascertain whether there is cause for concern.

None of the novel proteins in the New Leaf® Plus potatoes are considered to be hazardous therefore a dietary exposure assessment is unnecessary for determining if there is cause for concern. However, such information can provide additional assurance that exposure to the novel protein is low and/or that the novel protein is likely to be present in the diet at levels well below those found to be safe in animal toxicity studies.

The concentration of Cry3Aa and CP4 EPSPS in the New Leaf® Plus potatoes is known but the concentration of the NPTII and PLRV replicase proteins was unable to be quantified, therefore it is possible to only estimate the dietary intake for Cry3Aa and CP4 EPSPS.

Cry3Aa is expressed in the New Leaf® Plus potato tubers at levels ranging from 0.11 to 0.9 µg protein/g fresh weight and CP4 EPSPS is expressed at levels ranging from 0.21 to 0.78 µg protein/g fresh weight (see Table 4, Section 5.2).

Australian and New Zealand consumption data is available for potato crisps, instant mashed potato, and potato fries, although no data is currently available for potato flour and potato starch. The consumption data is presented in Table 11 below.

Table 11: Estimated consumption of processed potato products in Australia and New Zealand.

	All respondents (g/day)	Consumers only (g/day)

Food	Country	mean	mean	median	95th percentile
Potato crisps	Aus	2.8	38.8	25	100
	NZ	2.9	48.4	40	150
Instant mashed	Aus	-	-	-	-
potato	NZ	0.007	34.6	34.6	34.6
Potato fries,	Aus	16.6	132.5	113	264
commercial	NZ	18.6	141.2	142	300
Total potato	Aus	19.4	-	-	-
products	NZ	21.5	118	112.2	300

For calculation of the dietary intake of the novel proteins, the highest potato consumption figure (300 g/day) and the highest protein concentration was used. This represents a 'worst case' estimate and also makes allowances for the lack of consumption data for potato flour and potato starch.

To do the calculation, assumptions about the proportion of processed potato products derived from the New Leaf® Plus potatoes must be made. Data on market penetration of the New Leaf® Plus potatoes is not available. In the absence of information about market penetration, two estimates are made — one using a very worst case estimate where it is assumed that all potato products are derived entirely from New Leaf® Plus potatoes and the other, probably more realistic estimate, where it is assumed that 10% of potato products are derived from New Leaf® Plus potatoes. The two estimates of dietary intake for Cry3Aa and CP4 EPSPS are presented in Table 12 below.

Table 12: Estimate of dietary intake of Cry3Aa and CP4 EPSPS

Novel protein	Estimated dietary intake 100 % market penetration		10 % market penetration	
	µg /day	µg/kg BW/day[1]	µg /day	µg/kg BW/day
Cry3Aa (0.11-0.9µg/g FW)	33-270	0.51-4.2	3.3-27.0	0.05-0.42
CP4 EPSPS (0.21-0.78 µg/g FW)	63-234	0.97-3.6	6.3-23.4	0.096-0.36

[1] assuming a body weight of 65 kg.

For Cry3Aa, the very worst-case estimate is at least 1.2 million times less than the dose found to have no adverse effects in mice (5220 mg Cry3Aa/kg BW, administered as two doses in a single day). For CP4 EPSPS, the estimate is at least 150 000 times less than the dose found to have no adverse effects in mice (572 mg CP4 EPSPS/kg BW, administered as a single dose). Therefore, even if all processed potato products were to be derived from the New Leaf® Plus potatoes, a very large margin of safety exists for both proteins.

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