FOOD DERIVED FROM INSECT AND POTATO VIRUS Y-PROTECTED (NEW LEAF® Y) POTATO LINES RBMT15-101, SEMT15-02 AND SEMT15-15

A Safety Assessment

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SUMMARY

Food derived from GM potato lines RBMT15-101, SEMT15-02 and SEMT15-15 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 and Shepody potatoes (RBMT15-101, SEMT15-02 and SEMT15-15) were protected against Colorado potato beetle (CPB) and potato virus Y (PVY) 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 coat protein gene (*PVYcp*) from PVY. The insect and virus-protected potatoes are known as New Leaf® Y 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 *PVYcp* gene is responsible for the production of the PVY coat protein. The coat protein forms a protective coat around the RNA genome of the virus and comprises 95 % by mass of the virus particle. It has been found that plants can be protected from viral infection through the expression of one of a number of viral genes, including the coat protein gene, in the plant. The exact mechanism by which the viral protection occurs is unknown.

Other genes transferred to the New Leaf® Y potatoes were the *nptII* gene and the *aad* gene (in the Shepody lines only). The *nptII* gene is 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 *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® Y 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® Y 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® Y potatoes express three novel proteins — Cry3Aa, the PVY coat protein and NPTII. The Cry3Aa protein is expressed in tubers at levels ranging from 0.08 to 0.38 μg/g fresh weight (equivalent to 0.0005 to 0.0019 % of total tuber protein). The PVY coat protein was unable to be detected in the plants, indicating that if expressed it is at levels less than 1 μg/g fresh weight (equivalent to < 0.005% total tuber protein). NPTII is expressed in the tuber at levels ranging from 0.003 to 0.01 μg/g fresh weight (equivalent to < 0.001% of the total tuber protein).

Acute oral toxicity testing had been done previously in mice for the Cry3Aa and NPTII proteins where it was concluded that both proteins are non-toxic to humans. No additional evidence has emerged that would alter this conclusion. Dietary intake assessments indicate that exposure to both proteins from the consumption of New Leaf® Y potatoes will be low.

The potential toxicity of the PVY coat protein had not previously been considered. Human beings have a long history of exposure to the PVY coat protein through the consumption of PVY-infected plants. In addition, the data indicates that expression levels of the PVY coat protein are likely to be much lower in New Leaf® Y potatoes than in PVY-infected potatoes. Therefore, human populations consuming New Leaf® Y potatoes will most likely have lower exposure levels to the PVY coat protein than they would through the consumption of PVY-infected potatoes. Overall, it was concluded that the PVY coat protein, as expressed in the New Leaf® Y potatoes, is non-toxic to humans.

In terms of the potential allergenicity of the three novel proteins, it has previously been concluded that both Cry3Aa and NPTII are unlikely to be allergenic to humans. No additional data or evidence has emerged which would necessitate revising this conclusion. Despite the long history of human consumption of PVY-infected potatoes there are no recorded instances of allergenicity therefore it can be concluded that the PVY coat protein is unlikely to be allergenic to humans.

Comparative analyses

Detailed compositional analyses were done to establish the nutritional adequacy of the New Leaf® Y 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, 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 commercial potato varieties. On the basis of this information it was concluded that the New Leaf® Y potatoes are similar to other commercial potato varieties in terms of these key constituents.

The levels of naturally occurring toxins in New Leaf® Y potatoes were also assessed. The only naturally occurring toxins in potatoes are the glycoalkaloids. The glycoalkaloid levels in the New Leaf® Y potatoes are equivalent to those of the non-transformed control lines and are within the literature reported ranges for commercial potato varieties.

Conclusion

Based on the currently available data, food from New Leaf® Y potato lines RBMT15-101, SEMT15-02 and SEMT15-15 is as safe and nutritious as food from other commercially available potato cultivars.

FOOD DERIVED FROM INSECT AND POTATO VIRUS Y PROTECTED (NEW LEAF® Y) POTATO LINES RBMT15-101, SEMT15-02 and SEMT15-15:

A SAFETY ASSESSMENT

BACKGROUND

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

PVY is an RNA virus belonging to the potyvirus group of plant viruses. The virus is aphid transmissible and commonly infects potatoes, causing serious disease. Protection against PVY is produced through expression, in the plant, of a gene derived from PVY that encodes the viral coat protein. The coat protein forms a protective coat around the RNA genome of the virus. The expression of plant virus 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 this protection is conferred is unknown.

New Leaf® Y 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 are most likely to 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 and Shepody potatoes were transformed with the plasmid PV-STMT15 using *Agrobacterium*-mediated transformation of potato stem sections.

Function and regulation of novel genes

Agrobacterium-mediated transformation of potatoes with plasmid PV-STMT15 resulted in the transfer of three gene expression cassettes — *cry3Aa*, *PVYcp* and *nptII*. Each of these expression cassettes is described in Table 1 below.

Table 1: Description of the gene expression cassettes in PV-STMT15

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' non-translated 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.
PVYcp	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.
	PVYcp	Coding region of the coat protein gene derived from PVY strain O, a naturally occurring strain of PVY (Lawson *et al* 1990).	The coat protein forms a protective coat around the RNA genome of the virus. When expressed in plants it can confer protection against infection by PVY.
	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	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' non-translated region of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens*	Contains signals for termination of transcription and directs polyadenylation.

(Depicker *et al* 1982, Bevan *et al* 1983).

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 PVYcp gene

The *PVYcp* gene was isolated from potatoes infected with PVY strain O, a naturally occurring strain of PVY. The gene is identical to the coat protein gene present in PVY (Lawson *et al* 1990).

The strategy of expressing viral genes in plants to protect 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 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). It is derived from the bacterial transposon Tn5 isolated from *Escherichia coli* (Beck *et al* 1982). 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 *PVYcp* genes, enabling those plant cells successfully transformed with the *cry3Aa* and *PVYcp* genes to grow in the presence of kanamycin. Those cells who lack the *nptII* gene, and hence the *cry3Aa* and *PVYcp* genes, will not grow and divide in the presence of kanamycin.

Other genetic elements

The plasmid vector PV-STMT15 is a double border binary plant transformation vector. It contains 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 *aad* gene lies outside the T-DNA. The genetic elements are described in Table 2 below.

Table 2: Description of other genetic elements contained within PV-STMT15

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 containing the 24 bp nopaline-type T-DNA right border (RB) region. (Depicker *et al* 1982).	The RB region is used to initiate T-DNA 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 *Escherichia coli* strain MV1190, a derivative of the common laboratory *E. coli* K-12 strain (Bachmann 1987).

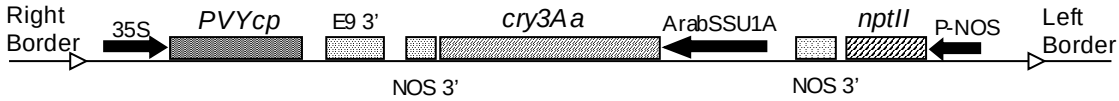
Characterisation of the genes in the plant

Studies evaluated:

Rogan, G.J. *et al* (1998). Characterization of T-DNA inserts present in New Leaf® Y potato line Nos RBMT15-101 and SEMT15-15 by Southern blot analysis. Monsanto Study No. 98-01-37-26.

Rogan, G.J. *et al* (1999). Characterization of T-DNA inserts present in New Leaf® Y potato line No. SEMT15-02 by Southern blot analysis. Monsanto Study No. 98-01-37-29.

Seven lines of transformed Russet Burbank and Shepody potatoes were produced but only three have been commercialised as New Leaf® Y potatoes. All lines were transformed with PV-STMT15 containing the *nptII* gene as a selectable marker. A map of the T-DNA in PV-STMV15 is given below.



The transferred genes in the New Leaf® Y potatoes were characterised using the technique of Southern blotting (Southern 1975). Southern blotting is a sensitive technique enabling the detection of specific sequences among DNA fragments that have been separated using gel electrophoresis. 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 loci in the genome has the T-DNA have inserted into, whether the inserted copies are intact, etc).

Genomic DNA was isolated from Russet Burbank and Shepody control plants and from the New Leaf® Y lines RBMT15-101, SEMT15-15 and SEMT15-02. Southern analysis was used to estimate the number of integration sites and evaluate the integrity of the inserted genes i.e., to determine if there had been any detectable deletions, insertions or rearrangements of the T-DNA. The results of the Southern analyses indicate the following:

(i) line RBMT15-101 — insertion of the T-DNA occurred at three to four loci. At least one locus contains two copies of the T-DNA organised in inverted orientations. For two copies of the T-DNA, transfer was incomplete at the right border resulting in an incomplete copy of the FMV 35S promoter associated with the *PVYcp* gene. One of the cry3Aa genes also lacks the *Arabidopsis* small subunit promoter and a portion of the 5' end of the gene. The NOS terminator region of this gene cassette is intact. One of the T-DNAs also has an incomplete NOS promoter region associated with an intact *nptII* coding region. The coding regions of all the other genetic elements are intact. The analyses also showed that no plasmid sequences beyond the left and right borders were transferred;

(ii) line SEMT15-02 — insertion of the T-DNA occurred at four to five loci. At least one locus contains two copies of the T-DNA organised in inverted orientations and one locus contains two T-DNAs linked by a complete copy of the plasmid backbone. For seven copies of the T-DNA, transfer of the T-DNA resulted in incomplete resolution of the right border leaving incomplete copies of the FMV promoter associated with the *PVYcp* coding region. One of the T-DNAs in this line has an incomplete NOS promoter region associated with an intact *nptII* coding region. One of the *nptII* genes has a truncation within the coding region. All full length and less than full-length copies of the *nptII* gene are associated with NOS terminators. The coding regions of all other genetic elements are intact. Plasmid sequences beyond the left and right borders, which include the *aad* gene and the *oriV* and *ori322* plasmid elements, were inserted into this line. Integration of complete backbone elements occurred in two different ways: at one locus two T-DNAs are linked by a complete copy of the backbone; at two other loci, backbone integration is not associated with the left border flanking the NOS promoter of the *nptII* gene; and

(iii) line SEMT15-15 — insertion of the T-DNA occurred at four to five loci. At least one locus contains copies of the T-DNA organised in inverted orientations. For two copies of the T-DNA, transfer of the T-DNA resulted in incomplete resolution of the right border leaving incomplete copies of the FMV promoter associated with the *PVYcp* coding region. One of the T-DNAs in this line has an incomplete NOS promoter region associated with an intact *nptII* coding region. The coding regions of all the genetic elements are intact. Plasmid sequences

beyond the left and right borders, which include the *aad* gene and the *oriV* and *ori322* plasmid elements, were inserted into this line.

Conclusion

The following intact genetic elements have been transferred to New Leaf® Y potato lines.

Table 3: Intact genetic elements transferred to the New Leaf® Y potatoes

Line	*PVYcp*	*nptII*	*cry3Aa*	*aad*	*oriV*	*ori322*
RBMT15-101	√	√	√			
SEMT15-02	√	√	√	√	√	√
SEMT15-15	√	√	√	√	√	√

Stability of genetic changes

The New Leaf® Y potatoes have been planted in field trials since 1994. Extensive testing was conducted over a five-year period to select the most efficacious lines for commercialisation. Selection was on the basis of continued high-level expression of the Cry3Aa protein to control Colorado potato beetle as well as continued protection against PVY. Lines RBMT15-101, SEMT15-02 and SEMT15-15 have therefore been selected for commercialisation on the basis that they have continued to display protection against Colorado potato beetle and PVY over a five-year period. It can therefore be concluded that both genes are stably integrated into the genome and maintained and expressed throughout multiple generations of vegetative propagation.

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® Y potatoes to microorganisms present in the human digestive tract.

In the New Leaf® Y potato lines, two antibiotic resistant genes have been transferred — the *nptII* gene and the *aad* gene. 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.

All three lines contain the *nptII* gene, under the control of the NOS promoter, meaning it will be expressed in plant cells. Lines SEMT15-02 and SEMT15-15 also contain a copy of the *aad* gene under the control of a bacterial promoter therefore it will not be expressed in plant cells.

The first issue that must be considered in relation to the presence of the *nptII* and *aad* genes in the New Leaf® Y 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 therefore 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 lines SEMT15-02 and SEMT15-15, there may be a slightly higher probability of horizontal gene transfer of the *aad* and *nptII* genes 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® Y 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 the amino acid 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*.

PVY coat protein

The PVY coat protein has a molecular mass of 32 kDa (Lawson *et al* 1990) and is used by the virus to encapsidate and protect its RNA genome. This is achieved by the aggregation of the coat protein monomers around the viral RNA. The PVY virion is composed of 95% coat protein and 5% nucleic acid (RNA) by mass (Lindbo and Dougherty 1994). The PVY coat protein is encoded by the *PVYcp* gene which was derived from PVY strain O, a naturally occuring isolate of PVY. The *PVYcp* gene introduced into the New Leaf® Y potatoes is identical to the native viral gene therefore the coat protein produced will be identical to the native viral coat protein.

Neomycin phosphotransferase II

Neomycin phosphotransferase II (NPTII) is an enzyme with a molecular weight of 29 kDa and catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics, thereby inactivating them (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).

Protein expression analyses

Studies evaluated:

Rogan, G.J. *et al* (1997). Expression levels of *B.t.t.* and NPTII proteins in tissues derived from Russet Burbank, Shepody and Hi-Lite potato plants resistant to Colorado potato beetle and potato virus Y. Monsanto Study No. 97-01-37-01.

Seitsinger, H. (1998). Determination of the relative amounts of coat protein mRNA in genetically modified NewLeaf Y potato varieties compared with coat protein mRNA from non-modified potato varieties naturally infected by PVY. Monsanto Study No. 98-01-37-21.

Bookout, J. *et al* (1998). Expression levels of potato virus Y coat protein in genetically modified and potato virus Y infected potato plants. Monsanto Study No. 98-01-37-10.

Rogan, G. *et al* (1999). Expression levels of potato virus Y coat protein in genetically modified New Leaf® Y potato tubers. Monsanto Study No. 98-01-37-28.

Cry3Aa and NPTII expression

The New Leaf® Y and non-transformed Russet Burbank and Shepody control plant lines were grown in field trials during 1995 and 1996 at several locations in the United States. Leaf samples were collected at approximately six to ten weeks post-planting and tuber samples were collected at harvest. The field trials were performed using randomised complete block design with four to ten replicates per line. Samples were obtained from at least four plots from each site for estimation of the protein expression levels. Expression levels were estimated using an enzyme-linked immunosorbent assay (ELISA). Background levels are common with ELISAs hence some of the controls will occasionally demonstrate detectable levels of novel protein. The results are summarised in Table 4 below.

Table 4: Expression levels for Cry3Aa and NPTII in the New Leaf® Y and control potato lines

Line	Cry3Aa Mean (µg/g FW)	Cry3Aa Range (µg/g FW)	Cry3Aa % total protein[1]	NPTII Mean (µg/g FW)	NPTII Range (µg/g FW)	NPTII % total protein[1]
RBMT15-101:						
Leaf	20.444	9.26-33.26	0.058-0.21	0.004	0.003-0.006	<0.0001
Tuber	0.246	0.13-0.38	0.0007-0.0019	0.005	0.004-0.006	< 0.0001
RB control:						
Leaf	0.154	0.00-2.94	-	Not detected	-	-
Tuber	0.060	0.04-0.09	-	Not detected	-	-
SEMT15-02:						
Leaf	22.51	8.66-35.20	0.054-0.22	0.009	0.007-0.010	<0.0001
Tuber	0.194	0.13-0.28	0.0007-0.0014	0.009	0.008-0.010	<0.0001
SEMT15-15:						
Leaf	28.486	5.96-47.35	0.037-0.296	0.003	0.003-0.005	<0.0001
Tuber	0.126	0.08-0.18	0.0005-0.0011	0.004	0.003-0.005	<0.0001
Shepody control						
Leaf	0.066	0.00-1.90	-	Not detected	-	-
Tuber	0.038	0.01-0.09	-	Not detected	-	-

[1] using total protein levels of 1.6 and 2.0% for leaf and tuber, respectively

PVY coat protein expression

Leaf and tuber samples from New Leaf® Y and control plants grown at various locations in the United States and Canada in 1995 and 1996 were analysed in two separate studies. In the first study, only the leaf samples were analysed for PVY coat protein expression using a western blot procedure. For comparison, PVY coat protein expression was also estimated in PVY-infected non-transformed Russet Burbank and Shepody potatoes using a commercially available ELISA. In the second study, only the tuber samples were analysed using a western blot procedure. Concentrations of purified virion (made up of 95 % coat protein) ranging from 1 – 10 ng were used in both studies as the reference standard.

In the first study using Western blot analysis, the detection limit for PVY coat protein derived from purified virion was estimated to be approximately 2 μg/g tissue fresh weight (2 ppm). Using this method, PVY coat protein in leaves of PVY-infected Russet Burbank potatoes was detected easily, its concentration estimated to be approximately 5 – 10 μg/g tissue fresh weight. This compares to an estimate of 25 μg/g tissue fresh weight obtained using a commercial ELISA kit. In contrast, PVY coat protein could not be detected by western blot of leaf tissue from any of the New Leaf® Y potato lines. ELISA was not used for the New Leaf® Y lines because previous attempts to use an ELISA assay had been unsuccessful.

In the second study, the detection limit for PVY coat protein derived from purified virion was 1 ng which equates to 1 μg/g tissue fresh weight. Approximately 1 mg of tuber tissue was assayed from New Leaf® Y and parental controls. Virion-derived PVY coat protein was easily detected at the lowest level of virion assayed in the western blot (1 ng). In tuber samples from the New Leaf® Y lines, however, no coat protein could be detected. It can be concluded that the level of expression of PVY coat protein in the New Leaf® Y lines is
< 1 μg/g tissue fresh weight.

Leaf tissue from the New Leaf® Y potato lines was also analysed using Northern blot analysis to quantify the levels of *PVYcp* mRNA produced in the plants. Northern blotting is a sensitive technique similar to Southern blotting except it is used for detecting specific RNA transcripts. Leaf tissue from PVY-infected non-transformed potatoes was also similarly analysed. *In vitro* synthesised *PVYcp* RNA transcript, at concentrations ranging from 0.25 to 50 pg was used as the reference standard. Messenger RNA was detected in leaf tissue from all three New Leaf® Y potato lines. The mRNA levels were 2.6, 2.7 and 2.7 pg/μg total RNA for lines RBMT15-101, SEMT15-02, and SEMT15-15, respectively. This compares to mean virion RNA levels of 16 and 23 pg/μg total RNA for PVY-infected Russet Burbank and Shepody potatoes, respectively.

Conclusion

Cry3Aa expression levels in the tuber (the edible portion of the plant) are low and range from 0.08 to 0.38 μg/g fresh weight or 0.0005 to 0.001% of the total protein. The NPTII expression levels are even lower, and were consistently measured to be <0.0001% of the total protein. PVY coat protein could not be detected in any of the New Leaf® Y lines, although the *PVYcp* mRNA is readily detected indicating that the transferred gene is transcribed. If it is assumed that the *PVYcp* mRNA is translated, but at levels which are below the current limit of detection, it can be concluded that the level of PVY coat protein in the New Leaf® Y tubers is less than 1 μg/g tissue fresh weight or < 0.005% total protein. This level is well below the level of coat protein found in PVY-infected potato plants.

Potential toxicity of novel protein

All three New Leaf® Y potato lines express the Cry3Aa protein and the data suggests, although there is no direct evidence, that they may also express the PVY coat protein. In addition to these two proteins, all three lines also express the NPTII protein. This section of the report will therefore assess the potential toxicity of these three proteins.

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 previously assessed for New Leaf® potatoes 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® Y 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 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 demonstrate 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 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 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 to humans.

PVY coat protein

Studies evaluated:
Naumovich, L. and Kaniewski, W. (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.

Evidence from Northern analyses demonstrates that the *PVYcp* gene is transcribed to give rise to mRNA. Expression studies using Western blot analysis, however, are unable to detect the presence of the PVY coat protein in the New Leaf® Y potatoes. In the absence of any evidence to show that coat protein is not produced it must be assumed that coat protein is expressed in the New Leaf® Y potatoes but at levels which are below the level of current detection methods.

PVY is a common pathogen of potatoes. PVY infection of potatoes is controlled today through the use of seed certification programs. Despite these seed certification programs, data from a recent survey of potato tubers grown in the United States and available in grocery stores indicates that between 19 and 38% of potatoes are infected with PVY. This indicates that even today humans are continually exposed to PVY coat protein through the consumption of PVY-infected potatoes. There have been no reports of any adverse health effects resulting from this exposure.

The coat protein gene transferred to the New Leaf® Y potatoes was derived from an isolate of PVY obtained from a naturally infected potato in the United States. Therefore, the PVY coat protein expressed in the New Leaf® Y potatoes identical to that which is present in PVY-infected potatoes. As there is a history of safe human exposure to PVY coat protein and the protein expressed in the New Leaf® Y potatoes is identical to that which is found in naturally infected potatoes, toxicity testing of the protein in animals is considered unnecessary.

PVY coat protein is detectable in PVY-infected potato plants at levels up to at least 10µg/g tissue fresh weight whereas the level of coat protein expression in the New Leaf® Y potatoes is below the limit of detection (<1 µg/g tissue fresh weight). Therefore exposure to PVY coat protein from the consumption of New Leaf® Y potatoes is likely to be much less than from the consumption of PVY-infected potatoes.

Conclusion

There is a long history of safe human exposure to the PVY coat protein through the consumption of PVY-infected potatoes. In addition, the evidence indicates that exposure to the PVY coat protein from the consumption of New Leaf® Y potatoes is likely to be much lower than from the consumption of PVY-infected potatoes. As there is a long history of safe human exposure to the PVY coat protein without any reported adverse health effects it can be concluded that the PVY coat protein, as expressed in the New Leaf® Y potatoes, is non-toxic to humans.

Neomycin phosphotransferase II

The potential toxicity of NPTII was previously assessed for New Leaf® potatoes 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 *nptII* gene used in the New Leaf® Y 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 be 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 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 potentially three new proteins expressed in the New Leaf® Y potatoes – Cry3Aa, PVY coat protein, and NPTII. The potential allergenicity of Cry3Aa and NPTII was previously considered for the New Leaf® potatoes. The findings of those assessments are briefly summarised below.

Cry3Aa and NPTII

For Cry3Aa and NPTII it was concluded that both proteins are within the size range of known allergens, however, neither of the proteins is glycosylated and both 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 and NPTII are unlikely to be allergenic to humans. No additional data or evidence has emerged which would necessitate revising this conclusion.

PVY coat protein

The potential allergenicity of the PVY coat protein has not previously been considered. The same considerations that apply to the toxicity of the PVY coat protein also apply to a consideration of its allergenicity. The consumption of PVY-infected tubers appears to be quite widespread among the human population. Despite this widespread consumption there have been no reports of any adverse health effects, including allergenicity, which can be attributed to the presence of the virus, including its coat protein, which accounts for 95% by mass of the virus particle. The PVY coat protein is expressed in the New Leaf® Y potatoes at very low levels and therefore is even less likely than coat protein expressed in PVY-infected potatoes to have potential for allergic sensitisation. On the basis of this information, it can be concluded that the PVY coat protein, as expressed in the New Leaf® Y potatoes, 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 transgenic 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. 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. *et al* (1997). Compositional analyses of potato tubers derived from cvs. Russet Burbank, Shepody and Hi-Lite potato plants resistant to Colorado potato beetle and potato virus Y. Monsanto Study No. 96-01-37-24.

Rogan, G.J. *et al* (1999). Composition analysis of potato tubers from New Leaf® Y and New Leaf® 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).

Two separate field studies of the New Leaf® Y potatoes were conducted. The first study was conducted in 1995 and 1996 at three locations in the United States and two locations in Canada. At each location, eight to fifteen replicated plots were grown per line. Compositional analyses were done of total solids, dextrose, sucrose, vitamin C, soluble protein, and proximate composition (total protein, fat, crude fibre, ash, total carbohydrates and calories). 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, and vitamin and mineral content.

Key potato constituents

Summaries of the results of proximate and other major constituent analyses are presented in Tables 5 and 6.

Table 5: Mean levels of major constituents in New Leaf® Y Russet Burbank potatoes

Constituent	RBMT15-101	RB control	Literature range
Total solids (% FW)	20.7 (0.81)	20.7 (0.81)	16.8-24.5
Sugars (% FW):			
Dextrose	0.24#	0.21	0.04-0.52
Sucrose	0.18	0.18	0.10-0.8
Soluble protein (% DW)	5.1#	5.4	3.4-7.3
Proximate[1]:			
Moisture	1.21#	2.26	-
Total protein	11.75	12.30	7.1-14.6
Fat	0.19	0.21	0.1-0.8
Ash	5.81	6.04	2.2-9.5
Crude fibre	1.69	1.66	0.2-3.5
Total carbohydrate	82.25	81.44	84.5 (average)
Calories	377.7	376.9	350 (average)

[1] 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.
[#] underlined values are significantly different from the control at the 5% level ($p<0.05$)

Table 6: Mean levels of major constituents in New Leaf® Y Shepody potatoes

Constituent	SEMT15-02	SEMT15-15	Shepody control	Literature range[2]
Total solids (% FW)	22.3	22.6	22.7	16.8-26.8
Sugars (% FW):				
Dextrose	0.22	0.21#	0.23	0.03-0.52
Sucrose	0.28	0.31	0.29	0.05-0.88
Soluble protein (% DW)	6.6#	6.4	6.3	3.3-7.3
Proximate[1]:				
Moisture	1.52	1.56	1.54	-
Total protein	11.43	10.76	11.03	7.1-14.6
Fat	0.17	0.19#	0.14	0.1-0.8
Ash	4.63	4.64	4.69	2.2-9.5
Crude fibre	1.33	1.42	1.53	0.2-3.5
Total carbohydrate	83.77	84.4	84.1	84.5 (average)
Calories	382.3	382.4	381.9	350 (average)

[1] 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.
[2] Literature ranges are not available for Shepody potatoes therefore the values reported are for Russet Burbank, Atlantic, Gemchip and Norchip varieties, combined.
[#] underlined values are significantly different from the control at the 5% level ($p<0.05$)

In line RBMT15-101, dextrose content is slightly elevated compared to the control, whereas soluble protein and moisture content are slightly decreased compared to the control. These differences are minor and have no biological or nutritional significance therefore they do not represent a cause for concern. The values reported are also well within the literature reported ranges for the Russet Burbank cultivar. All other values reported for major constituents of the New Leaf® Y Russet Burbank line are equivalent to those of the control.

In Shepody line SEMT15-02, soluble protein content is slightly elevated compared to the control. For line SEMT15-15, dextrose content is slightly decreased compared to the control, whereas fat content is slightly elevaterd compared to the control. Once again, the differences reported are not large and do not have any biological or nutritional significance. The values reported are also well within the literature reported ranges for common commercial varieties of potatoes. All other values reported for major constituents of the New Leaf® Y Shepody lines are equivalent to those of the control.

Amino acid content

The concentration of 18 out of a total of 20 amino acids was measured for the New Leaf® Y potato lines. The two amino acids not analysed were asparagine and glutamine. The data obtained on the amino acid composition of the New Leaf® Y potato lines are summarised in Tables 7 and 8.

Table 7: Mean levels (range) of amino acids in New Leaf® Y Russet Burbank line

Amino acid	RBMT15-101	RB control	Literature range
	(mg/200 g tuber fresh weight)		
Aspartic acid	1194 (1020-1346)	1250 (728-1630)	677-1476
Threonine	138 (125-148)	147 (119-173)	102-214
Serine	145 (136-157)	155 (124-185)	125-255
Glutamic acid	751 (644-826)	793 (516-1055)	583-1207
Proline	117 (97-134)	119 (88-160)	89-366
Glycine	114 (106-117)	121 (107-143)	92-195
Alanine	107 (102-114)	117 (99-135)	87-238
Cysteine	59 (54-65)	62 (57-70)	48-93
Valine	208 (193-225)	218 (175-284)	196-363
Methionine	54 (50-58)	56 (41-84)	57-100
Isoleucine	131 (119-141)	139 (117-178)	119-238
Leucine	207 (183-220)	220 (176-263)	171-346
Tyrosine	121 (100-134)	144 (117-178)	114-236
Phenylalanine	158 (144-167)	168 (133-208)	138-272
Histidine	79 (67-89)	82 (66-100)	33-117
Lysine	227 (203-243)	233 (193-291)	154-342
Arginine	187 (170-194)	200 (145-254)	175-362
Tryptophan	43 (36-52)	42 (34-54)	29-70

Table 8: Mean levels (range) of amino acids in New Leaf® Y Shepody lines

Amino acid	SEMT15-02	SEMT15-15	Shepody Control	Literature range
	(mg/200 g tuber fresh weight)			
Aspartic acid	919 (615-1152)	994 (702-1404)	1002 (671-1325)	677-1476
Threonine	186 (142-221)	202 (158-279)	183 (139-226)	102-214
Serine	191 (148-232)	202 (149-287)	188 (141-230)	125-255
Glutamic acid	866 (663-1073)	977 (857-1174)	966 (773-1181)	583-1207
Proline	172 (127-232)	181 (130-272)	165 (119-202)	89-366
Glycine	166 (133-197)	179 (148-250)	162 (133-185)	92-195
Alanine	149 (118-172)	163 (134-220)	146 (119-172)	87-238
Cysteine	79 (72-90)	84 (76-109)	76 (67-87)	48-93
Valine	219 (187-272)	249 (223-346)	226 (201-248)	196-363
Methionine	75 (63-85)	84 (72-106)	72 (55-84)	57-100
Isoleucine	160 (130-208)	184 (160-259)	164 (137-187)	119-238
Leucine	304 (227-363)	332 (252-461)	292 (214-359)	171-346
Tyrosine	146 (128-171)	171 (152-228)	151 (137-161)	114-236
Phenylalanine	200 (162-240)	226 (193-315)	202 (165-228)	138-272
Histidine	85 (73-94)	94 (82-128)	87 (76-97)	33-117
Lysine	277 (231-318)	304 (266-410)	275 (226-315)	154-342
Arginine	220 (174-259)	251 (201-340)	242 (172-314)	175-362

Tryptophan	43 (39-49)	46 (36-67)	43 (35-49)	29-70

The values reported for amino acids are all comparable to the literature reported ranges.

Vitamin and mineral content

Data obtained for the vitamin and mineral composition of the New Leaf® Y potato lines are summarised in Tables 9 and 10.

Table 9: Mean levels (range) of vitamins and minerals in New Leaf® Y Russet Burbank line

Constituent	RBMT15-101	RB control	Literature range[1]
	(mg/200 g fresh weight, except for vitamin C which is reported as mg/100 g fresh weight)		
Vitamin C	14.5[#] (NA)	13.4 (NA)	10.3-22.0
Vitamin B6	0.52 (0.46-0.54)	0.52 (0.45-0.56)	0.26-0.82
Niacin	4.11 (3.34-4.46)	4.06 (3.49-4.60)	0.18-6.2
Copper	0.30 (0.11-0.42)	0.32 (0.14-0.50)	0.03-1.4
Magnesium	49.8 (48.0-52.3)	51.5 (47.1-66.1)	22.5-110
Potassium	996.6 (826.5-1151.9)	1080.7 (979.2-1202-7)	700-1250

[1] The values reported are for Russet Burbank, Atlantic, Gemchip and Norchip varieties, combined.
[#] underlined values are significantly different from the control at the 5% level ($p<0.05$)

Table 10: Mean levels (range) of vitamins and minerals in New Leaf® Y Shepody lines

	SEMT15-02	SEMT15-15	Shepody control	Literature range[1]
	(mg/200 g fresh weight, except for vitamin C which is reported as mg/100 g fresh weight)			
Vitamin C	22.7 (NA)	23.9 (NA)	23.8 (NA)	10.3-22.0
Vitamin B6	0.56 (0.49-0.62)	0.50 (0.32-0.72)	0.52 (0.40-0.62)	0.26-0.82
Niacin	4.55 (4.14-5.05)	4.78 (3.98-5.86)	4.43 (3.73-5.15)	0.18-6.2
Copper	0.41 (0.20-0.61)	0.48 (0.23-1.10)	0.39 (0.20-0.53)	0.03-1.4
Magnesium	53.1 (48.2-67.2)	56.9 (47.9-90.0)	54.2 (48.9-65.5)	22.5-110
Potassium	1097 (997-1327)	1135 (972-1634)	1162 (1106-1259)	700-1250

[1] Literature ranges are not available for Shepody potatoes therefore the values reported are for Russet Burbank, Atlantic, Gemchip and Norchip varieties, combined.

Line RBMT15-101 has a slightly elevated vitamin C content compared to the control. The difference, however, is minor and of no biological significance. The value reported is also within the literature reported range for vitamin C content of Russet Burbank potatoes. No other significant differences in vitamin and mineral content were observed between the New Leaf® Y potatoes and the control line and the values reported were all within the literature reported ranges for potato varieties.

Conclusion

Based on the data submitted in the present application, the New Leaf® Y potato lines are compositionally equivalent to other commercial varieties of potato.

Key toxicants

Studies evaluated:

Lavrik, P.B. *et al* (1997). Compositional analyses of potato tubers derived from cvs. Russet Burbank, Shepody and Hi-Lite potato plants resistant to Colorado potato beetle and potato virus Y. Monsanto Study No. 96-01-37-24.

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).

Analyses for total glycoalkaloids (solanines and chaconines) were done on tubers collected from field trials of New Leaf® Y and control lines grown in 1995 and 1996 at three locations in the United

States and two locations in Canada. At each location, eight to fifteen replicated plots were grown per line. A summary of the results is presented in Table 11 below.

Table 11: Mean levels of total glycoalkaloids in tubers from New Leaf® Y and Russet Burbank and Shepody control lines grown in 1995 and 1996.

Line	Total glycoalkaloids[1] (standard error)
RBMT15-101	10.6 (2.54)
Russet Burbank control	11.7 (2.54)
Literature range	3.1-16.1
SEMT15-02	5.5 (1.10)
SEMT15-15	5.3 (1.09)
Shepody control	4.6 (1.07)
Literature range	Not available (2.5-16.1 for Russet Burbank, Atlantic, Gemchip , Norchip)

[1] values are mg/100 g fresh weight

Conclusion

The glycoalkaloid levels of the New Leaf® Y potato tubers are equivalent to those of the non-transformed control lines and are within the literature reported ranges for commercial potato varieties.

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 occurring allergens

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® Y 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 and 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® Y potatoes to support typical growth and well-being. Additional 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 are 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® Y potatoes are considered to be hazardous therefore a dietary exposure assessment is unnecessary for determining their safety. 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 which have been found to be safe in animal toxicity studies.

The concentration of Cry3Aa and NPTII in the New Leaf® Y potatoes is known but the concentration of the PVY coat protein was unable to be quantified, therefore it is possible to only estimate the dietary intake for Cry3Aa and NPTII.

Cry3Aa is expressed in the New Leaf® Y potato tubers at levels ranging from 0.08 to 0.38 µg protein/g fresh weight and NPTII is expressed at levels ranging from 0.003 to 0.01 µg protein/g fresh weight (see Table 4, Section 5.2).

Australian and New Zealand consumption data are 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 are presented in Table 12 below.

Table 12: 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® Y potatoes must be made. Data on market penetration of the New Leaf® Y potatoes are 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® Y potatoes and the other, probably more realistic estimate, where it is assumed that 10% of potato products are derived from New Leaf® Y potatoes. The two estimates of dietary intake for Cry3Aa and NPTII are presented in Table 13 below.

Table 13: Estimate of dietary intake of Cry3Aa and NPTII

	Estimated dietary intake	
Novel protein	**100 % market penetration**	**10 % market penetration**

	µg /day	µg/kg BW/day[1]	µg /day	µg/kg BW/day[1]
Cry3Aa (0.08-0.38 µg/g FW)	24-114	0.37-1.75	2.4-11.4	0.037-0.18
NPTII (0.003-0.01 µg/g FW)	0.9-3	0.014-0.046	0.09-0.3	0.009-0.03

[1] assuming a body weight of 65 kg.

For Cry3Aa, the very worst-case estimate is nearly 3 million times less than the dose found to have no adverse effects in mice (5220 mg Cry3Aa/kg BW). For NPTII, the estimate is at least 10 million times less than the dose found to have no adverse effects in mice (5000 mg NPTII/kg BW). Therefore, even if all processed potato products were to be derived from the New Leaf® Y potatoes, a very large margin of safety exists for both proteins.

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