FOOD DERIVED FROM INSECT-PROTECTED (NEW LEAF®) POTATO LINES BT-06, ATBT04-06, ATBT04-31, ATBT04-36, AND SPBT02-05

A Safety Assessment

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SUMMARY

Food derived from GM potato lines BT-06, ATBT04-06, ATBT04-31, ATBT04-36 and SPBT02-05 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

Fives lines of three different potato cultivars (Russet Burbank line BT-06, Atlantic lines ATBT04-06, ATBT04-31, and ATBT04-36, and Superior line SPBT02-05) were protected against the Colorado potato beetle through the *Agrobacterium tumefaciens* mediated transfer of the *cry3Aa* gene from the soil bacterium *Bacillus thuringiensis* subspecies *tenebrionis* (*B.t.t.*). The insect-protected potato lines are known as New Leaf® 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. A number of microbial pesticide products based on Cry3Aa are commercially available in the United States, with some being in use since 1989.

Other genes transferred along with the *cry3Aa* gene to the New Leaf® potatoes were the *nptII* gene (in all but line SPBT02-05) and the *aad* gene (in line ATBT04-36 only). The *nptII* gene is a marker used for selection of transformed plant lines during the potato transformation procedure. It codes for the enzyme neomycin phosphotransferase II (NPT II) 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 *cry3Aa* gene in the New Leaf® potatoes appears to be stably integrated and all lines are phenotypically and genotypically stable 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® 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® 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

Lines BT-06, ATBT04-06, ATBT04-31, and ATBT04-36 each express two novel proteins —Cry3Aa and NPT II — whereas line SPBT02-05 only expresses the Cry3Aa protein. The expression levels of both proteins are variable between lines but are consistently low. Expression levels range from between 0.00025 and 0.006% total tuber protein for Cry3Aa (0.05 – 1.29 µg/g fresh weight) and from <0.0002 to 0.02% total protein for NPTII (0.01 – 3.82 µg/g fresh weight). Line SPBT02-05 exhibited the highest Cry3Aa expression level and line ATB04-06 the highest NPTII expression level.

Acute oral toxicity testing in mice demonstrated that both Cry3Aa and NPTII have very low oral toxicity, with no adverse signs being seen in mice at doses up to 5220 mg/kg body weight. Human dietary exposure to both proteins is estimated to be well below this level. In terms of their allergenicity, neither of the proteins possess any of the physical characteristics which are common to allergens, neither have any significant similarity to known allergens, nor are they present at high levels in potato tubers and both proteins are readily degraded in conditions that simulate mammalian digestion. Furthermore, humans have a prior history of exposure to these proteins with no recorded instances of allergenicity. Therefore it was concluded that Cry3Aa and NPTII are unlikely to be allergenic to humans.

Comparative analyses

Compositional analyses were done to establish the nutritional adequacy of the New Leaf® 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), and vitamin C content. These analyses showed that the levels of key constituents in the New Leaf® potato lines is comparable to other commercial potato cultivars.

The levels of naturally occurring toxins in New Leaf® potatoes were also assessed. The only naturally occurring toxins in potatoes are the glycoalkaloids. For the majority of New Leaf® lines, the glycoalkaloid levels were either equivalent to or slightly lower than the glycoalkaloid levels found in the control. For one of the lines, BT-06, glycoalkaloid levels were slightly elevated compared to the control however the level reported was still at the lower end of the normal range reported for commercial varieties of Russet Burbank potatoes. The slightly elevated glycoalkaloid level in line BT-06 does not raise any safety concern.

Nutritional impact

Two feeding studies – one in birds and one in rats – were provided as additional supporting data for New Leaf® Russet Burbank line BT-06. The results of these studies confirm that this line is nutritionally adequate.

Conclusion

Based on the currently available data, food from New Leaf® potato lines BT-06, ATBT04-06, ATBT04-31, ATBT04-36, and SPBT02-05 is as safe and nutritious as food from other commercially available potato cultivars.

FOOD DERIVED FROM INSECT-PROTECTED (NEW LEAF®) POTATO LINES BT-06, ATBT04-06, ATBT04-31, ATBT04-36, AND SPBT02-05

A SAFETY ASSESSMENT

INTRODUCTION

A safety assessment has been conducted on potatoes that have been genetically modified to be protected against the Colorado potato beetle (*Leptinotarsa decemlineata* Say.), one of the principle pests of potatoes in North America. The potatoes are known commercially as New Leaf® potatoes.

Protection against the 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 microbial pesticide products (M-One® and Foil®), which are based on *B.t.t,* are commercially available in the United States and have been in use 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.

New Leaf® potatoes are not grown in Australia or New Zealand and are currently not permitted to be imported into Australia or 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

A total of fourteen transformed potato lines were produced using *Agrobacterium*-mediated transformation of stem tissue. Plasmid PV-STBT02 was used to generate seven Russet Burbank and two Superior potato lines and plasmid PV-STB-04 was used to generate five Atlantic potato lines. The two plasmids differ only in the promoter region for one of the genes.

Function and regulation of the novel genes

The transformation of the potatoes with either plasmid PV-STBT02 or PV-STBT04 resulted in the transfer of two gene expression cassettes — *cry3Aa* and *nptII*.

The gene expression cassettes are described in Table 1 below.

Table 1: Description of the gene expression cassettes in PV-STBT02 and PV-STB-04

Cassette	Genetic element	Source	Function
cry3Aa	Enhanced 35S promoter (PV-STBT02)	The cauliflower mosaic virus (CaMV) 35S promoter region (Odell *et al* 1985) with duplicated enhancer region (Kay *et al* 1987).	A promoter of high-level constitutive gene expression in plant tissues.
	ArabSSU1A promoter (PV-STB04)	The *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.
	E9 3' terminator	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	35S promoter	The 35S promoter region of CaMV (Gardner *et al* 1981, Sanders *et al* 1987).	A promoter of high-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.

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 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* gene, enabling those plant cells successfully transformed with the *cry3Aa* gene to grow in the presence of kanamycin. Those cells that lack the *nptII* gene, and hence the *cry3Aa* gene, will not grow and divide in the presence of kanamycin.

Other genetic elements

The plasmid vectors, PV-STBT02 and PV-STBT04, are double border binary plant transformation vectors, which differ only in the non-translated promoter region of the *cry3Aa* gene. 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-STBT02 and PV-STBT04

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 derived from the bacterial transposon Tn7 (Fling *et al* 1985) and 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. It encodes the enzyme streptomycin adenyltransferase, which confers resistance to the antibiotics spectinomycin and streptomycin. Only those bacterial cells that have been transformed with the plasmid containing the *aad* gene, and hence the gene of interest (in this case the *cry3Aa* gene) 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 MM-294, a derivative of the common laboratory *E. coli* K-12 strain.

Characterisation of the genes in the plant

Studies evaluated:

Keck, P.J. (1993). Molecular characterisation of CPB resistant Russet Burbank potatoes. Monsanto Study No. MSL-12784

The technique of Southern blotting was used to characterise the genes that had been transferred to the potato plants. Southern blotting is a sensitive technique that enables the detection of specific sequences among DNA fragments separated using gel electrophoresis (Southern 1975). The size and overall pattern of hybridising bands can be used to characterise the nature of the T-DNA insertion into the genome (e.g., how many sites in the genome the T-DNA has inserted into, whether the inserted T-DNA copies are intact).

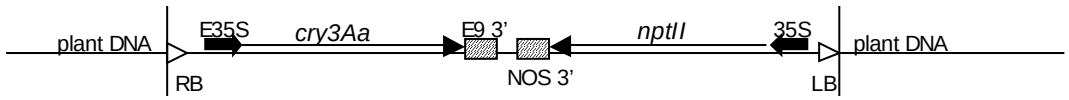
Southern blotting was done on genomic DNA isolated from the fourteen lines of transformed potatoes in order to characterise the inserted T-DNA in terms of the number of integration sites in the genome and the number of T-DNA copies inserted at a particular site. Five of the fourteen lines of transformed potato were selected for commercialisation on the basis that they contained single copies of the inserted T-DNA at a single site within the potato genome. These lines are listed in Table 3 below.

Table 3: Commercialised New Leaf® potato lines

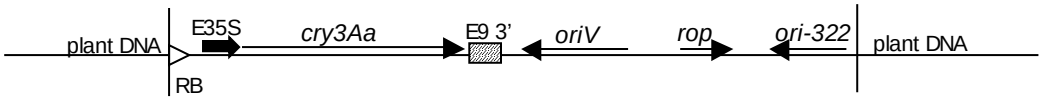
Name	Plasmid vector	Cultivar
BT-06	PV-STBT02	Russet Burbank
ATBT04-06	PV-STBT04	Atlantic
ATBT04-31	PV-STBT04	Atlantic
ATBT04-36	PV-STBT04	Atlantic
SPBT02-05	PV-STBT02	Superior

Each of these lines were further analysed to determine the gene organisation as well as confirm the absence of DNA from outside the T-DNA borders. A diagram of the gene organisation in the insertion site for each line is presented below.

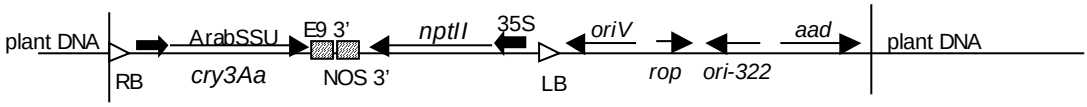
BT-06, ATBT04-06 and ATBT04-31



SPBT02-05



ATBT04-36



Although DNA residing outside the T-DNA generally does not get transferred into the plant genome, occasionally, insertion of DNA beyond the classically defined T-DNA region is known to occur (Zambryski 1992). The *aad* gene, the *oriV* and *ori322* regions are all located outside the T-DNA borders therefore they would not be expected to be transferred in the transformation. Southern blotting of the transformed potato lines indicates, however, that some or all of these three elements have been transferred into the genome of lines SPBT02-05 (*oriV* and *ori322*) and ATBT04-36 (*oriV*, *ori322* and *aad*).

Conclusion

The genetic elements that have been transferred to each of the New Leaf® lines are summarised in Table 4 below.

Table 4: Genetic elements transferred to the New Leaf® lines

	Transferred genetic elements				
Line No.	***cry3Aa***	***nptII***	***oriV***	***ori-322***	***aad***
BT-06	√	√			
SPBT02-05	√		√	√	
ATBT04-06	√	√			
ATBT04-31	√	√			
ATBT04-36	√	√	√	√	√

Stability of the genetic changes

The New Leaf® potato lines were evaluated over several generations of vegetative propagation used to generate potato seed to determine if they expressed consistent levels of the Cry3Aa protein in leaf tissue. Expression of the Cry3Aa protein was found to be highly stable across multiple generations in all the lines tested. The results of the expression studies are summarised in Table 5 below.

Table 5: Cry3Aa expression in New Leaf® potatoes over multiple generations

	Mean protein expression levels (µg/g tissue fresh weight)		
Potato line	**Generation 1**	**Generation 2**	**Generation 3**
ATBT04-36	12.8	13.3	14.6
ATBT04-06	52.2	42.7	ND
ATBT04-31	ND	7.1	8.3
SPBT02-05	ND	8.3	7.9
BT-06	5.7	6.2	7.5

New Leaf® Russet Burbank line BT-06 exhibited insect-resistance under field conditions when it was first planted in 1991. The applicant reports that this trait has been stably maintained through subsequent generations of plant propagation and breeding under different environmental conditions in potatoes grown in subsequent field trials and on a commercial scale beginning in 1996 in the United States and Canada. The Superior variety of New Leaf® potato (SPBT02-05) and the Atlantic varieties (ATBT04-36, ATBT04-06, ATBT0-31) were first planted in 1992 and the applicant reports that the trait in these lines has also been consistently expressed since that time.

Conclusion

The *cry3Aa* gene in the New Leaf® potatoes is stably integrated and all lines appear to be phenotypically and genetically stable 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® potatoes to microorganisms present in the human digestive tract.

The two plasmids used to transform the potato lines – STBT02 and PV-STBT04 – both contained a copy of 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.

Line SPBT02-05 was shown, by Southern blotting, not to contain either of the antibiotic resistance genes (see Section 3.3). Lines BT-06, ATBT04-06, ATBT04-31 and ATBT04-36, however, all contain the *nptII* gene, under the control of the 35S promoter, meaning it will be expressed in plant cells, and line ATBT04-36 also contains a copy of the *aad* gene, under the control of a bacterial promoter, meaning it will not be expressed in plant cells.

The first issue that must be considered in relation to the presence of an intact *nptII* and *aad* gene in the New Leaf® potatoes is the probability that this genes would be successfully transferred to, and expressed in, microorganisms present in the human digestive tract. The following steps would be 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 highly unlikely because of the number and complexity of the steps that would need to take place consecutively.

In the case of line ATBT04-36, 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 the linked *Escherichia coli* origin of replication (*ori322*) and the origin of replication for the broad host-range *Agrobacterium* plasmid (*oriV*). Depending on the integrity of these components, the presence of these elements on the same DNA fragment could lead to the reconstitution of almost the entire PV-STBTO4 plasmid. 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® 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 weight 73 kDa), which is produced by *B. thuringiensis* during sporulation. The protein is encoded by the *cry3Aa* gene, which is 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 protein.

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*.

Neomycin phosphotransferase II

Neomycin phosphotransferase II (NPT II; also known as aminoglycoside 3'-phosphotransferase II) is an enzyme with a molecular weight 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).

Protein expression analyses

In planta expression of Cry3Aa

Studies evaluated:

Bartnicki, D.E. *et al* (1993). Characterisation of the major tryptic fragment from Colorado potato beetle active protein from *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*). Monsanto Study No. 92-01-37-15.

Bartnicki, D.E. *et al* (1993). Equivalence of microbially-produced and plant-produced *B.t.t.* protein also called Colorado potato beetle active protein form *Bacillus thuringiensis* subsp. *tenebrionis*. Monsanto Study No. 92-01-37-07.

Rogan, G.J. and Lavrik, P.B. (1994). Compositional comparison of Colorado potato beetle (CPB) active *Bacillus thuringiensis* subsp. tenebrionis (*B.t.t.*) proteins produced in CPB resistant potato plants and commercial microbial products. Monsanto Study No. 92-01-37-17.

An initial study was done, using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to analyse the physical properties of potato tuber-expressed Cry3Aa using immunoaffinity chromatography purified Cry3Aa from New Leaf® Russet Burbank tubers. When the tuber-purified Cry3Aa was run on a polyacrylamide gel it was possible, using Coomassie blue stain, to visualise three polypeptides. The major protein band had an apparent molecular weight of 68 kDa and a minor band just below it had an apparent molecular weight of 63 kDa. The third band represented about 20-30% of the tuber-purified Cry3Aa protein and had an apparent molecular weight of 55 kDa.

Western blotting was used to further characterise the major polypeptides. Western blotting is an extremely specific immunological technique that allows for comparisons of apparent molecular weights of proteins possessing immunological cross-reactivity. Western blotting, using a rabbit polyclonal antibody against Cry3Aa isolated from *B.t.t*, demonstrated similar apparent molecular weights and staining for the 68 kDa protein, and similar staining intensity of the 55 kDa fragment. The presence of the 55 kDa band did not appear to be an artefact of the purification process as the 55 kDa band has previously been reported following digestion of the 68 kDa Cry3Aa protein by incubation in insect gut fluid or trypsin, and shown to retain full bioactivity against target insects (Carroll and Ellar 1989).

In further experiments, Western blotting was used to examine extracts of tuber and leaf tissues from Russet Burbank line BT-06 and compare them to extracts of commercial *B.t.t* preparations (M-One® and Foil®). Two major protein bands were detected in the extract from Russet Burbank line BT-06. The more abundant of the two proteins co-migrated with a 68 kDa size marker and the second band, which was about half as abundant, co-migrated with the 55 kDa size marker. Identical bands to those observed in the potato extract were also seen in the extract prepared from the M-One® microbial preparation except in this preparation the 55 kDa species was the more abundant. The extract from the other microbial preparation (Foil®) only contained the 68 kDa band.

These data confirm that the 68 kDa protein product of the *cry3Aa* gene transferred into potato plants undergoes processing or degradation in the plant cell into a smaller protein of 55 kDa. The two protein products seen in the plant cell appear identical to that observed in commercial *B.t.t* preparations.

Protein expression levels

Studies evaluated:

Rogan, G.J. *et al* (1993) Determination of the expression levels of *B.t.t* and NPTII proteins in potato tissues derived from field grown plants. Monsanto Study No. 92-01-37-02.

Duff, D.A. (1993). Development and validation of an enzyme-linked immunosorbent assay (ELISA) for detection and quantitation of *Bacillus thuringiensis* subsp. *tenebrionis* Colorado potato beetle active protein in genetically modified potato plants. Monsanto Study No. 92-01-37-02, Report No. 12735.

Anderson, J.S and Rogan, G.J. (1993). Development and validation of an enzyme-linked immunosorbent assay (ELISA) for detection and quantitation of neomycin phosphotransferase II in Colorado potato beetle resistant potatoes. Monsanto Study No. 92-01-37-02, Report No. 12330.

Lavrik, P.B. and Grace, A.M. (1995). Expression levels of *B.t.t.* and NPTII proteins in Colorado potato beetle resistant potato tissues derived from Superior potato plants grown under field conditions. MSL No. 14415.

Lavrik, P.B. and Grace, A.M. (1996). Expression levels of *B.t.t.* and NPTII proteins in Colorado potato beetle resistant potato tissues derived from Atlantic potato plants grown under field conditions. MSL No. 14659.

The levels of Cry3Aa and NPTII proteins expressed in the leaf and tuber tissue of all five New Leaf® lines were determined by 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. Novel protein expression in individual New Leaf® potato lines was determined using tissues isolated from a number of different field trials conducted in different years.

Tissues from Russet Burbank line BT-06 were collected from plants grown in field trials during the summer of 1992 at seven locations. A randomised non-replicated arrangement of treatments was used at five of the field locations, and a six replicate randomised complete block design was used at the remaining two field locations. The sampling regimen was designed to obtain tissue samples of the same relative physiological stage from all of the field locations. One whole plant was taken per plot at replicated sites and three plants per plot were taken at non-replicated sites. Protein expression levels were determined for leaves, whole plant and tubers and are summarised in Table 6 below.

Table 6: Protein expression data for Cry3Aa and NPTII in New Leaf® Russet Burbank line BT-06

Sample	Mean expression (µg/g tissue fresh weight)	Range	% total protein[1]
Cry3Aa:			
Whole plant (early season)	3.33	2.025-6.258	
Whole plant (late season)	4.297	0.526-5.644	
First leaf sample	16.364	12.15-20.823	0.076-0.13%
Second leaf sample	11.617	7.868-15.635	0.049-0.098%
Third leaf sample	11.094	5.395-13.111	0.034-0.082%
Tubers	0.664	0.404-0.955	0.002-0.005%
NPTII:			
Whole plant (late season)	0.446	0.038-0.862	
First leaf	2.063	1.344-2.431	0.008-0.015%
Tubers	0.353	0.173-0.599	0.0009-0.003%

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

These data show that Cry3Aa and NPTII are expressed at higher concentrations in leaf tissue compared to tubers — a maximum of 0.13% total protein in leaf for Cry3Aa compared to a maximum of 0.005% total protein in tubers, and a maximum of 0.015% in leaf for NPTII compared to 0.003% in tubers.

Tissues from Superior line SPBT02-05 and one control Superior plant line were collected from plants grown in two field locations in the United States during 1994. A four replicate randomised complete block design was used at both locations. Leaf samples were collected at approximately 6 weeks post planting from one of the field locations and tuber samples were collected at harvest from the other site

from three of the four replicated plots. Protein expression data for Cry3Aa and NPTII are summarised in Table 7 below.

Table 7: Cry3Aa and NPTII expression[1] in New Leaf® Superior and control lines

	Cry3Aa expression			NPTII expression		
Tissue	**Mean**	**Range**	**% total protein[2]**	**Mean**	**Range**	**% total protein**
SPBT02-05[3]:						
Leaf	11.542	8.90-15.49	0.055-0.097	0.003	0.00-0.01	< 0.0001
tuber	1.146	1.00-1.29	0.005-0.006	0.016	0.01-0.03	<0.0002
Superior control:						
Leaf	0.038	0.00-0.01		0.004	0.00-0.01	
tuber	0.042	0.03-0.05		0.034	0.03-0.04	

[1] values are µg/g tissue fresh weight
[2] using total protein levels of 1.6 and 2.0% for leaf and tuber fresh weight, respectively
[3] line SPBT02-05 does not contain a copy of the *nptII* gene

These data confirm, as expected, that NPTII expression in line SPBT02-05 is equivalent to the background levels found in the control line. Cry3Aa expression is highest in the leaves, at a maximum representing nearly 0.1% of total leaf protein. In tubers, Cry3Aa expression is considerably lower, representing at a maximum only 0.006% of total tuber protein. Some background levels of both NPTII and Cry3Aa are seen in the controls however this is commonly observed with ELISA.

Tissues from Atlantic lines ATBT04-36, ATBT04-06, and ATBT04-31 and a control Atlantic line were collected from plants grown at six field locations in the United States and Canada in 1993 through to 1995. The field trials were arranged either in a four, six, twelve or fifteen replicate randomised complete block design. Leaf samples were collected at approximately six, ten and fourteen weeks post planting from a single location in the 1993 field trials with each line replicated six times and at approximately six weeks post planting from a single location in the 1994/1995 field trials with each line replicated four times. Tuber samples from each line were collected at harvest from four different locations in the 1995 field trials. Four replicated plots for each line were harvested from each site. Protein expression data for Cry3Aa and NPTII are summarised in Table 8 below.

Table 8: Cry3Aa and NPTII expression[1] in New Leaf® Atlantic and control lines

	Cry3Aa expression			NPTII expression		
Tissue	**Mean**	**Range**	**% total protein[2]**	**Mean**	**Range**	**% total protein**
ATBT04-06:						
Leaf	59.336	29.48-88.67	0.18-0.55	36.564	21.54-47.63	0.13-0.3
Tuber	0.528	0.26-0.71	0.001-0.004	2.864	2.06-3.82	0.01-0.02
ATBT04-31						
Leaf	15.694	8.23-19.81	0.05-0.12	4.994	3.23-5.59	0.02-0.035
Tuber	0.140	0.07-0.26	0.0003-0.001	0.726	0.27-1.75	0.0013-0.009
ATBT04-36						
Leaf	20.278	8.90-32.22	0.06-0.2	12.156	5.46-23.02	0.034-0.14
Tuber	0.126	0.05-0.27	0.00025-0.001	0.583	0.29-0.80	0.0015-0.004
Atlantic control:						
Leaf	0.050	0.01-0.13		0.093	0.01-0.41	
Tuber	0.029	0.01-0.07		0.035	0.00-0.18	

[1] values are µg/g tissue fresh weight
[2] using total protein levels of 1.6 and 2.0% for leaf and tuber fresh weight, respectively

Expression of Cry3Aa and NPTII is quite variable both within each line and between lines but in all cases is highest in the non-edible portion of the plant – the leaf tissue. Cry3Aa expression in the tuber ranges from 0.00025 to 0.004% total protein and NPTII tuber expression ranges from 0.0013 to 0.02% total protein. Line ATBT04-06 exhibited the highest tuber expression levels for both proteins.

Conclusion

The expression levels of Cry3Aa and NPTII in potato tubers from the New Leaf® lines are quite variable but consistently low. Expression levels range from between 0.00025 and 0.006% total tuber protein for Cry3Aa and from <0.0002 to 0.02% total protein for NPTII. Line SPBT02-05 exhibits the highest Cry3Aa expression level and line ATB04-06 the highest NPTII expression level.

Potential toxicity of novel proteins

Cry3Aa

The New Leaf® potato lines all express the Cry3Aa protein (see Section 5.2).

Studies evaluated:

Bartnicki, D.E. *et al* (1993). Equivalence of microbially-produced (*Escherichia coli*) and plant-produced (Colorado potato beetle resistant potato) neomycin phosphotransferase II (NPTII). Monsanto Study No. 92-01-37-08.

Naylor, M.W. (1993). Acute oral toxicity study of *B.t.t.* protein in albino mice. Study No. ML-92-407.

Lavrik, P.B. *et al* (1993). Colorado potato beetle active *Bacillus thuringiensis* subsp. *tenebrionis* protein dose formulation, dose confirmation, and dose characterisation for albino mice acute toxicity study. Monsanto Study No. ML-92-407.

Lavrik, P.B. (1993). Characterisation of Colorado potato beetle active *Bacillus thuringiensis* subsp. *tenebrionis* protein produced in *Escherichia coli*. Monsanto Study No. 92-01-37-10.

Bartnicki, D.E. *et al* (1993). Equivalence of microbially-produced and plant-produced *B.t.t.* protein also called Colorado potato beetle active protein form *Bacillus thuringiensis* subsp. *tenebrionis*. Monsanto Study No. 92-01-37-07.

Cyr3Aa 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.

An acute oral toxicity study was done to confirm the absence of mammalian toxicity of Cry3Aa. 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). The test protein was produced in a single fermentation batch of *E. coli* (Batch No. 5192101) and purified to greater than 95% protein. The test protein 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 a suitable substitute for plant produced Cry3Aa for the purposed of toxicity testing.

The Cry3Aa protein (Batch No. 5192101) was administered by gavage to groups of 10 CD-1 mice (10/sex/dose) at levels of 0, 500, 1000 and 5220 mg/kg body weight. Another group of mice were dosed with 5000 mg/kg bovine serum albumin and controls were administered the same volume of dosing vehicle given to the high dose mice. Mice were observed twice daily for signs of toxicity and food consumption was recorded daily. Body weights were recorded pre-test and on day 7 after dosing. All animals were necropsied 7 days after dosing.

Two animals died during the test — a vehicle control female on day 1 and a low dose male on day 3. The death of the control female was attributed to accidental gavaging of the vehicle into the lung. No cause of death could be determined for the low dose male. As there were no deaths in other treated mice, or at higher exposure levels, the death is not considered to be treatment related.

No abnormal clinical signs were observed in any of the other 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. The minor pathologic changes were randomly distributed among all groups and the applicant reports that these are commonly seen for the strain of mice used by the testing laboratory.

Conclusion

No adverse effects were observed when Cry3Aa was administered by gavage to mice at doses up to 5220 mg/kg. Cry3Aa is therefore considered to have low oral toxicity in mice.

Neomycin phosphotransferase II

All lines, with the exception of SPBT02-05, express the NPTII protein (see Section 5.2).

Studies evaluated:

Bartnicki, D.E. *et al* (1993). Equivalence of microbially-produced (*Escherichia coli*) and plant-produced (Colorado potato beetle resistant potato) neomycin phosphotransferase II (NPTII). Monsanto Study No. 92-01-37-08.

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 No. ML-91-409.

Naylor, M.W. (1992). Acute oral toxicity study of neomycin phosphotransferase (NPT II) in albino mice. Monsanto Study No. ML-91-409.

An acute oral toxicity study was done to assess the potential mammalian toxicity of neomycin phosphotransferase II. The test protein was produced in a single fermentation batch of *E. coli* (Batch No. NBP 4821020) and purified to greater than 95% protein. The test protein 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 *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. This study has also been published (Fuchs *et al* 1993). The *E. coli* produced NPTII is therefore a suitable substitute for plant produced NPTII in toxicity testing.

The NPTII protein was administered by gavage to groups of CD-1 mice (10/sex/dose) at doses of 0, 100, 1000, and 5000 mg/kg body weight. The test material was dissolved in a 0.1M carbonate buffer vehicle and given as a solution. Control mice were administered vehicle only. Clinical observations, body weights and food consumption measurements were performed. All animals were necropsied 8-9 days after dosing.

When compared to controls, there were no statistically significant differences between group mean body weights or cumulative body weight gain in any of the treated groups. Among males, there were no statistically significant differences in food consumption but among females food consumption was

significantly lower compared to controls. However, food consumption by the female control mice was nearly twice that of any male group. The applicant speculates that the high apparent food consumption by the control females reflects spillage due to digging behaviour. This being the case, the observed differences in food consumption among females could not be attributed to any toxicity of the test material. No abnormal clinical signs were noted and there were no unscheduled deaths. There were no differences between group mean terminal body weights and no gross lesions were observed at necropsy.

Conclusion

No adverse effects were observed when NPTII was administered by gavage to mice at doses up to 5000 mg/kg. NPTII is considered to have low oral toxicity in mice.

Potential allergenicity of novel proteins

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 molecular weights 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 two new proteins expressed in the New Leaf® potatoes – Cry3Aa and NPTII. The potential allergenicity of these two proteins will therefore be considered.

Studies evaluated:

Keck, P.J. and Sims, S.R. (1993). Assessment of the metabolic degradation of the Colorado potato beetle (CPB) active proteins in simulated mammalian digestive models. Monsanto Study No. 389270.

Ream, J. (1993) Assessment of degradation of neomycin phosphotransferase II in *in vitro* mammalian digestion models. Monsanto Study No. IRC-91-ANA-06.

Cry3Aa

Source of the protein

Cry3Aa is encoded by the *cry3Aa* gene, which is derived from the soil bacterium *B. thuringiensis* subsp. *tenebrionis* (*B.t.t.*) and is expressed in the bacterium during sporulation. Microbial formulations of *B.t.t.* have been in use since 1989 where they are used on a wide variety of crops such as eggplant, potato and tomato. These formulations have been shown to contain the Cry3Aa protein (see Section 5.2) and to date there have been no reports of any allergic reactions.

Physical and chemical characteristics

The Cry3Aa protein has a molecular weight of 68 kDa, which is in the size range of known allergens. Cry3Aa is not glycosylated, however, as it is expressed in the plant cell cytoplasm. For glycosylation of Cry3Aa 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 *cry3Aa* gene construct. The absence of glycosylation of Cry3Aa has also been determined experimentally (see section 5.3).

Digestibility

If proteins are to be allergenic they must be stable to the peptic and tryptic digestion and acid conditions of the digestive system if they are to pass through the intestinal mucosa to elicit an allergenic response.

The digestibility of Cry3Aa was determined experimentally using *in vitro* mammalian digestion models. *In vitro* studies with simulated digestion solutions have been used as models for animal digestion for a number of years and have had wide application. For example, they have been used to investigate the digestibility of plant proteins (Nielsen 1988, Marquez and Lajolo 1981), milk proteins (Zikakis *et al* 1977), and flavouring substances (Tilch and Elias, 1984); to assess protein quality (Akeson and Stahmann 1964); and to study digestion in pigs and poultry (Fuller 1991).

Purified Cry3Aa protein (68 kDa - Batch No. 5192101) was added to simulated gastric and intestinal fluids and incubated at 37ºC. The 55 kDa protein of Cry3Aa was prepared by trypsinization of the purified 68 kDa protein. The protein used was from the same batch that had been produced in *E.coli* for acute toxicity testing in mice. This protein has been shown experimentally to be equivalent to plant expressed Cry3Aa protein (see Section 5.3). The degradation of the protein in the digestion fluid was assessed over time by Western blot analysis. An insect bioassay was used as an additional means of monitoring Cry3Aa degradation in the digestion fluids. The simulated digestion fluids were prepared according to procedures outlined in the United States Pharmacopeia (1990). The simulated gastric fluid contained 3.2g pepsin/L and 2.0g NaCl/L at pH 1.2 and the simulated intestinal fluid contained 10g pancreatin/L in phosphate buffer at pH 7.5.

The 68 kDa and 55 kDa species of Cry3Aa were shown to be readily degraded in simulated gastric fluid, with neither protein species able to be detected by Western blot after thirty seconds of incubation. Samples were removed from the incubation at 0 and 10 minutes for bioassay with Colorado potato beetle. Intermediate time points were not taken. The results of the bioassay correlate with those of the Western blot where the level of bioactivity of the Cry3Aa proteins was reduced from between 60-100% mortality of Colorado potato beetle at 0 minutes to background levels (2-4% mortality) after 10 minutes of incubation.

Western blot analysis of samples incubated with simulated intestinal fluid show that about 10-20% of the 68 kDa species of Cry3Aa is converted to two smaller proteins of about 63 and 55 kDa within 30 seconds. The smaller proteins are consistent with the protein bands seen on Western blots of tuber extracts from line BT-06 (Section 5.2). After two hours of incubation, most of the 68 kDa protein had degraded to the 55 kDa protein product which persisted for up to 14 hours. The experiment was repeated with the isolated 55 kDa protein species which was also shown to persist for up to 14 hours, although a small amount of degradation could be observed. No decrease in bioactivity of the Cry3Aa protein was observed over the 14 hours of incubation, as would be expected from the Western blot data. The predominant proteolytic component of simulated intestinal fluid is trypsin. It has been previously reported that a 55 kDa protein product is generated from the 68 kDa protein upon incubation with trypsin or insect gut fluids (Carroll and Ellar 1989). The 55 kDa protein product therefore appears to be trypsin resistant and so would be expected to persist in the simulated intestinal fluid. Other Cry proteins are also known to have trypsin-resistant cores (Hofte and Whitely 1989).

Similarity to known allergens

The amino acid sequence of the Cry3Aa protein was compared to the 121 amino acid sequences that have been reported for allergens in the three current protein databases – Genpept, Pir protein and Swissprot. No significant similarity to any of the 121 amino acid sequences was found. There was

also no greater similarity of the Cry3Aa protein to any of the 121 amino acid sequences for the allergenic proteins than for a scrambled sequence of the same amino acids that comprise the Cry3Aa protein.

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 Cry3Aa protein, in contrast, is expressed at very low levels in the tubers of New Leaf® potatoes. For example, in Russet Burbank line BT-06 the levels of Cry3Aa in the tuber range between 0.4 and 0.95 µg/g tissue fresh weight (equivalent to 0.002 - 0.005% of total protein).

Neomycin phosphotransferase II

The allergenicity of NPTII has previously been considered by the WHO (1993) and by the Nordic Council of Ministers (Kärenlampi 1996).

In relation to the potential allergenicity of proteins derived from marker genes, the conclusion of the WHO Workshop was that *unless the marker gene is derived from a source known to cause food allergy, there is no reason to believe that marker gene proteins per se would cause allergenic reactions*. The Nordic Council of Ministers concluded that NPTII is not allergenic to humans because it has no significant similarity to known allergens, it is not glycosylated, it is rapidly degraded in the gastrointestinal tract thereby minimising its potential for absorption by the gut, and importantly, it is a protein which is not novel to humans as NPTII producing kanamycin resistant bacteria are present in normal gut flora.

The applicant also provided additional studies confirming that the NPTII protein is not glycosylated *in vivo* in New Leaf® potato plants (see Section 5.3) and that it is rapidly degraded in simulated gastric and intestinal digestion fluids.

Purified NPTII (Batch No. NBP4821020) was added to simulated gastric and intestinal fluids and incubated at 37ºC. The protein used was from the same batch that had been produced in *E.coli* for acute toxicity testing in mice. The *E.coli* produced NPTII protein has been shown experimentally to be equivalent to the plant expressed protein (see Section 5.3). The degradation of the NPTII protein in the digestion fluid was assessed over time by Western blot analysis. Measurements of NPTII enzymatic activity were used as an additional means to monitor the degradation of NPTII. The simulated digestion fluids were prepared according to procedures outlined in the United States Pharmacopoeia (1990). The simulated gastric fluid contained 3.2g pepsin/L and 2.0g NaCl/L at pH 1.2 and the simulated intestinal fluid contained 10g pancreatin/L in phosphate buffer at pH 7.5.

NPTII was readily digested in simulated gastric fluid with no protein able to be detected by Western blotting after only ten seconds of incubation. In simulated intestinal fluid, NPTII was shown to have a half-life of between 2 and 5 minutes. The enzymatic activity of NPTII had been completely destroyed by two minutes in the simulated gastric fluid and by 15 minutes in the simulated intestinal fluid. These data correlate with the Western blot observations.

Conclusion

Both the Cry3Aa and the NPTII proteins are within the size range of known allergens, however, neither are glycosylated and both are rapidly degraded in the proteolytic and acid conditions of simulated gastric fluid suggesting neither would survive mammalian digestion. Neither of the

proteins has 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 can be concluded that Cry3Aa and NPTII are unlikely to be allergenic to humans.

COMPARATIVE ANALYSES

There are concerns that transformation 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 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 and Love, S.L. (1994). Composition and quality analysis of potato tubers derived from field-grown Colorado potato beetle resistant potato plants. Monsanto Study No. 92-01-37-19.

Dodson, H.C. *et al* (1998). Composition analysis of potato tubers from Colorado potato beetle resistant Atlantic potato plants grown under field conditions. Monsanto Study No. 98-01-37-01.

Dodson, H.C. *et al* (1998). Composition analysis of potato tubers from Colorado potato beetle resistant Superior potato plants grown under field conditions. Monsanto Study No. 98-01-37-12.

Lavrik, P.B. (1996). Proximate analysis of potato tubers from Colorado potato beetle resistant Russet Burbank potatoes grown under field conditions. Monsanto Study No. 96-02-37-23.

Dodson, H.C. (1998). Proximate analysis of potato tubers from Colorado potato beetle resistant Superior line SPBT02-05 potato plants grown under field conditions. Monsanto Study No. 98-01-37-13.

Dodson, H.C. (1998). Proximate analysis of potato tubers from Colorado potato beetle resistant Atlantic potato plants (lines ATBT04-06, ATBT04-31 and ATBT04-36) grown under field conditions. Monsanto Study No. 98-01-37-15.

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 compositional analyses of tubers from New Leaf® potato lines grown in a series of separate field trials across the United States.

Russet Burbank line BT-06

New Leaf® Russet Burbank line BT-06 and Russet Burbank non-transformed control plants were grown during the 1992 field season. The study was done it two parts. In Part I, tubers were grown in six locations using six replicated plots per line for two of the locations, and a single plot per line at the remaining four locations. Tubers harvested from these sites were analysed for total solids, sugars (dextrose and sucrose), vitamin C, and total protein. In part II, tubers were grown in two locations using six replicated plots per line at each site. Composite tuber samples from each of the two sites were analysed for proximate composition (total protein, fat, carbohydrate, total dietary fibre, calories and ash) and for minor constituents — thiamine (vitamin B_1), pyridoxine (vitamin B_6), folic acid, niacin (vitamin B_3), riboflavin (vitamin B_2), and minerals (calcium, copper, iodine, iron, magnesium, phosphorous, sodium, zinc and potassium). The compositional data from all locations was combined. A summary of the data appears in Table 9 below.

Table 9: Compositional data[1] for Russet Burbank potato line BT-06

Constituent	Line BT-06	Control line	Normal range
values reported below are means, with ranges in brackets (n=6)			
Total solids (%)	20.5 (16.0-23.9)	19.9 (16.2-22.3)	16.8-24.5
Sugars:			
Dextrose (%FW)	0.18[#] (0.11-0.26)	0.15 (0.05-0.21)	0.04-0.52
Sucrose (%FW)	0.13 (0.03-0.20)	0.12 (0.06-0.18)	0.1-0.88
Vitamin C (mg/100g FW)	8.8 (6.2-11.7)	9.0 (5.9-12.3)	10.3-22.0
Protein (%DW)	4.9 (4.1-6.0)	5.2 (3.7-7.5)	3.4-7.3
values reported below are means of two analyses from two composite samples			
Proximate data:			
Protein (g/100g)	10.45, 10.8	10.10, 10.7	7.1-14.6
Moisture (g/100g)	4.55, 4.6	3.3, 5.2	Not available
Fat (g/100g)	0.3, 0.3	0.3, 0.25	0.2-0.8
Ash (g/100g)	4.35, 5.2	4.35, 5.25	2.2-9.5
Total dietary fibre (g/100g)	6.65, 7.5	6.25, 7.1	5-13
Carbohydrates (g/100g)	80.35, 79.1	81.95, 78.6	84.5 (avg)
Calories (kcal/100g)	366, 362.5	371, 359.5	350 (avg)
Minor constituents:			
Thiamine-HCl (mg/100g)	0.38, 0.42	0.35, 0.41	0.35-0.70
Pyridoxine-HCl (mg/100g)	0.42, 0.55	0.45, 0.53	0.7-1.4
Folic acid (mg/100g)	0.031, 0.023	0.044, 0.026	0.02-0.1
Niacin (mg/100g)	6.72, 8.95	6.58, 8.31	4-8
Riboflavin (mg/100g)	0.085, 0.135	0.080, 0.125	0.05-0.45
Calcium (mg/100g)	68.7, 58.9	64.85, 59.4	30-90
Copper (mg/100g)	0.38, 0.3	0.38, 0.67	0.4-1.0
Iron (mg/100g)	5.19, 517	5.24, 4.96	2.5-10
Iodine (µg/100g)	<10.0, <10.0	<10.0, 13.65	2-60
Magnesium (mg/100g)	87.2, 68.1	96.5, 67.1	60-140
Phosphorous (mg/100g)	91.6, 139.5	118, 149.5	150-300
Sodium (mg/100g)	18.4, 17.6	20.0, 15.8	4-26
Potassium (mg/100g)	1960, 2175	1965, 2190	1700-3000
Zinc (mg/100g)	0.98, 1.15	0.99, 1.20	0.6-2.4

[1] composition based on dry matter, except for moisture content
[#] statistically significantly different compared to the control

The level of dextrose in line BT-06 was slightly elevated compared to the control line, however, the difference is minor and the level reported is within the normal range for Russet Burbank cultivars. There were no significant differences between line BT-06 and the control line for any of the other analysed constituents and for the majority of constituents the levels reported were comparable to the normal ranges for Russet Burbank cultivars. The exceptions to this were for vitamin C, pyridoxine-HCl and phosphorous content, which were all low, compared to the literature reported values, for both the transformed and control lines.

Atlantic potato lines ATBT04-06, ATBT04-31, and ATBT04-36

The New Leaf® Atlantic potatoes and non-transformed Atlantic potatoes were grown during the summer of 1997 at three locations in the United States and Canada. Four replicate plots per line were grown at two of the sites, and eight replicated plots per line were grown at the remaining site. Tubers were collected at harvest and analysed for total solids, dextrose, sucrose, vitamin C and proximate composition (total protein, fat, crude fibre, ash, total carbohydrate and calories). A summary of the results of the compositional analyses is presented in Table 10 below.

Table 10: Compositional data[1] from Atlantic potato lines ATBT04-06, ATBT04-31, and ATBT04-36

Constituent	ATBT04-06	ATBT04-31	ATBT04-36	Control	Literature Range
Total solids (%FW)	24.6 (23.1-26.5)	24.6 (23.1-25.9)	23.3 (21.9-25.0)	24.0 (22.5-26.9)	22.0-26.8
Sugars:					
Dextrose (%FW)	0.31 (0.12-0.51)	0.35 (0.10-0.60)	0.37 (0.15-0.65)	0.35 (0.09-0.58)	0.03-0.14
Sucrose (%FW)	0.66 (0.34-1.02)	0.69 (0.35-1.14)	0.66 (0.38-1.01)	0.57 (0.39-0.70)	0.11-0.62
Vitamin C (mg/100 g FW)	8.3 (7.7-9.3)	9.4 (8.4-11.7)	8.9 (7.8-10.2)	8.3 (7.8-8.9)	15.4-19.4
Proximate data[2]:					
Protein	9.7 (8.2-10.7)	10.0 (9.5-10.9)	9.9 (8.6-10.4)	9.3 (8.6-10.2)	7.1-14.6
Fat	0.32 (0.15-0.40)	0.33 (0.27-0.44)	0.37 (0.29-0.46)	0.37 (0.26-0.45)	0.2-0.8
Ash	4.9 (4.1-5.6)	4.6 (3.7-5.4)	4.84 (4.1-5.5)	4.7 (3.9-5.4)	2.2-9.5
Crude Fibre	1.4 (1.3-1.6)	1.5 (1.3-1.7)	1.4 (1.3-1.5)	1.5 (1.1-1.7)	Not available
Carbohydrates	85.1 (84.0-86.1)	85.1 (84.1-86.3)	84.9 (84.2-85.6)	85.6 (84.5-86.4)	84.5 (avg)
Calories/100g	382 (379-386)	383 (380-387)	383 (379-385)	383 (379-386)	350 (avg)

[1] values reported as means, range in brackets (n=16)
[2] Except for calories, reported values are in g/100 g dry weight.

No significant differences in the key constituents were found between the Atlantic potato lines and the non-transformed control. With the exception of vitamin C, the values reported were comparable to the normal ranges for Atlantic cultivars. The levels of vitamin C were reduced compared to literature reported values in both the New Leaf® lines and the control lines.

Superior potato line SPBT02-05

New Leaf® Superior potato line SPBT02-05 and a control (non-transformed) Superior potato line were grown at eight locations in the United States and Canada during the summer of 1997. Tubers were grown in non-replicated plots at each of the eight sights. Tubers were collected at harvest from four areas of each plot at each site and analysed for total solids, sugars, vitamin C and proximate (total protein, fat, ash, crude fibre, total carbohydrates and calories). A summary of the results of the compositional analyses is presented in Table 11 below.

Table 11: Compositional data[1] from Superior potato line SPBT02-05

Constituent	SPBT02-05	Control line	Literature range
Total solids (%FW)	19.5 (16.6-23.8)	19.7 (18.0-21.7)	Not available
Sugars:			
Dextrose (%FW)	0.62 (0.30-0.98)	0.60 (0.51-0.72)	"
Sucrose (%FW)	0.23 (0.08-0.34)	0.20 (0.10-0.32)	
Vitamin C (mg/100 g FW)	8.42# (7.28-9.44)	9.33 (8.49-10.55)	"
Proximate data[2]:			
Total protein	10.9# (7.1-13.2)	12.0 (9.2-14.0)	7.1-14.6
Fat	0.55 (0.38-0.76)	0.51 (0.33-0.70)	0.2-0.8
Ash	4.98# (4.1-5.4)	4.70 (3.7-5.1)	2.2-9.5
Crude fibre	2.31 (1.67-2.92)	2.10 (1.77-2.43)	Not available
Total carbohydrate	83.6 (80.3-87.5)	82.8 (80.6-85.5)	84.5 (avg)
Calories	383 (381-387)	384 (382-388)	350 (avg)

[1] values reported as means, range in brackets (n=8)
[2] Except for calories, reported values are in g/100 g dry weight. Calories are reported in calories/100 g.
statistically significantly different compared to the control

Literature ranges for total solids, sugars and vitamin C are not available for the Superior cultivar of potato. The literature ranges for these constituents determined for other potato cultivars is presented in Table12 below.

Table 12: Literature ranges for major constituents of potato cultivars

Constituent	Atlantic	Gemchip	Norchip	Russet Burbank
Total solids	22.0-26.8	19.3-25.4	17.3-22.7	16.8-24.5
Sugars:				
Dextrose	0.03-0.14	0.08-0.28	0.03-0.39	0.04-0.52
Sucrose	0.11-0.62	0.05-0.44	0.09-0.64	0.10-0.88
Vitamin C	15.4-19.4	15.9-18.0	15.9-20.3	10.3-22.0

Significant differences between the New Leaf® Superior line and the non-transformed control line were noted for vitamin C, total protein and ash. The level of total protein and vitamin C in SPBT02-05 was slightly decreased compared to the control and the level of ash was slightly elevated. The slight differences in total protein and ash content in line SPBT02-05 compared to the control are not biologically significant and the values reported are within the literature reported ranges.

In relation to the differences in vitamin C content, this vitamin is reported to decrease during storage (Burton 1987), therefore, the low levels in both the transformed and control Superior lines may be explained if the tubers used for the analyses had been stored for prolonged periods. The New Leaf® and control Atlantic lines also exhibited decreased vitamin C levels compared to literature reported ranges (see above). An alternative explanation for the lower vitamin C content could be that the Superior cultivar naturally has lower levels of vitamin C compared to other potato cultivars. The applicant reports (data not provided) that in reviewing its previous studies using the Superior cultivar, both the transformed and non-transformed controls typically produced low levels of vitamin C compared to other varieties. Therefore, it appears that lower vitamin C content could be a varietal characteristic of the Superior cultivar. The small but significant difference in vitamin C content between the New Leaf® Superior line and the Superior control does not pose a safety concern and, although potatoes are a significant source of vitamin C, the levels in line SPBT02-05 are still considered to be nutritionally adequate.

Conclusion

On the basis of the data provided, New Leaf® lines BT-06, ATBT04-06, ATBT04-31, and ATBT04-36 are compositionally equivalent to other commercial lines of potatoes. The New Leaf® Superior line had a slightly lower vitamin C content compared to its non-transformed control however the control line also had a vitamin C content that was significantly lower than the normal range seen in commercial potato varieties. Lower vitamin C content may be a characteristic of the Superior cultivar. The slightly reduced level of vitamin C observed in line SPBT02-05 is not a cause for concern and the levels of vitamin C in this line are considered to be nutritionally adequate.

Key toxicants

Studies evaluated:

Dodson, H.C. *et al* (1998). Composition analysis of potato tubers from Colorado potato beetle resistant Atlantic potato plants grown under field conditions. Monsanto Study No. 98-01-37-01.

Dodson, H.C. *et al* (1998). Composition analysis of potato tubers from Colorado potato beetle resistant Superior potato plants grown under field conditions. Monsanto Study No. 98-01-37-12.

Lavrik, P.B. and Love, S.L. (1994). Composition and quality analysis of potato tubers derived from field-grown Colorado potato beetle resistant potato plants. Monsanto Study No. 92-01-37-19.

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).

Atlantic potato lines ATBT04-06, ATBT04-31, and ATBT04-36 were grown in field trials during the 1997 season at three locations in the United States. Superior potato line SPBT02-05 was grown in field trials during the summer of 1997 at eight locations in the United States and Canada. Russet Burbank potato line BT-06 was grown during the 1992 season at six locations in the United States. In all cases, tubers were collected from four areas of each plot at each site and analysed for glycoalkaloid content. The controls were tubers isolated from the non-transformed potato line grown at the same field location. A summary of the results is present in Table 13 below.

Table 13: Glycoalkaloid content[1] in New Leaf® potato lines.

Line	Total glycoalkaloids	Literature range
Atlantic:		4.2-10.5
ATBT04-06 (n=16)	8.7 (5.58-13.09)	
ATBT04-31 (n=16)	8.1 (3.82-15.53)	
ATBT04-36 (n=16)	7.6[#] (5.00-12.52)	
Control (n=16)	9.4 (5.27-16.78)	
Superior:		Not available
STBT02-05 (n=9)	5.2 (2.5-10.4)	
Control (n=6)	4.8 (1.9-7.3)	
Russet Burbank:		3.1-16.1
BT-06 (n=6)	6.6[#] (3.4-14.3)	
Control (n=6)	4.5 (2.7-6.7)	

[1] the values provided are mg/100g fresh weight and are the mean values with the range in parentheses
[#] significantly different compared to the control

Lines ATB04-06, ATBT04-31, and STBT02-05 have glycoalkaloid levels that are equivalent to the non-transformed controls and line ATBT04-36 has glycoalkaloid levels that are decreased compared to the control. Slightly decreased glycoalkaloid levels do not raise any safety concerns and all the values reported are within the literature reported range for glycoalkaloids. Line BT-06 has glycoalkaloid levels that are slightly elevated compared to the control. The increase however is minor and the value reported is at the lower end of the normal range reported in the literature for glycoalkaloids, therefore the slight increase does not raise any safety concerns.

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 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® 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.

In the case of the New Leaf® potatoes, the extent of the compositional and other data provided in the application is considered adequate to establish the safety of the food. Nonetheless, the applicant also provided two animal feeding studies to compare the wholesomeness of New Leaf® Russet Burbank line BT-06 with control lines. Although not considered essential for establishing safety in this instance, these animal feeding studies have been reviewed as additional supporting data.

Studies evaluated:

Cambell, S.M. *et al* (1993). A dietary toxicity study with Russet Burbank potatoes in the Northern Bobwhite. Wildlife International Ltd Study No. 139-356.

Cambell, S.M. *et al* (1993). A dietary toxicity study with Russet Burbank potatoes in the Northern Bobwhite. Wildlife International Ltd Study No. 139-357.

Naylor, B.S. (1993). One month feeding study with CPB (Colorado potato beetle) control potatoes in Sprague-Dawley rats. Monsanto Study No. 92209.

Bird feeding study

Potato tubers were obtained from 1992 field trials, conducted with Russet Burbank New Leaf® line BT-06 and a non-transformed Russet Burbank line, in the United States.

Ten-day-old northern bobwhite quail chicks (30/group of mixed sex) were randomly assigned to either the New Leaf® line or the parental control line. The chicks were fed dietary concentrations of 50,000 ppm (5% w/w) lyophilised potato powder mixed into a basal ration. The applicant has calculated that this is equivalent to approximately 90 g potatoes/kg body weight. In addition to the parental control line, a group of 30 chicks were also fed the basal ration without any potato supplementation. Each group was fed the diet for 5 days and then switched to basal diets for the last 3 days of the study. Food consumption was recorded for each pen, with food and water provided *ad libitum*. Individual body weights were recorded at the commencement of the study, on study day 5 and at study termination. Birds were observed twice daily for mortality or signs of toxicity.

Results

No mortality and no differences in food consumption or body weight gains between the treated and control groups were observed. One bird in the negative control group (basal diet only) was observed as nostril picked on day 6 and one bird in the Russet Burbank (control) group (fed parental line of potatoes) was noted as toe picked on day 8. Two birds fed New Leaf® line BT-06 were observed to be toe picked on days 4 and 5. All other birds were reported as appearing normal.

Conclusion

No treatment related mortality or differences in food consumption, body weight gain or behaviour occurred between birds fed 50 000ppm lyophilised New Leaf® Russet Burbank potato powder in the diet and birds fed the same level of Russet Burbank control potatoes or birds fed basal diets. New Leaf® Russet Burbank line BT-06 is equivalent to Russet Burbank control potatoes in its ability to support the typical growth and well-being of bobwhite quail chicks over the time period measured.

Rat feeding study

The origin of the potato tubers used in this study is not clearly specified in the report provided but appear to originate from the 1992 field trial of the New Leaf® Russet Burbank potatoes (line BT-06).

Six-week-old Sprague-Dawley rats (10/sex) were each given one fresh raw potato approximately every 2-3 days of the study for one month. Overall study mean consumption of the potatoes was 25.2 mg/rat/day for males and 21.0 mg/rat/day for females. The consumption rate was thus calculated to be approximately 84 g potato/kg body weight/day. The applicant stated this consumption rate is equivalent to the consumption of 40 potatoes per day for an adult human (assuming an average mass of 150 g per potato). The form in which potato was fed to the rats is not apparent from the data provided in the study. Rodent Chow was continuously available to the test and control groups. Clinical observations and determinations of body weights and food consumption were done. All animals were necropsied at the termination of the study (days 29-31). Liver, kidneys and testes were weighed and tissues retained.

Results

Consumption of potatoes (both control and test group) was slightly reduced for the first 3 days of the study, however, both group's potato consumption increased rapidly and remained high for the rest of the study. Consumption of Rodent Chow was similar for both the test and control group. Cumulative weight gains were normal and equivalent to the control group. No adverse clinical signs were observed. Gross pathology revealed a number of abnormal findings, such as enlarged lymph nodes, hydronephrosis and enlarged adrenals, however, these findings were observed in both the test and control groups and could not be related to the test substance. There were no significant differences in absolute or relative organ weights of the kidney, liver or testes for the test group when compared to the control.

Conclusion

There are no significant differences in the measured parameters between control animals fed the parent line of Russet Burbank potatoes and those fed Russet Burbank line BT-06. Therefore, Russet Burbank line BT-06 is equivalent to control Russet Burbank potato lines in its ability to support typical growth and well-being.

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.

The two novel proteins expressed in the New Leaf® potatoes are not 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 which have been found to be safe in animal toxicity studies.

The concentration of Cry3Aa and NPTII in the New Leaf® Plus potatoes is known, therefore it is possible to estimate their dietary intake.

Cry3Aa is expressed in New Leaf® potato tubers at levels ranging from 0.05 to 1.29 μg protein/g fresh weight and NPTII is expressed at levels ranging from 0.01 to 3.82 μg protein/g fresh weight (see Tables 6 – 8, 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 are presented in Table 14 below.

Table 14: 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 potato	Aus	-	-	-	-
	NZ	0.007	34.6	34.6	34.6
Potato fries, commercial	Aus	16.6	132.5	113	264
	NZ	18.6	141.2	142	300
Total potato products	Aus	19.4	-	-	-
	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® potatoes must be made. In 1998, the New Leaf® potatoes comprised approximately 4% of the United States potato acreage (USEPA/USDA 1999) although this has since declined to less than 1% as this variety has been superseded by other New Leaf® varieties.

Therefore it is possible to make two dietary intake estimates — one using a very worst case estimate where it is assumed that all potato products on the market are derived entirely from New Leaf® potatoes and the other, more realistic estimate, where it is assumed that 10% of potato products are derived from New Leaf® potatoes. The two estimates of dietary intake for Cry3Aa and NPTII are presented in Table 15 below.

Table 15: Estimate of dietary intake of Cry3Aa and NPTII

Novel protein	**Estimated dietary intake**			
	100 % market penetration		**10 % market penetration**	
	µg /day	**µg/kg BW/day[1]**	**µg /day**	**µg/kg BW/day[1]**
Cry3Aa (0.05-1.29µg/g FW)	15-387	0.23-5.95	1.5-38.7	0.02-0.60
NPTII (0.01-3.82 µg/g FW)	3-1146	0.046-17.6	0.3-114.6	0.005-1.76

[1] assuming a body weight of 65 kg.

For Cry3Aa, the very worst case estimated intake is at least 0.9 million times less than the dose found to have no adverse effects in mice (5220 mg Cry3Aa/kg BW). For NPTII, the estimated dietary intake is at least 0.3 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® potatoes, an incredibly large margin of safety exists for both proteins.

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