Application to Food Standards Australia and New Zealand for the Inclusion of Potatoes with Low Acrylamide Potential and Reduced Black Spot in Standard 1.5.2 Food Produced Using Gene Technology

Submitting Company:
SPS International Inc.
999 W Main St #1300, Boise, ID 83702, United States of America

Submitted By:
[redacted]

Submitted On: 25th February 2016
SPS International Inc.

OECD Unique identifiers:
SPS-ØØE12-8

VOLUME 1 of 10

PART 1: General Requirements

PART 2: Specific Data Requirements for Safety Assessment

This document does not contain confidential information

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***Corresponding Author:**
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SPS International Inc.
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Boise, ID 83702,
United States of America
[redacted]

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Statutory Declaration – Australia

Statutory Declarations Act 1959

I, [redacted] SPS International Inc., 999 W Main St #1300, Boise, ID 83702, United States of America, make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

..... [redacted]

Signa

Declared at Boise ID on 16 of February 2016
[place] [day] [month] [year]

Before [redacted]

.....

Signature of person before whom this declaration is made

[redacted]

[Full name, qualification and address of person before whom this declaration is made]

Part 1 General Requirements

Executive Summary

SPS International Inc. (SPSII) has pioneered a new approach that marks a significant and vital advance in plant breeding. Innate® technologies transform potato plants with potato genomic DNA, without the incorporation of selectable markers or vector backbone sequences.

Innate® technologies were used to address two critical potato quality issues: (1) an acrylamide by-product that is produced when potatoes are heated, causing reducing sugars to react with asparagine through the Maillard reaction, that also results in a variety of desired compounds contributing to flavour, aroma, and browning; and (2) susceptibility to enzymatic darkening and discoloration, which happens when polyphenol oxidase leaks out of damaged plastids of bruised and cut potatoes. In the cytoplasm, the enzyme oxidises phenols, which then rapidly polymerise to produce dark pigments.

The potato variety Russet Burbank was transformed with a DNA insert containing two RNA interference cassettes in an *Agrobacterium* vector (pSIM1278). The resulting event (E12) has reduced expression of four potato enzymes (asparagine synthetase, polyphenol oxidase, water dikinase and phosphorylase). No new polypeptides are produced from this construct as the RNA catalyses the degradation of specific mRNA that down-regulates the target genes within the plant. The E12 event with the desired modified traits was characterised and is the subject of this submission (OECD Unique Identifier: SPS-∅∅E12-8).

In addition to the analysis of the desired traits, molecular characterisation of the event was performed to determine the number of copies, arrangement and stability of the inserted DNA. The event was confirmed to be free of *Agrobacterium*-derived backbone DNA. In the United States confined field trials were undertaken over three years, with the conventional variety and other cultivated varieties as controls. Observations at these trials looked for changes which could have an impact on the environment or affect genetic stability. Compositional analysis was performed on field-grown tubers to compare nutritional and anti-nutritional compounds and to determine if any biologically relevant differences existed that could result in increased risk to humans or other non-target organisms. There are no new polypeptides produced from the inserted DNA and thus there are no new potential toxins or allergens produced in Innate® potatoes.

Analysis of the E12 potato event has not revealed any biologically relevant differences compared to the conventional variety, except for the intended low free asparagine, low reducing sugars, and low polyphenol oxidase activity. Collectively, results of the molecular characterisation, agronomic assessment, and composition analysis support our application for amendment to the *Australia New Zealand Food Standards Code* to allow inclusion of the Innate® potato event E12 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

Applicant Details

(a)	Applicant's name/s	[redacted]
(b)	Company/organisation name	SPS International Inc.
(c)	Address (street and postal)	999 W Main St #1300, Boise, ID 83702, USA
(d)	Telephone number	[redacted]
(e)	Email address	[redacted]
(f)	Nature of the applicant's business	Regulatory Manager
(g)	Details of other individuals, companies or organisations associated with the application	PTM Solutions Australia Pty Ltd 11 Moras Court Gisborne, VICTORIA 3437 [redacted]

Purpose of the Application

This application seeks to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of potato event E12 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

SPSII has developed and tested potato events that have reduced expression of four specific enzymes. The Innate® event described in this application has the unique OECD code: SPS-∅∅E12-8, and is referred to as E12 within this submission. SPSII currently does not intend to import the Innate® potatoes into Australia or New Zealand. The primary aim of this application is to obtain a food approval in case of inadvertent presence in imported foods. This submission is consistent with SPS membership in the Excellence Through Stewardship® (ETS) program, adhering to stewardship and industry best practice by obtaining regulatory approvals across the global supply chain.

Relevant Overseas Approvals

Applications for approval of SPS-∅∅E12-8 have been submitted to other jurisdictions (Table 1).

Responsible environmental stewardship and deployment of biotechnology-derived products are important to SPS International Inc., its parent company, J.R. Simplot Company, and Simplot Plant Sciences (SPS). SPS is a member of Excellence Through Stewardship® (ETS), an industry-coordinated initiative that promotes the global adoption of stewardship programs and quality management systems for the full life cycle of biotechnology-derived plant products. The ETS "*Guide for Product Launch Stewardship of Biotechnology-Derived Products*" (ETS, 2013) also references and is consistent with the product launch policies of the Biotechnology Industry Organisation and Crop Life International.

Table 1: Current Applications and Approval Status for SPS-ØØE12-8.

Country	Competent National Authority	Type of Authorisation	Application Status	Approval Date
United States	United States Department of Agriculture (USDA)	Determination of non-regulated status	Approved	10 Nov 2014
	Food and Drug Administration (FDA)	Consultation completed; no further questions regarding use in food or feed	Completed	20 Mar 2015
	Environmental Protection Agency (EPA)		Not Applicable	
Canada	Canadian Food Inspection Agency (CFIA)	Unconfined environmental release	Under review	
		Use in livestock feed	Under review	
	Health Canada	Food approval	Under review	
Mexico	Department of Health (COFEPRIS)	Authorisation for food and feed use	Under review	
Japan	Ministry of Health, Labour and Welfare (MHLW)	Internal review of food safety assessment	Under review	
	Ministry of Agriculture, Forestry and Fisheries (MAFF)	Approval for feed use	Under review	
Korea	Ministry of Food and Drug Safety	Authorisation for food and feed use	Under review	

Justification for the Application

SPS International Inc. has developed a new potato event, E12. The new potato event was created using an insert containing potato DNA sequences that confers lower levels of free asparagine and reducing sugars, which together contribute to reduced acrylamide potential, and lower levels of polyphenol oxidase which reduces black spot.

Reduced Black Spot in Potatoes: Black spot refers to the black or greyish colour that may form in damaged or cut potatoes. It is a post-harvest physiological effect resulting from the handling of potato tubers during harvest, transport, processing, and storage and it contributes to waste experienced by growers, consumers, and processors. The enzymatic discoloration is associated with polyphenol oxidase (PPO) and occurs when the enzyme leaks out of the plastids of potatoes (Vaughn et al., 1988). Potatoes with black spot are typically either trimmed or rejected before processing, resulting in quality control challenges and economic loss. Lowering PPO levels in potatoes reduces the occurrence of black spot and this reduces grower, consumer, and processor waste.

Reduced Acrylamide Potential: Lowering the acrylamide potential of potatoes is important because acrylamide presents a potential health risk for consumers (FDA, 2013). Although acrylamide is not present in fresh potatoes, it forms in carbohydrate-rich foods when the amino acid asparagine and the reducing sugars, glucose and fructose, are heated at temperatures above 120 °C (O'Brien and Morrissey, 1989). Lowering the concentrations of free asparagine, glucose, and fructose in potatoes reduces the acrylamide potential of cooked potatoes. The biochemical basis of acrylamide formation has been published by Stadler, 2005.

Potatoes with lower acrylamide may address a potential health concern for consumers, especially in light of recent toxicology studies (Food Drink Europe , 2014; Health Canada, 2012; NTP, 2012). Various governments have responded to the findings of acrylamide in food by providing guidance documents and conducting surveys to assess the source and intake of acrylamide in foods (EFSA CONTAM Panel, 2015; FDA, 2006; Food Drink Europe, 2014). Through one such survey, the United States FDA determined that potato products contribute 35% of the acrylamide exposure through diet in the United States (FDA, 2006). Along with studies to mitigate the levels of acrylamide in food, the scientific community has also been working to understand the health implications of acrylamide consumption (NTP, 2012).

The United States FDA has proposed guidance for industry on the reduction of acrylamide levels in food products (FDA, 2013). In their *Draft Guidance for Industry on Acrylamide in Foods*, the FDA notes "Reducing acrylamide in foods may mitigate potential human health risks from exposure to acrylamide." An extensive list of potential mitigation techniques were summarised in the guidance document (FDA, 2013), focusing primarily on the reducing sugar levels in potatoes. Many of the methods in FDA's Guidance document are consistent with those reported in the *Acrylamide Toolbox* published by Food Drink Europe (Food Drink Europe, 2014).

Estimated dietary exposures of Australian consumers to acrylamide in food were investigated as a part of the first phase of the 24th Australian Total Diet Study (FSANZ, 2014). The study found that the levels of acrylamide were generally lower than, or comparable to, those reported in previous Australian and international studies. However, the estimated dietary exposures of Australian consumers were in the range considered to be of possible concern to human health by the Joint Expert Committee on Food Additives.

In New Zealand, the Ministry for Primary Industries (MPI) reassessed dietary exposure with a survey of foods contributing to acrylamide intake in New Zealand (Cressey et al., 2012). The survey found that dietary exposure estimates have remained fairly constant since a previous survey in 2006.

International food regulators are working with industry to reduce acrylamide levels. New farming and processing techniques are being investigated to produce lower levels of acrylamide, for example, lowering cooking temperatures, using enzymes that reduce acrylamide formation, and obtaining raw materials with lower reducing sugar levels. However, reducing acrylamide in some foods, such as coffee, is difficult without changing its taste.

FSANZ is encouraging and supporting industry to use enzymes that reduce acrylamide formation and urging industry to adopt an *Acrylamide Toolbox* produced by the Confederation of the Food and Drink Industries of the EU (CIAA, 2013). A Codex working group has created a Code of Practice for reducing acrylamide in food (Codex, 2009). Both FSANZ and MPI contributed to the development of this Code of Practice.

Assessment Procedure

SPSII is anticipating that this application will be considered under the **General Procedure** for Administrative Assessment process by Food Standards Australia New Zealand.

Exclusive Capturable Commercial Benefit

SPSII acknowledges that the proposed amendment to the Standard will likely result in an exclusive capturable commercial benefit being accrued to the parent company (J.R. Simplot Company) as defined in Section 8 of the *FSANZ Act*.

[1] Food Drink Europe, 2014 (http://www.fooddrinkeurope.eu/publications/category/toolkits/)

International and other National Standards

The Codex *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants* (CAC/GL 45-2003, 2006) is applicable to the assessment of this application to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of event SPS-∅∅E12-8 in **Standard 1.5.2**–Food Produced Using Gene Technology.

Costs and Benefits

Today, one of the main global challenges is how to ensure food security for a growing population whilst ensuring long-term sustainable development. According to the FAO, food production will need to grow by 70% to feed world population which could reach 9 billion by 2050 (Alexandratos and Bruinsma, 2012). Further trends, such as increasing urban population, shift of lifestyle and diet patterns of the rising middle class in emerging economies, along with climate change, put considerable pressure on the earth's resources. Freshwater resources and biodiversity may suffer decline and loss of fertile land occurs with urbanisation trends.

In the meantime, while the number of food insecure population remains unacceptably high, each year, worldwide, massive quantities of food are lost due to spoilage and infestations on the journey from farm to consumers. One of the major ways of strengthening food security is by reducing these post-harvest losses.

The term 'post-harvest loss' refers to measurable quantitative and qualitative food loss in the post-harvest supply chain. The supply chain comprises interconnected activities from the time of harvest through crop processing, marketing and food preparation, to the final decision by the consumer to eat or discard the food.

Nowadays, post-harvest loss reduction interventions are seen as an important component of efforts to reduce food insecurity. Post-harvest loss reduction is increasingly recognised as part of an integrated approach to realising agriculture's full potential to meet the world's increasing food and energy needs. Therefore, reducing post-harvest loss by making more effective use of today's crops, improving productivity on existing farmland, and bringing additional acreage into sustainable production, is critical to facing the challenge of feeding an increasing world population.

As a global staple food crop, post-harvest losses in the potato supply chain due to black spot, enzymatic darkening, have been a major cause of waste and economic loss. These issues occur in the fresh and processed food supply chains in both industrial and third world countries across the globe.

Enzymatic darkening is a widespread colour reaction occurring in fruits and vegetables, which involves the interaction of oxygen, phenolic compounds and polyphenol oxidases (PPOs). Darkening is usually initiated by bruising of the potato caused by impact and pressure during harvest and storage. It also is initiated by slicing/dicing/juicing fresh fruit and/or vegetables for use in fresh consumption or as part of preparation for further processing. As a result, PPO catalyses the enzymatic oxidation and conversion of monophenols to o-diphenols and o-dihydroxyphenols to o-quinones. The quinone products polymerise and react with amino acid groups of cellular proteins, resulting in black or brown pigment deposits ('darkening').

A variety of fruits and vegetables, such as apple, pear, banana, peach, lettuce and potato, are especially susceptible to enzymatic darkening during storage and processing. Darkening has a negative effect on appearance and may impair other sensory properties including taste, odour and texture.

With E12's potential to reduce black spot by up to 44%, significant savings can be realized both economically and environmentally. For example, if E12 replaced all Russet Burbank's in the United States fresh market, this would save 635 million kg of potato waste annually, resulting in a reduction of $90 million in producer costs. This amount of waste savings would translate to an annual reduction of 27 million kg of CO_2 emissions, 25 billion fewer litres of water and up to 69,000 hectare applications of pesticides. These estimates are based

on the reduced acres that would need to be planted to achieve the same marketable yield that would result from E12 replacing conventional Russet Burbank potatoes in the fresh market.

E12 potatoes utilise RNAi gene silencing technology to regulate the expression of the genes responsible for the enzymatic darkening process. As a result, Innate® potatoes are less susceptible than conventional potatoes to darkening and the onset of black spot from bruising caused by impact and pressure during harvest, storage and food preparation.

Research has demonstrated potential for the following benefits to be captured by United States potato farmers, supply chain participants and consumers following the introduction of Innate® technology in a range of potato varieties.

- E12 potatoes bruise up to 44% less than conventional varieties
- Using Innate® technology, it is possible for packers to experience an estimated 15% increased pack-out of fresh-grade potatoes, providing better utilisation, improved processing efficiencies and less waste.
- Because E12 potatoes show virtually no signs of bruise, there will be fewer rejected loads by processors and a reduction in price discounts based on quality downgrades due to bruising.
- Consumers will throw away fewer fresh potatoes up to 28% by some estimates in the United States alone, representing 1.48 billion kg per year which has been estimated to cost upwards of $1.44 billion annually according to the United States Journal of Consumer Affairs.
- It is worth noting that food approvals in influential countries like Australia, increase the likelihood that these potatoes can become available in developing countries, where they can be even more valuable in helping alleviate hunger in resource-scarce parts of the world. Simplot is working to help ensure this can happen by partnering with Michigan State to develop varieties for Bangladesh and Indonesia farmers. This initiative is part of the United States Government's Feed the Future initiative.

In addition to reduced black spot, E12 potatoes have lower levels of asparagine and reducing sugars, which decreases the potential for acrylamide formation. Acrylamide is a chemical compound that occurs when potatoes, wheat, coffee, and other foods are cooked at high temperatures. It is classified as a probable carcinogen by the United States FDA and the European Food Safety Authority. Guidance from many regulatory agencies advises limiting dietary intake of acrylamide.

Most potatoes consumed in Australia are grown domestically. Domestic production of potato in Australia (2013/14 - 1,171,300 metric tonnes) is supplemented by imports of processed potato, predominantly from the United States (2012/13 108,623 metric tonnes). Australian potato exports were 52,371 tonnes for the year ending June 2013. This was made up of 37,766 tonnes fresh product and 14,605 tonnes processed product.

Of the total domestic production 53% is sent to processing while the remaining 47% is sold in fresh form. When local processed volumes are combined with the 108,622 tonnes of imported product, a total of 412,343 tonnes is supplied to the processing sector. The majority (79%) of this is distributed to foodservice outlets, while 19% is distributed to retail outlets and 4% is exported.

Potatoes enjoy consistent market penetration year round at 52-58%, confirming their role as a staple Australian product in both summer and winter menus. Family households typically buy more potatoes at each purchase than smaller households, while households with lower discretionary income are more likely to purchase lower priced pre-packed products compared to those with higher discretionary income. (AusVeg[2], August 2015)

[2] Ausveg's Potato Consumer Research http://ausveg.com.au/potatoes/potato-consumer-research.htm.

In Australia, consumer research has confirmed that all major uses of potato involve some level of cooked preparation. The versatility of potato has led to a range of uses, with the majority being centred around dinner, and to lesser degree lunch time occasions. Seasonal influences have little bearing on the main uses of potato, reflecting patterns of consistent consumption year round. Within the Australian diet potatoes are prepared and consumed in the following forms:

- Boiled, Microwaved, Steamed;
- Deep fried;
- Mashed;
- Roasted;
- Baked/Grilled;
- Salad cooked;
- Soup/Sauce;
- Stir fry;
- Juiced;
- Sandwich/burger/wrap; and
- Snacks potato chips / straws.

In Australia, for the year ending June 2013, per capita consumption for fresh potatoes purchased through retail for home consumption was 14.56 kg, and for fresh potatoes purchased and consumed in food service away from home was 3.24 kg. Per capita consumption for processed potato products purchased through retail for home and foodservice consumption was 17.73 kg. Therefore, total annual consumption was 35.53 kg per capita.

The importance of potato and the impact of black spot darkening in the fresh potato market is demonstrated in Ausveg's Potato Consumer Research of August 2015 that reported that - "Consumers are concerned about wastage and are seeking information on freshness (best before dates). This could be in the form of providing estimated freshness for loose potatoes at the point of sale, such as 'will last for 2 weeks in your cupboard."

Release of Information

SPSII is submitting the information in this application for review by Food Standards Australia New Zealand (FSANZ) for amendment to the Food **Standard 1.5.2** Food produced using gene technology. SPSII holds proprietary rights to the extent allowable by law to all such information and by submitting this information, SPSII does not authorise its release to any third party except to the extent it is duly requested under the Freedom of Information Act 1982 (*FOI Act*) or in compliance with the responsibility of FSANZ to publish documents required under Sections 8, 8(A), 8(C) and 8(D) of the *FOI Act*; and this information is responsive to the specific aforementioned request. Accordingly, except as specifically stated above, SPSII. does not authorise the release, publication or other distribution of this information (including website posting or otherwise), nor does SPSII authorise any third party to use, obtain, or rely upon any such information, directly or indirectly, as part of any other application or for any other use, without SPSII's prior notice and written consent. Submission of this information does not in any way waive SPSII's rights (including rights to exclusivity and compensation) to such information.

Confidential Commercial Information (CCI)

Confidential Commercial Information (CCI) has not been included in this submission document. However, the following supportive Study Reports do contain Confidential Commercial Information and are attached as separate CCI documents:

[redacted]

Table of Contents

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Abbreviations, Acronyms and Definitions[3]

Abbreviation/Acronym	Definition
ADP	Adenosine diphosphate
AGP	pAgp-derived probe used in DNA gel blot hybridization
ALA	Alanine
APV	Adjusted p-value
A. tumefaciens	*Agrobacterium tumefaciens*
ARG	Arginine
ASN	Asparagine
ASN1	*Asn1* gene-derived probe used in DNA gel blot hybridisation
Asn1	Asparagine synthetase-1 gene
ASP	Aspartic acid
Atl	Non-transformed Atlantic control
Backbone DNA	DNA associated with vector backbone
blastn	Basic Local Alignment Search Tool for nucleotide sequences
BLASTP	Basic Local Alignment Search Tool for proteins
bp	Base pair
BsrGI	Restriction enzyme
CFIA	Canadian Food Inspection Agency
chs	Chalcone synthase gene
CLR	Combined literature range, derived from scientific literature
Ct	Threshold Cycle time in Real-Time PCR
cwt	Hundredweight, equivalent to one hundred pounds
cwt/A	Unit of measure in hundredweight per acre
CYS	Cysteine
DIGII	Molecular weight markers
DIGIV	Molecular weight markers
DNA	Deoxyribonucleic acid
DNA insert	The DNA sequence from pSIM1278 located between the LB and RB intended to be integrated into the potato genome
dsRNA	Double-stranded RNA
EB	Ethidium bromide
*Eco*RI	Restriction enzyme
*Eco*RV	Restriction enzyme
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FG	Field grown
g	Gram
G0	First clonal cycle greenhouse-grown tuber seed
G1	First clonal cycle field-grown tuber seed
G2	Second clonal cycle field-grown tuber seed
G3	Third clonal cycle field-grown tuber seed
GBS1	*pGbss*-derived probe used in DNA gel blot hybridisation; = GBS
Gbss	Granule-bound starch synthase gene gene
GLN	Glutamine
GLU	Glutamic acid
GLY	Glycine
GM	Genetically modified
gus	β-glucuronidase gene

[3] NOTE: Abbrev at ons of un ts of measure and of phys ca and chem ca quant t es are used accord ng to the standard format descr bed n Instruct ons to Authors n the Journa of B o og ca Chem stry (http://www.jbc.org/).

Abbreviation/Acronym	Definition
HC	Health Canada
Hd	Restriction enzyme *Hin*d III
HD121F	Primer set
HD121R	Primer set
*Hind*III	Restriction enzyme
HIS	Histidine
IB	Internal bands
IgE	Immunoglobulin E antibody
ipt	Isopentyltransferase gene – produces cytokinin hormones associated with plant growth and development
ILE	Isoleucine
JC	Atlantic controls
JB	Junction band
kb	Kilobase
kcal	Kilocalorie
LB	Left Border (a 25-base pair sequence) similar to *A. tumefaciens* T-DNA border
Innate®	A branded biotechnology approach that uses plant genes to enhance desired traits
Left Border side	The region of the DNA insert near the Left Border, which may either extend up to the cleavage site in the Left Border or be truncated at a position of between a single base pair and 100s of base pairs downstream from that cleavage site
Left Border site	The 25-base pair sequence defined as Left Border, similar to *A. tumefaciens* T-DNA
LEU	Leucine
LYS	Lysine
µg	Microgram
MAFF	Japan Ministry of Agriculture, Forestry, and Fisheries
MET	Methionine
MfeI	Restriction enzyme
mg	Milligram
MHLW	Japan Ministry of Health, Labor, and Welfare
MSU	Michigan State University
N	Sample size
NBY	Nutrient broth-yeast extract
NC	Negative control
NCBI	National Center for Biotechnology Information
*Nde*I	Restriction enzyme
NGS	Next generation sequencing
NNPAS	National Nutrition and Physical Activity Survey
Non-coding DNA	DNA not coding for RNA that is translated into protein
*npt*II	Neomycin phosphotransferase II gene – used as/for marker for plant transformation, gene expression, and regulation studies
nr	Non-redundant
OB	Snowden endogenous bands
OECD	Organisation for Economic Cooperation and Development
ORFs	Open reading frames
*Pac*I	Restriction enzyme
pAgp	Promoter of the ADP glucose pyrophosphorylase gene
PB	Plasmid bands
pg	Pictogram
PCR	Polymerase chain reaction
pGbss	Promoter of the granule-bound starch synthase gene
PHE	Phenylalanine
PhL	Phospholrylase-L gene

Abbreviation/Acronym	Definition
ppb	Parts per billion
ppm	Parts per million
PPO	Polyphenol oxidase enzyme
Ppo5	Polyphenol oxidase-5 gene
Pro	Functionally-active promoter
PRO	Proline
R1	Water dikinase R1 gene
R1	Southern blot probe used to detect the R1 cassette
RB	Right Border (a 25-base pair sequence) similar to *A. tumefaciens* T-DNA border
Right Border side	The region of the DNA insert near the Right Border, which may either extend up to the cleavage site in the Right Border or be truncated at a position of between a single base pair and 100s of base pairs upstream from that cleavage site
Right Border site	The 25-base pair sequence defined as Right Border, similar to *A. tumefaciens* T-DNA
RNA	Ribonucleic acid
RNAi	RNA interference
*Sca*I	Restriction enzyme
SER	Serine
Sol t 1	42 kDa allergen known as patatin
Somaclonal Variation	Genetic and/ or phenotypic variation among clonally propagated plants of a single donor clone; generated by tissue culture and other forms of vegetative propagation
SPS-∅∅E12-8	Potato Event E12–pSIM1278 transformed into variety Russet Burbank
SPSII	SPS International Inc., the applicant
T-DNA	Transfer DNA from *A. tumefaciens* delineated by left and right border sequences
THR	Threonine
TRP	Tryptophan
TYR	Tyrosine
Ubi7	Polyubiquitin 7 promoter
USDA	United States Department of Agriculture
USDA-APHIS	United States Department of Agriculture-Animal and Plant Health Inspection Service
VAL	Valine
wt	Wild type, synonym for control
WT	Genomic DNA of Russet Burbank control
*Xba*I	Restriction enzyme

Part 2 Specific Data Requirements for Safety Assessment

The following information is provided to support an application for a new genetically modified food. The details presented are in accordance with Section 3.5.1. of the FSANZ Application Handbook as at 1 September 2013 (amended 1 June 2015).

A. Technical Information on the Genetically Modified Food

A1. Nature and Identity of the Genetically Modified Food

A1(a) A description of the GM organism from which the new GM food is derived. The description must include the nature and purpose of the genetic modification.

The potato event E12 was developed to confer lower levels of free asparagine and reducing sugars, and reduced polyphenol oxidase, which contribute to reduced acrylamide potential and reduced black spot.

The potato variety Russet Burbank was transformed with a DNA insert containing two cassettes from pSIM1278, an *Agrobacterium* vector. The resulting event has down-regulated expression of four specific enzymes (asparagine synthetase, polyphenol oxidase, water dikinase, and phosphorylase). No new polypeptides are produced from this construct as the RNA product catalyses the degradation of specific mRNA and thus down-regulates the target genes within the plant. Event E12 is the subject of this submission. This event has been extensively characterised and shows the desired modified traits.

A1(b) The name, number or other identifier of each of the new lines or strains of GM organism from which the food is derived.

In accordance with OECD '*Guidance for the Designation of a Unique Identifier for Transgenic Plants*', the OECD Unique Identification Code for the potato event E12 is SPS-∅∅E12-8.

A1(c) The name the food will be marketed under (if known).

Some E12 potatoes containing the Innate® technology are marketed as White Russet potatoes on the United States fresh potato market. Others are marketed under a variety of labels as fresh cut, fries or crisps, depending on the licenced user of the event.

A1(d) The types of products likely to include the food or food ingredient.

Potato products containing the Innate® technology will include both fresh and processed potatoes (i.e. fresh cut, fries, chips, dehydrated potato and starch).

A2. History of use of the host and donor organisms

A2(a) A description of all the donor organism(s) from which the genetic elements are derived, including:

A2(a) (i) Common and scientific names and taxonomic classification

Donor DNA in the insert consists of non-coding genetic elements as described in Table 2.

All sequences are from *Solanum tuberosum* var. Ranger Russet (Potato) except for 2 elements derived from *S. verrucosum,* a diploid (2n=24) wild potato species from Mexico.

ORDER: Solanales

FAMILY: Solanaceae

GENUS: *Solanum*

SPECIES: *S. tuberosum* and *S. verrucosum* Schltdl

COMMON NAME: Potato

A2(a) (ii) Information about any known pathogenicity, toxicity or allergenicity of relevance to the food

Details of the pathogenicity, toxicity or allergenicity of potato are described in the OECD Consensus Document on Compositional Considerations for New Varieties of Potatoes: Key Food and Feed Nutrients, Anti-nutrients and Toxicants (OECD, 2002).

Potatoes are not known to cause disease in humans or animals and have a long history of safe use as a food. Several features of this commodity relate to toxicity and allergenicity, and are briefly discussed below.

All potatoes contain natural toxins called glycoalkaloids of which the most prevalent are solanine and chaconine. Solanine is also found in other plants in the family Solanaceae, which includes such plants as the deadly nightshade (*Atropa belladonna*), henbane (*Hyoscyamus niger*), tobacco (*Nicotiana*), as well as eggplant and tomato.

Until recently potatoes were not considered a source of allergens. However, potato contains heat-labile proteins which can induce immediate hypersensitivity reactions when raw potatoes are consumed (Jeannet-Peter et al., 1999).

A study on patatin, the main storage protein in potatoes, reports induction of allergic reactions in sensitive children (Seppälä et al., 1999). These authors consider additional studies necessary in order to confirm the allergenicity of patatin. In addition to patatin, concomitant IgE binding to several proteins belonging to the family of soybean trypsin inhibitors was observed (Seppälä et al., 2000).

No sequences associated with either glycoalkaloids or patatin proteins were used in creating the potato event.

Table 2. Genetic Elements of pSIM1278, from Left Border to Right Border

Genetic Element	*Origin*	Accession Number	Position (pSIM1278)	Size (bp)	Intended Function
1. Left Border (LB) s te[1]	Synthet c	AY566555[2] (bases 1-25)	1 – 25	25	S te for secondary c eavage to re ease s ng e-stranded DNA nsert from pSIM1278 (van Haaren et a ., 1989)
2. Left Border reg on sequence	*S. tuberosum* var. Ranger Russet.	AY566555[2] (bases 26-187)	26 – 187	162	Supports secondary c eavage at LB
3. Interven ng Sequence	*S. tuberosum*	AF393847	188 –193	6	Sequence used for DNA c on ng
4. Promoter for the ADP g ucose pyrophosphory ase gene (pAgp), 1st copy	*S. tuberosum* var. Ranger Russet	HM363752	194-2,453	2260	One of the two convergent promoters that dr ves express on of an nverted repeat conta n ng fragments of Asn1 and Ppo5, espec a y n tubers
5. Fragment of the asparag ne synthetase-1 (Asn1) gene (1st copy ant sense or entat on)	*S. tuberosum* var. Ranger Russet	HM363759	2,454-2,858	405	Generates w th (11) doub e stranded RNA that tr ggers the degradat on of Asn1 transcr pts to mpa r asparag ne format on (Chaw a et a ., 2012[2])
6. 3'-untrans ated sequence of the po ypheno ox dase-5 gene (*Ppo5*) (1st copy, n ant sense or entat on)	*S. verrucosum*	HM363754	2,859-3,002	144	Generates w th (9) doub e stranded RNA that tr ggers the degradat on of Ppo5 transcr pts to b ock b ack spot deve opment
7. Interven ng Sequence	*S. tuberosum*	DQ478950	3,003-3,008	6	Sequence used for DNA c on ng
8. Spacer-1	*S. tuberosum* var. Ranger Russet	HM363753	3,009-3,165	157	Sequence between the 1st nverted repeats
9. 3'-untrans ated sequence of the po ypheno ox dase-5 gene (*Ppo5*) (2nd copy, n sense or entat on)	*S. verrucosum*	HM363754	3,166-3,309	144	Generates w th (6) doub e stranded RNA that tr ggers the degradat on of Ppo5 transcr pts to b ock b ack spot deve opment
10. Fragment of the asparag ne synthetase-1 (*Asn1*) gene (2nd copy, n sense or entat on)	*S. tuberosum* var. Ranger Russet	HM363759	3,310-3,715	406	Generates w th (5) doub e stranded RNA that tr ggers the degradat on of Asn1 transcr pts to mpa r asparag ne format on (Chaw a et a ., 2012[2])
11. Interven ng Sequence	*S. tuberosum*	X73477	3,716-3,721	6	Sequence used for DNA c on ng
12. Promoter for the granu e-bound starch synthase (*pGbss*) gene (1st copy, convergent or entat on re at ve to the 1st copy of pAgp)	*S. tuberosum* var. Ranger Russet	HM363755	3,722-4,407	686	One of the two convergent promoters that dr ves express on of an nverted repeat conta n ng fragments of Asn1 and Ppo5, espec a y n tubers
13. Interven ng Sequence	*S. tuberosum*	X95996 / AF393847	4,408-4,423	16	Sequence used for DNA c on ng
14. pAgp, 2nd copy	*S. tuberosum* var. Ranger Russet	HM363752	4,424-6,683	2260	One of the two convergent promoters that dr ves express on of an nverted repeat conta n ng fragments of the promoters of PhL and R1, espec a y n tubers

Genetic Element	*Origin*	Accession Number	Position (pSIM1278)	Size (bp)	Intended Function
15. Fragment of promoter for the potato phosphory ase-L (*PhL*) gene (1st copy, n ant sense or entat on)	*S. tuberosum* var. Ranger Russet	HM363758	6,684-7,192	509	Generates w th (21) doub e stranded RNA that tr ggers the degradat on of PhL transcr pts to m t the format on of reduc ng sugars through starch degradat on
16. Fragment of promoter for the potato R1 gene (1st copy, n ant sense or entat on)	*S. tuberosum* var. Ranger Russet	HM363757	7,193-7,724	532	Generates w th (20) doub e stranded RNA that tr ggers the degradat on of R1 transcr pts to m t the format on of reduc ng sugars through starch degradat on
17. Interven ng Sequence	*S. tuberosum*	DQ478950	7,725-7,730	6	Sequence used for DNA c on ng
18. Spacer-2	*S. tuberosum* var. Ranger Russet	U26831[3]	7,731-7,988	258	Sequence between the 2nd nverted repeat
19. Fragment of promoter for the potato R1 gene (2nd copy, n sense or entat on)	*S. tuberosum* var. Ranger Russet	HM363757	7,989-8,520	532	Generates w th (20) doub e stranded RNA that tr ggers the degradat on of R1 transcr pts to m t the format on of reduc ng sugars through starch degradat on
20. Fragment of promoter for the potato phosphory ase-L (*PhL*) gene (2nd copy, n sense or entat on)	*S. tuberosum* var. Ranger Russet	HM363758	8,521-9,029	509	Generates w th (16) doub e stranded RNA that tr ggers the degradat on of PhL transcr pt to m t the format on of reduc ng sugars through starch degradat on
21. pGbss (2nd copy, convergent or entat on re at ve to the 2nd copy of pAgp)	*S. tuberosum* var. Ranger Russet	X83220[4]	9,030-9,953	924	One of the two convergent promoters that dr ves express on of an nverted repeat conta n ng fragments of the promoters of PhL and R1, espec a y n tubers
22. Interven ng Sequence	*S. tuberosum*	AF143202	9,954 – 9,962	9	Sequence used for DNA c on ng
23. R ght Border reg on sequence	*S. tuberosum* var. Ranger Russet	AY566555[5] (bases 231-391)	9,963 – 10,123	161	Supports pr mary c eavage at RB s te
24. R ght Border (RB) sequence[1]	Synthet c	AY566555[5] (bases 392-416)	10,124 – 10,148	25	S te for pr mary c eavage to re ease s ng e stranded DNA nsert from pSIM1278 (van Haaren et a ., 1989)

[1]The LB and RB sequences (25 bp each) were synthet ca y des gned to be s m ar to and funct on ke T DNA borders from *Agrobacterium tumefaciens*
[2]ASN1 descr bed as genet c e ements 5 and 11 s referred to as StAst1 n Chaw a et a , 2012
[3] GenBank Access on HM363756 s rep aced w th a c tat on to GenBank Access on U26831 to proper y nc ude four 3' end nuc eot des present n the pGbss DNA e ement of the pS M1278 construct
[4] GenBank Access on HM363755 s rep aced w th a c tat on to GenBank Access on X83220 to proper y nc ude the fu pGbss (2nd copy) DNA nse t sequence present n the pS M1278 construct
[5]GenBank Access on AY566555 was rev sed to c ar fy the sources of DNA for the Border reg ons

A2(a) (iii) Information about the history of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant).

The potato is the world's fourth largest food crop, following rice, wheat, and maize. It has a long history in the diets of humans across the entire world.

The Inca Indians in Peru were the first to cultivate potatoes around 8,000 BC to 5,000 B.C.

In 1536 Spanish Conquistadors conquered Peru, discovered the flavours of the potato, and carried them to Europe. Before the end of the sixteenth century, families of Basque sailors began to cultivate potatoes along the Biscay coast of northern Spain. Sir Walter Raleigh introduced potatoes to Ireland in 1589 on the 40,000 acres of land near Cork. It took nearly four decades for the potato to spread to the rest of Europe.

Potatoes arrived in the Colonies in 1621 and the first permanent potato patches in North America were established in 1719. From there, the crop spread across the United States.

Source: International Year of the Potato (FAO, 2008)

A2(b) A description of the host organism into which the genes were transferred and its history of safe use for food, including:

A2(b) (i) Any relevant phenotypic information

The potato variety Russet Burbank has been transformed with pSIM1278. Russet Burbank represents significant value to the United States potato industry and relatively large percentage of the overall United States potato acreage (Figure 1).

Luther Burbank developed this variety in 1914 and it was introduced into Victoria, Australia, in the late 1970s[4]. Russet Burbank is a late maturing (140-150 day growing season) variety that is grown in the Northwest, north-central and north-eastern regions of the United States and in Canada. It is the standard for baking and processing quality. Russet Burbank tubers display white flesh, shallow eyes and have good long-term (around 9-10 months) storage characteristics. Plants are vigorous, spreading, thick stemmed and display an indeterminate growth habit. Blossoms are sparse, white, and infertile. This cultivar is tolerant to common scab (PAA, 2015b) and is one of Australia's major varieties for fries and wedges.

[4] Agr cu ture V ctor a (2010). http://agr cu ture.v c.gov.au/agr cu ture/hort cu ture/vegetab es/potatoes/potato-var et es

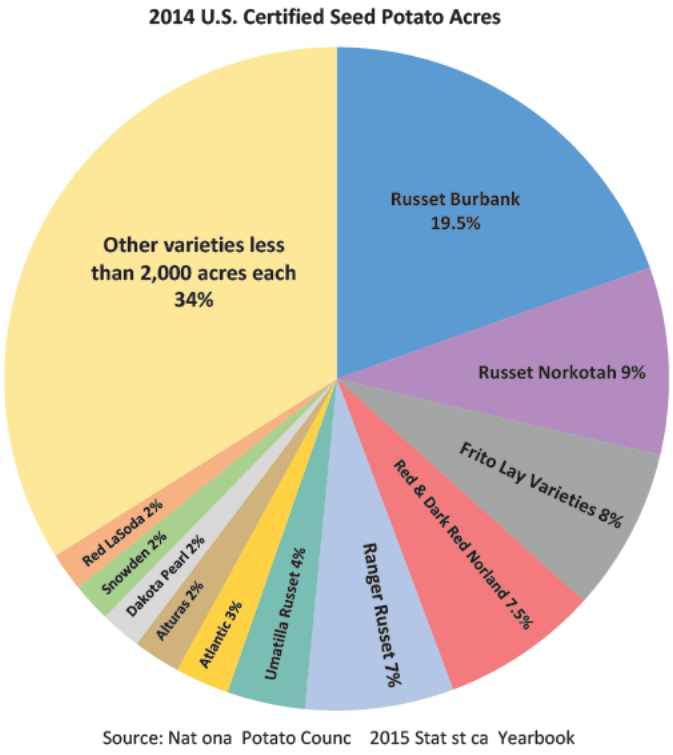


Figure 1. 2014 United States Potato Seed Acreage by Variety

This chart represents the top potato seed varieties grown in the United States during 2014. Varieties with less than 2,000 acres are combined to represent 34% of all other potato varieties planted in the United States in 2014. Data is sourced from NPC 2015 Statistical Yearbook http://yearbook2015.cloud-pages.com.

A2(b) (ii) How the organism is typically propagated for food use

Potato varieties do not have a high frequency of introduction and discontinuation compared to some other crops, such as corn or soybeans, due to challenges in potato breeding. Potato plants grown commercially are clones, produced clonally by mini-tubers or by tuber cuttings (seed pieces) that contain lateral buds or "eyes" (Figure 2). Thus, the genetic background of the initial parent is fixed within the subsequent clonal cycles.

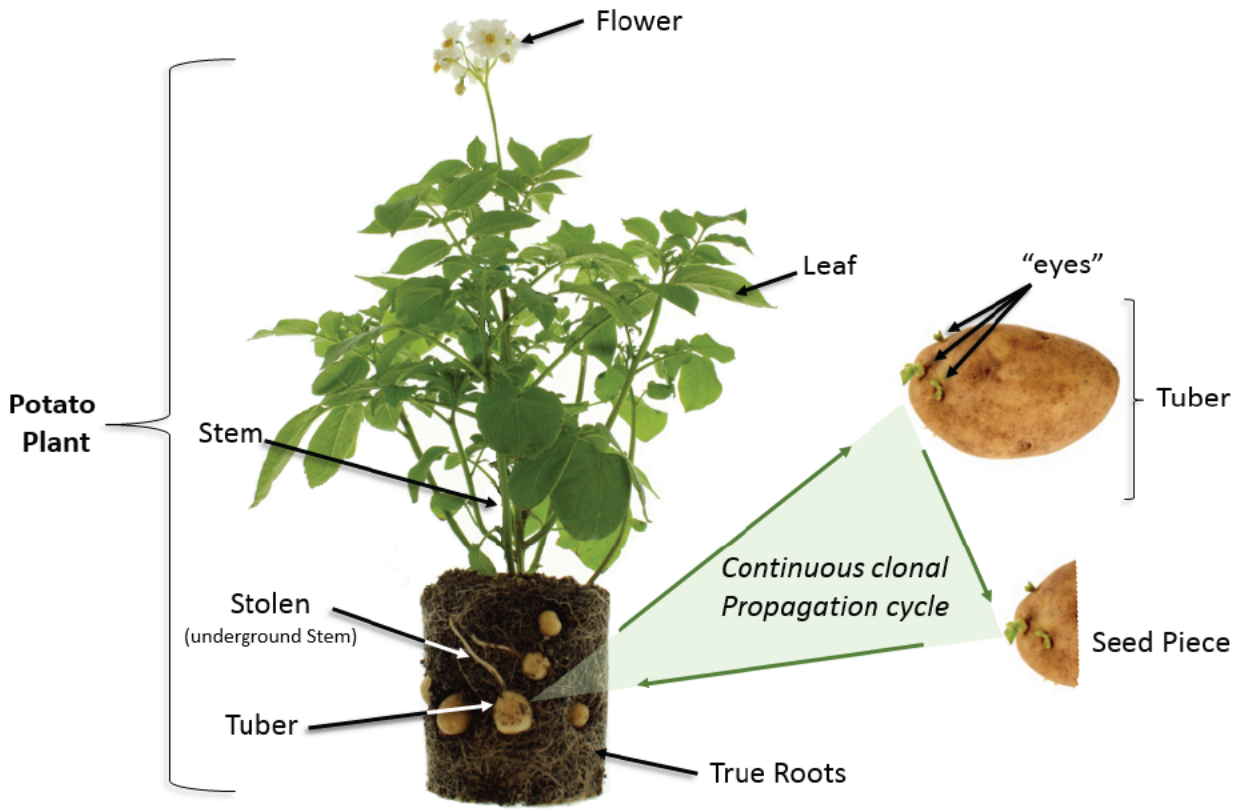


Figure 2. Potato Plant and Tuber Propagation Cycle

Image depicts mature potato plant and tuber propagation cycle used to clonally propagate the next clonal cycle of potato plants. Tubers are harvested and planted whole, or may be cut into seed pieces that contain lateral buds or "eyes". Once planted, sprouts emerging from the seed piece eyes develop into mature potato plants. This process of clonal propagation is repeated continuously throughout the life cycle of the variety. As a result of this clonal propagation, each potato clonal cycle is a genetic clone of the previous plant.

As a result of clonal propagation, each clonal cycle of potato is a genetic clone of its parent since the tuber (and not the true seed) is used to generate the next clonal cycle (Figure 2). An example of potato plant clonal cycles is shown in (Figure 3). Tuber production begins with "mother plants" containing the desired traits of interest. Cuttings from the mother plants are propagated in tissue culture (plantlets). These are transferred to greenhouses to grow mini-tubers (G0) for distribution to seed farmers. Mini-tubers are planted in fields to produce G1 tuber seed. After multiple seasons of re-planting tubers (generally 3 5 seasons), the tubers will be sold for commercial potato production and seed production continues with tissue culture mother plants that have been tested to confirm the presence of the desired traits and disease free status.

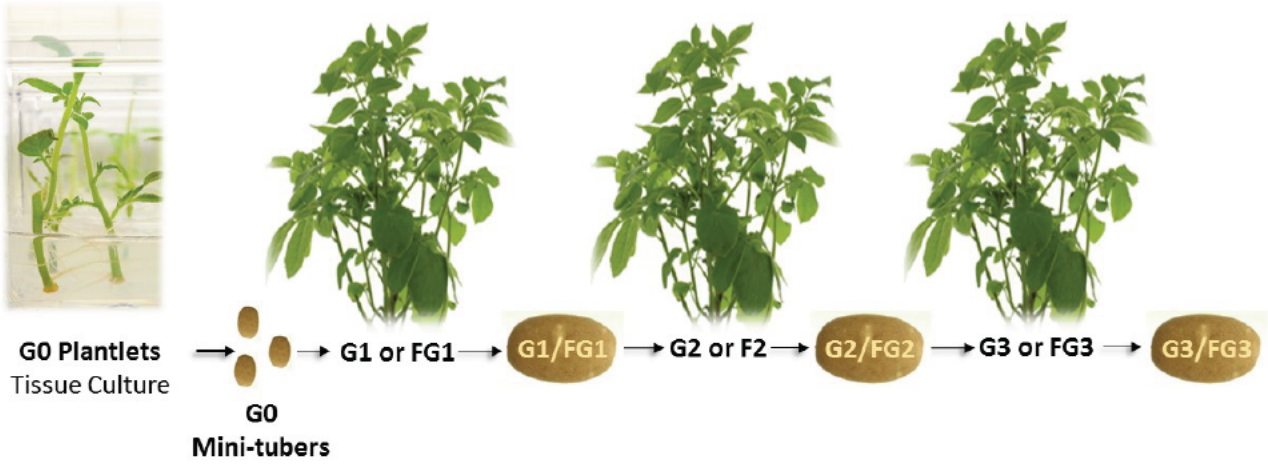


Figure 3. Commercial Production of Potatoes

For E12, plantlets are propagated from cuttings of the G0 tissue culture plantlet. Plantlets are transferred to soil or grown using Nutrient Film Technique (NFT) or hydroponics in greenhouses. Tubers from these G0 plants are referred to as mini-tubers. An entire mini-tuber is planted in either the greenhouse (G = greenhouse grown) or field (FG = field grown) to produce a new clonal potato plant. Tubers from G1/FG1 plants can be cut into 2-4 oz. (55-115 g) pieces, which contain lateral buds, and are used as "seed pieces" to produce G2/FG2 plants. The process of clonal propagation is repeated to generate additional clonal plants.

A2(b) (iii) What part of the organism is typically used as food

Potato tubers are the only part consumed as food.

A2(b) (iv) Whether special processing is required to render food derived from the organism safe to eat

Potato tubers for direct consumption should be cooked before eating because of the indigestibility of non-gelatinised starch and the presence of anti-nutritional proteins (OECD, 2002).

Potatoes are prepared and packaged fresh as well as processed for fries, chips and flakes.

Potatoes are prepared in many ways: skin-on or peeled, whole or cut up, with seasonings or without. The only requirement involves cooking to swell the starch granules. Most potato dishes are served hot, but some are first cooked, then served cold, notably potato salad and potato chips/crisps.

Other uses include:

- Used to brew alcoholic beverages such as vodka, potcheen, or akvavit;
- Feed for domestic animals;
- Potato starch is used in the food industry as, for example, thickeners and binders of soups and sauces, in the textile industry, as adhesives, and for the manufacturing of papers and boards; and
- Potato skins, along with honey, are a folk remedy for burns in India. Burn centres in India have experimented with the use of the thin outer skin layer to protect burns while healing.

A2(b) (v) The significance to the diet in Australia and New Zealand of food derived from the host organism.

Potatoes are a significant food in the diet of both Australians and New Zealanders.

Australia

According to the 2011-12 National Nutrition and Physical Activity Survey (Australian Bureau of Statistics 2013), Australians aged 2 years and over consumed an estimated 3.1 kg of foods and beverages (including water) per day, made up from a wide variety of foods across the major food groups. Vegetable products and dishes were consumed by 75% of the population, with potatoes making up around 25% (by weight) of all vegetables consumed. Around 52% of all potatoes consumed were boiled, baked, roasted, fried or grilled, with 32% eaten as chips, fries, wedges or similar products and 16% eaten in mixed dishes such as mashed potato or potato bake.

New Zealand

Potatoes are one of the most popular vegetables in New Zealand. In New Zealand there are over 200 potato growers and the growing area is 10,591 hectares. Typically, New Zealand produces between 500,000 and 550,000 metric tonnes of potatoes per annum (Potatoes.co.nz). Of this, up to 50% are processed for domestic consumption and a further 10% consumed fresh. The remaining crop is either retained as seed or exported (fresh or processed).

A3. The nature of the genetic modification

A3(a) A description of the method used to transform the host organism.

Event SPS-∅∅E12-8 (E12) was developed through *Agrobacterium*-mediated transformation (Figure 4). A C58-derived *Agrobacterium* strain, AGL1 (Lazo et al., 1991), carrying the pSIM1278 vector, was used to transform potato internode segments from *in vitro* grown Russet Burbank stock plants. *Agrobacterium*-mediated transformation was carried out using a modified procedure based on Richael et al., 2008. Potato stock plants were maintained in magenta boxes with half-strength M516 (PhytoTechnology) medium containing 3% sucrose and 2 g/l gelzan (propagation medium). Internode segments of 4 to 6 mm were cut from approximately four-week old plantlets and infected with *Agrobacterium* AGL1 strain carrying pSIM1278.

Infected internode segments were grown on regeneration medium M404 (PhytoTechnology) containing 3% sucrose and 2 g/l gelzan to foster callus formation with 1.2 ml/l Plant Protective Mixture (PhytoTechnology) and 300 mg/l timentin to inhibit *Agrobacterium* growth. Calluses were transferred every four weeks to fresh regeneration medium until visible shoots developed. Shoots were transferred to magenta boxes containing half strength hormone free medium with timentin to generate roots. Shoots containing the pSIM1278 backbone DNA and expressing *ipt* were discarded based on abnormal growth due to overproduction of the plant hormone cytokinin.

Once roots developed in approximately three weeks, leaf samples from the plantlets were collected and PCR analyses was performed to identify plantlets containing an insert from the pSIM1278 T-DNA. Tips from PCR positive plantlets were excised and transferred to fresh magenta boxes containing half strength medium and timentin to root plantlets and select against *Agrobacterium*.

Plantlets were allowed to develop for approximately three weeks and retested by PCR to confirm presence of T-DNA. Tips from PCR positive plantlets were excised and transferred to fresh magenta boxes containing half strength medium with timentin to continue selection against *Agrobacterium*.

Plantlets were allowed to mature and were retested a third time for the T-DNA insert by PCR. Leaf samples (4 to 6 mm) from mature plantlets testing positive for the T-DNA insert after the third test were collected and tested for the absence of *Agrobacterium*. Sterile, harvested leaves were removed and incubated in NBY

(nutrient broth-yeast extract) broth for 14-days. Leaf samples containing *Agrobacterium* resulted in visible cloudy growth in the NBY medium. Plants showing no bacterial growth in NBY broth after fourteen days were tested a second time to confirm absence of *Agrobacterium*.

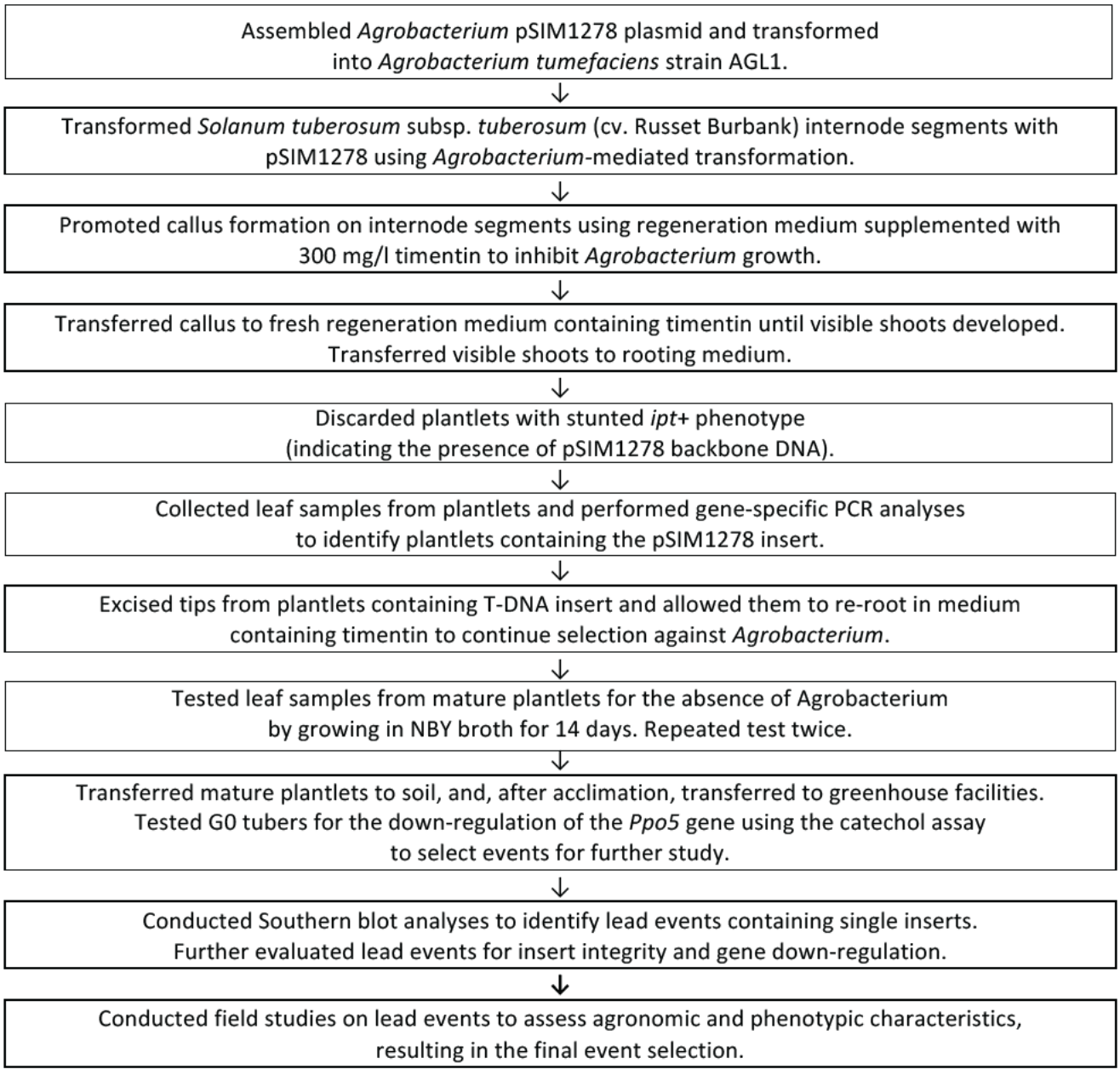


Figure 4. Schematic Diagram of Event Development and Selection

For the few plants that tested positive for presence of *Agrobacterium*, the tips of the plants were excised and re-cultured in fresh magenta boxes containing half strength medium and timentin to regenerate plantlets. After root formation, leaf samples from plantlets were re-tested in NBY broth to confirm absence of *Agrobacterium*. This process was continued until *Agrobacterium* could be confirmed absent via the NBY assay.

Agrobacterium-free plantlets were transferred to a peat-based soil mixture and slowly acclimated to the less humid conditions of the growth chamber or greenhouse.

A3(b) Information about the intermediate host organisms (e.g. bacteria) used for all laboratory manipulations prior to transformation of the host organism.

Standard *E. coli* laboratory strains were used to construct the transformation binary vector pSIM1278. This vector was transferred to *Agrobacterium tumefaciens* strain AGL1 for potato transformation.

A3(c) A description of the gene construct and the transformation vectors used.

The size, source, and function of each of the genetic components of pSIM1278 are provided in Table 2**.**

The plasmid, pSIM1278, is a 19.6 kb binary transformation vector used in the transformation of *in vitro* grown Russet Burbank stock plants to generate event SPS-∅∅E12-8. A plasmid map and a summary of the genetic elements in pSIM1278 are provided in Figure 5, Table 2 and Table 3.

Table 3. Genetic Elements of the pSIM1278 Plasmid Backbone

Genetic Element	Origin	Accession Number[1]	Position	Size (bp)	Function
1. Interven ng sequence	Synthet c DNA		10,149-10,154	6	Sequence used for c on ng
2. Overdr ve	*Agrobacterium tumefaciens* T -p asm d	NC_002377	10,155-10,184	30	Enhances c eavage of *A. tumefaciens* R ght Border s te [1]
3. Interven ng sequence	*Pseudomonas fluorescens* pVS1	AJ537514	10,185-11,266	1,082	pVS1 backbone[1]
4. pVS1 part t on ng prote n StaA (PVS1 Sta)	*P. fluorescens* pVS1	AJ537514	11,267-12,267	1,001	pVS1 stab ty [1]
5. Interven ng sequence	*P. fluorescens* pVS1	AJ537514	12,268-12,860	593	pVS1 backbone[1]
6. pVS1 rep con (pVS1Rep)	*P. fluorescens* pVS1	AJ537514	12,861-13,861	1,001	pVS1 rep cat on reg on n *Agrobacterium* [1]
7. Interven ng sequence	*P. fluorescens* pVS1	AJ537514	13,862-14,099	238	pVS1 backbone[1]
8. Interven ng sequence	pBR322	AF234297	14,100-14,270	171	pCamb a1301 backbone[1]
9. pBR322 bom	pBR322	AF234297	14,271-14,531	261	pBR322 reg on for rep cat on n *E. coli* [1]
10. Interven ng sequence	pBR322	AF234297	14,532-14,670	139	pBR322 backbone[1]
11. Or g n of rep cat on for pBR322 (pBR322 or)	pBR322	AF234297	14,671-14,951	281	Bacter a or g n of rep cat on [1]
12. Interven ng sequence	pBR322	AF234297	14,952-15,241	290	pCamb a1301 backbone[1]
13. Neomyc n phosphotransferase II (*nptII*) gene	Tn*5* transposon	FJ362602	15,242-16,036	795	Am nog ycos de phosphotransferase[1] (S mpson et a ., 1985)
14. Interven ng sequence	Vector DNA	FJ362602	16,037-16,231	195	pCAMBIA vector backbone[1]
15. Term nator of the ub qu t n-3 gene (*tUbi3*)	*S. tuberosum*	GP755544	16,232-16,586	355	Term nator for *ipt* gene transcr pt on (Garbar no and Be knap, 1994)
16. Interven ng sequence	*A. tumefaciens* T -p asm d	NC_002377	16,587-16,937	351	Sequence used for DNA c on ng
17. Isopenteny transferase (*ipt*) gene	*A. tumefaciens* T -p asm d	NC_002377	16,938-17,660	723	Condensat on of AMP and sopenteny -pyrophosphate to form sopenteny -AMP, a cytok n n n the p ant. Resu ts n abnorma growth phenotypes n p ant (Sm gock and Owens, 1988)
18. Interven ng sequence	Synthet c DNA		17,661-17,672	12	Sequence used for DNA c on ng
19. Po yub qu t n promoter (Ub 7)	*S. tuberosum* var. Ranger Russet	U26831	17,673-19,410	1,738	Promoter to dr ve express on of the *ipt* backbone marker gene (Garbar no et a ., 1995)
20. Interven ng sequence	Vector DNA	U10460	19,411-19,660	250	pZP200 vector backbone[1]

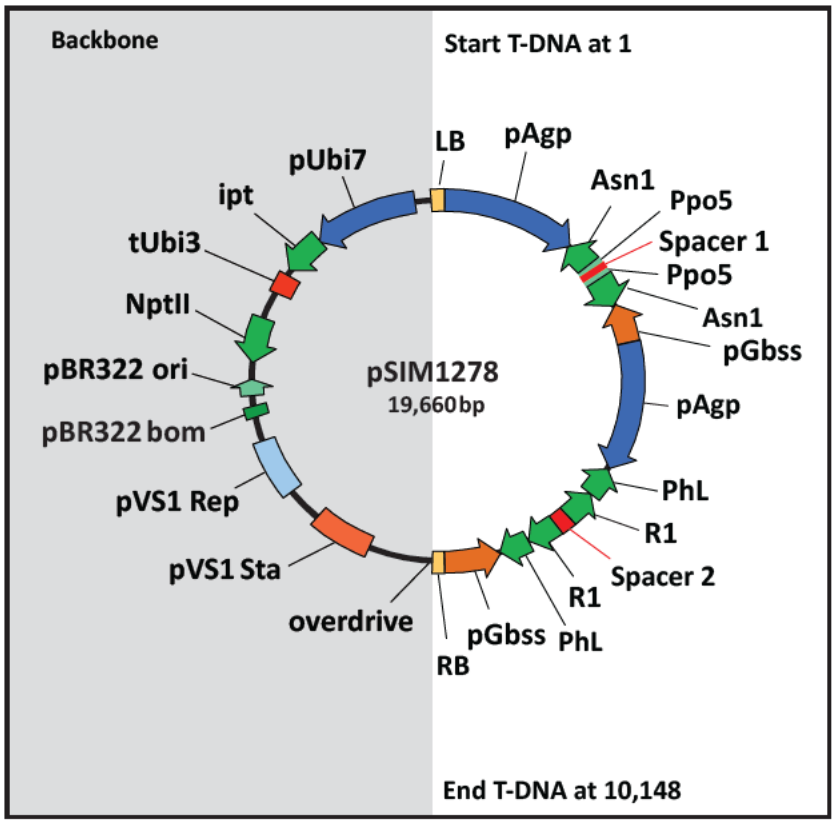


Figure 5. Plasmid Map of pSIM1278

Map shows the plasmid backbone sequence highlighted in grey and the T-DNA region flanked by the LB and RB sequences.

The backbone region is required for replication and selection of the plasmid in bacteria and is not intended for transfer into the plant genome. The T-DNA region, flanked by the LB and RB, comprises 10,148 bp and is intended for transfer into the plant genome. This region consists of two cassettes with RNAi sequences for the four targeted potato genes and the regulatory elements necessary for their expression (e.g. promoter) (Figure 5 and Figure 6).

The first cassette results in down-regulation of *Asn1* and *Ppo5* in the transformed potato variety. It is comprised of two identical 405-bp fragments of *Asn*1 and two identical 144-bp fragments of *Ppo5* (Figure 6). The fragments of *Asn*1 and *Ppo5* are arranged as inverted repeats separated by a non-coding 157-bp Ranger Russet potato nucleotide spacer element (Figure 6).

The *Asn1* and *Ppo5* fragments are arranged between the two convergent potato promoters; the *Agp* promoter of the ADP glucose pyrophosphorylase gene (*pAgp*) and the *Gbss* promoter of the granule-bound starch synthase gene (*pGbss*) (Figure 6). These promoters drive expression of the inverted repeats to generate double-stranded RNA and down-regulate *Asn1* and *Ppo5*. The *pAgp* and *pGbss* promoters have been well characterised and are known to be highly active in potato tubers (Nakata et al., 1994; Visser et al., 1991).

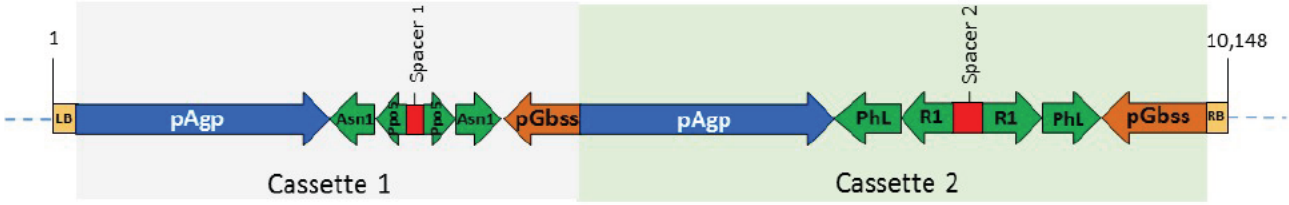


Figure 6. Design of pSIM1278 T-DNA

T-DNA region showing the flanking Left and Right Borders and the two cassettes. See Table 2 for genetic element descriptions.

The second cassette results in down-regulation of *PhL* and *R1* in the transformed potato variety. It is comprised of two identical 509-bp fragments of the *PhL* promoter region (*pPhL*) and two identical 532-bp fragments of *R1* promoter region (*pR1*). The fragments of *PhL* and *R1* are arranged as inverted repeats separated by a non-coding 258-bp fragment of the Ranger Russet potato polyubiquitin gene. Like the first cassette, the *pPhL* and *pR1* fragments are arranged between and transcribed by the potato *Agp* and *Gbss* promoters (Figure 6). The function of the second cassette is to down-regulate expression of the water dikinase gene (*R1*) and the phosphorylase-L gene (*PhL*).

A3(d) A full molecular characterisation of the genetic modification in the new organism

Event SPS-∅∅E12-8 was created by transformation with pSIM1278 that contains a T-DNA region consisting of two cassettes designed to down-regulate four potato genes, *Asn1*, *Ppo5*, *R1*, and *PhL*, in tubers. The Asn1/Ppo5 down-regulation cassette is comprised of two inverted repeat fragments of the potato asparagine synthetase-1 gene (*Asn*1) and two inverted repeat fragments of the potato polyphenol oxidase-5 gene (*Ppo5*) separated by an associated non-coding potato nucleotide spacer element (spacer 1). The PhL/R1 down-regulation cassette is comprised of an inverted repeat fragment of the promoter region of the potato phosphorylase-L gene (*PhL*) and an inverted repeat fragment of the water dikinase gene (*R1*) promoter separated by an associated non-coding fragment of the potato polyubiquitin gene (spacer 2). Both cassettes are driven by two convergent potato promoters; the potato *pAgp* of the ADP glucose pyrophosphorylase gene (*Agp*) and the potato *pGbss* of the granule-bound starch synthase gene (*Gbss*). These promoters drive expression of the inverted repeats to generate double-stranded RNA that results in the down-regulation of the four native potato genes *Asn1, Ppo5, R1, and PhL,* in tubers.

Molecular characterisation was undertaken for event E12 demonstrating:

1. A single insertion site with one copy of the T-DNA;
2. The DNA insert is structurally equivalent to the original T-DNA with small deletions at the ends of the left border (LB) and right border (RB);
3. The organisation of the inserted genetic material at the insertion site, as mapped.
4. No pSIM1278 plasmid backbone is present;
5. Characterisation of junction regions with all possible open reading frames defined and examined to verify that they do not show homology to toxic or allergenic proteins; and
6. The identification and characterisation of open reading frames created at the junctions of the E12 insert, including possible fusion proteins or protein expression products created as a result of the transformation.

Southern Blot Presentation Notes

Southern blots are presented for insert structure determination with tables that indicate the approximate size of the bands associated with the T-DNA insert and any digested pSIM1278 plasmid bands used as controls. The bands associated with inserts are distinguished as either junction bands (JB), plasmid bands (PB), or internal bands (IB) depending on their location. Plasmid bands refer to digestion products of predictable sizes and are associated with digested pSIM1278 plasmid, while internal bands refer to digestion products of predictable sizes that are contained entirely within the boundaries of the T-DNA insert. Junction bands are those bands that extend from the T-DNA insert into the adjacent, potato DNA. The size of these bands is only known when restriction sites fall within identified flanking sequences. However, the minimum size of these bands can be calculated for a simple T-DNA insert based upon the distance from a given restriction site to the end of the identified flanking sequences (Study 15-29-SPS-MOL).

Junction bands were useful for confirming the number of integration sites in the genome as each integration event possesses two junction bands. Since the T-DNA sequence is largely derived from potatoes, Southern blot probes targeting the insert also detect a variable number of endogenous bands, depending upon the frequency of that sequence in the genome. These bands were not labelled in the Southern blots for simplicity, but consist of all bands common in size and intensity between the Russett Burbank control and E12 samples.

Occasionally, bands greater than 3 kb migrated faster than expected due to the presence of residual polysaccharides in the isolated DNA. The size of these bands were verified where possible, by including control samples spiked with pSIM1278 plasmid DNA that produces the same sized bands for comparison.

Characterisation of Event SPS-ØØE12-8

Southern blot analysis was conducted to determine the E12 insert copy number and structure. Full characterisation details are presented in Study 15-29-SPS-MOL and a summary of the findings is presented here.

Characterisation of E12 Insert Copy Number

The pSIM1278 T-DNA consists of an Asn1/Ppo5 down-regulation cassette flanked by converging *Agp* and *Gbss* promoters and a PhL/R1 down-regulation cassette flanked by the same set of converging promoters (Figure 7). The number of inserts in E12 was determined by Southern blot analysis of genomic DNA digested with the *Nde*I restriction enzyme and was hybridised using a series of seven probes that span the length of the T-DNA (Figure 7). *Nde*I cuts frequently within the potato genome, but does not cut within the boundaries of the T-DNA itself. Thus, a single contiguous band is expected for each insert. Moreover, an intact insert will have the same size when using any of the probes. The presence of additional bands would indicate the presence of additional inserts. Since *Nde*I cuts frequently in the potato genome, it is unlikely that two inserts would have the same size.

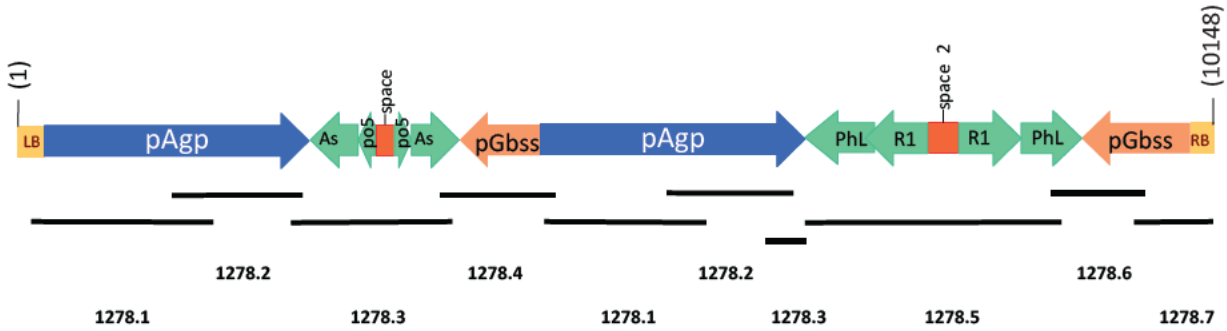


Figure 7. pSIM1278 T-DNA Probes used to Characterise Insert Number
A schematic diagram of the full structure of the pSIM1278 T-DNA is shown with the binding sites for each of the seven probes (1278.1 1278.7). The probes were designed to overlap and cover the T-DNA sequence. Due to redundancy in the insert sequence, some probes hybridised to more than one location within the T-DNA. Probes 1278.4 and 1278.6 are only shown once in the figure, but overlap in their coverage of the insert.

Following digestion with *Nde*I, DNA was separated on agarose gels using extended electrophoresis to ensure adequate separation and resolution of high-molecular weight bands. Southern blots were hybridised with each of the probes described in Figure 7. In each case, a single, high molecular weight band (about 12 kb) was detected using each of the seven probes (Figure 8). The band unique to E12 was indicated in each gel with a black arrow. All other bands existed in both the WT and E12 samples indicating that they are endogenous bands not related to the transformation.

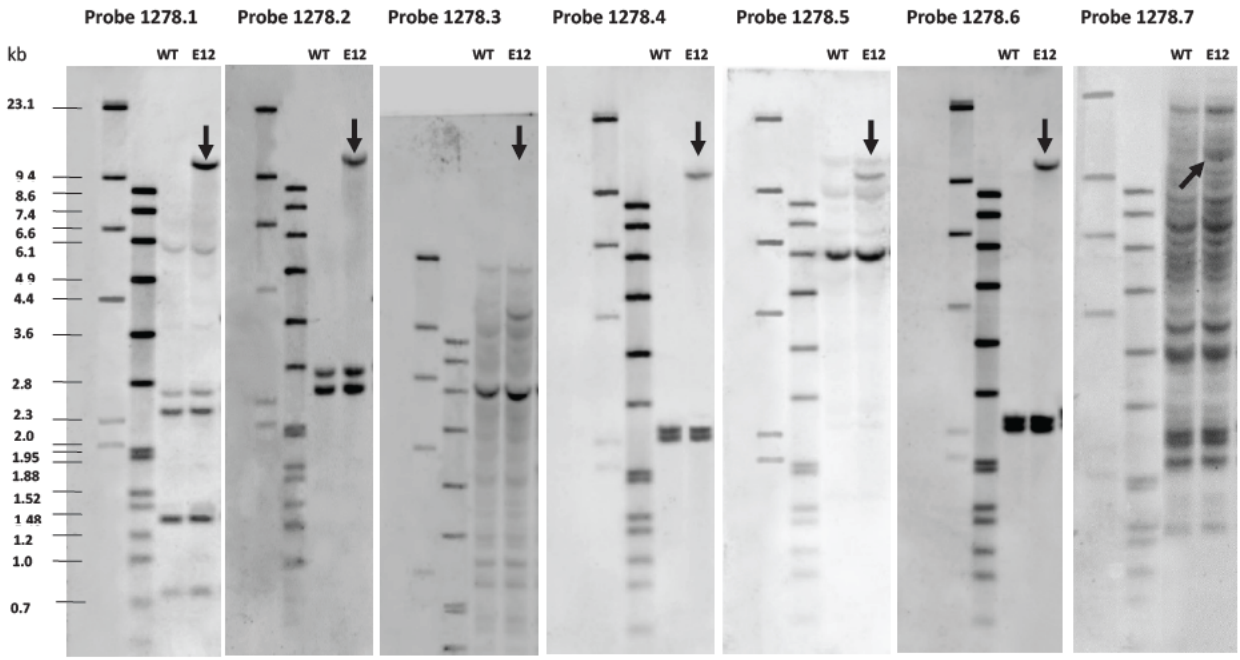


Figure 8. Southern Blots Showing a Single Insert in E12

Southern blots of *Nde*I-digested genomic DNA isolated from Russet Burbank and E12 plants. *Nde*I does not cut within the T-DNA insert, but cuts frequently in the potato genome. The single T-DNA insert is indicated in each gel (Probes 1278.1–1278.7) by a black arrow. The molecular weight markers, DIGII and DIGVII, were included in each gel and are labelled in kilobases (kb) at the left of the first gel.

Since the insert is a single copy of the T-DNA in a tetraploid potato genome, the detection of this band establishes that the sensitivity of the assay is sufficient to detect a single insert copy in the genome. Collectively, these data show the pSIM1278 insert was integrated at a single locus within the Russet Burbank genome.

Characterisation of the Structure of the E12 Insert

A series of Southern blots were performed to determine the structure of the single insert. These Southern blots were performed using a different set of probes, because the probes described in Figure 7 would result in complex banding patterns due to the number of hybridisation sites in the genome and their juxtaposition to the restriction sites. Instead, a combination of probes and restriction digests were chosen that would result in an informative mixture of digestion products with predicable sizes based upon the structure of the T-DNA in pSIM1278 (Figure 9). Probes hybridising to the *pAgp*, Asn1, *pGbss*, and R1 elements were used following digestion with: (1) *Eco*RV, (2) *Hind*III, or (3) a double digest with *Eco*RI and *Sca*I.

The location of the binding site for each probe and a schematic of the restriction patterns of the final structure (grey box) are provided in Figure 9 for ease of comparison with the Southern blot results. The expected bands, their sizes, and a coloured marker indicating probes expected to hybridise with each band are provided. Genomic DNA of non-transformed Russet Burbank was used as a wild-type comparison. A second wild-type sample was spiked with pSIM1278 plasmid prior to digestion to serve as a copy number control and size marker when appropriate. The plasmid was spiked at a target concentration of roughly one copy per genome equivalent. As genomic DNA isolated from potato plants does not always migrate true to size when compared to a molecular weight marker, presumably due to the presence of polysaccharides, the plasmid control provided a valuable size comparison.

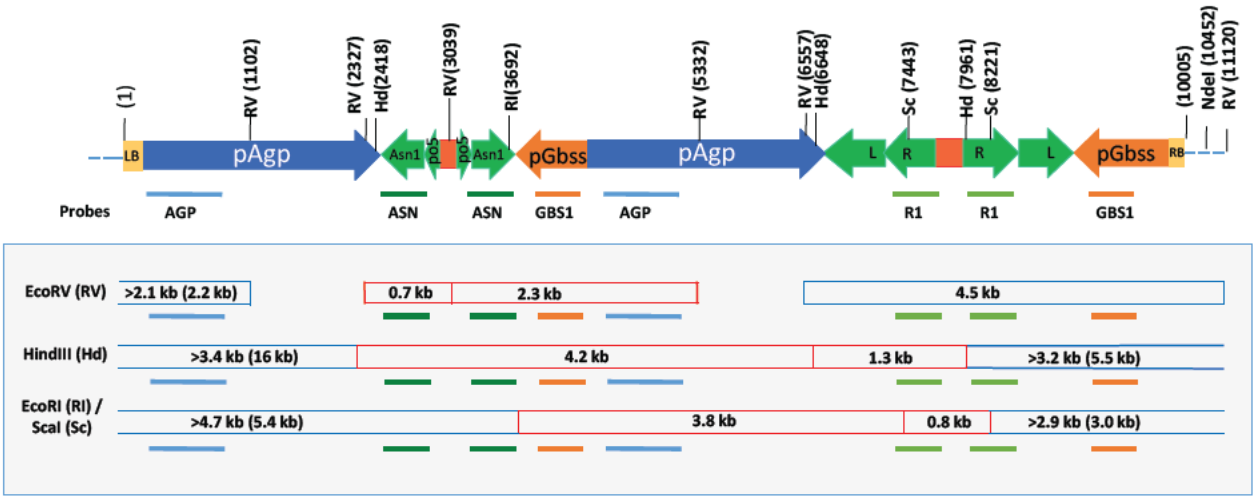


Figure 9. Structure of the E12 Insert with Digestion Patterns and Probe Binding Sites

A schematic diagram of the pSIM1278 T-DNA insert shown with the binding sites for each of the four probes (AGP, ASN, GBS1, and R1). The probes all bind to two different locations within the insert. The restriction products recognised by the probes are shown in the grey box where the expected sizes are indicated. The actual size of junction bands is shown in parenthesis (sizes of junction bands are estimates, except the 4.5 kb EcoRV band).

All Southern blots (Figures 10 13) are presented adjacent to a table indicating the observed bands and their molecular weights to simplify comparison with Figure 9. The tables adjacent to blots denote internal bands (IB) and junction bands (JB) in red; whereas bands associated with the pSIM1278 plasmid (PB) DNA are

indicated in black.The red boxes in Figure 9 show six bands that are completely contained within the boundaries of the T-DNA (internal bands, IB). These same bands are expected for the pSIM1278 plasmid DNA control (WT p1278) and provide an ideal molecular weight comparison.

Each digest resulted in two internal bands; 0.7 kb and 2.3 kb for *Eco*RV, 4.2 kb and 1.3 kb for *Hind*III, and 3.8 and 0.8 kb for *Eco*RI/*Sca*I. Southern blots hybridised separately with the AGP, ASN, GBS1 or R1 probes confirmed the presence and size of each of the bands (Figures 10 13).

- In the *Eco*RV digests, the 0.7 kb band was uniquely detected by the ASN probe (Figure 11), whereas the 2.3 kb fragment was detected by the ASN, GBS1, and AGP probes (Figures 11 13).
- In the *Hind*III digests, the 4.2 kb fragment was detected by the AGP, ASN, and GBS1 probes (Figures 10 12), whereas the 1.3 kb fragment was detected by the R1 probe (Figure 13). The 4.2 kb band had a higher intensity when hybridised with the ASN probe (Figure 11) due to multiple binding sites associated with the inverted repeat (Figure 9).
- In the *Eco*RI/*Sca*I digests, the 3.8 kb fragment was detected in the AGP, GBS1, and R1 blots (Figures 10, 12, and 13), whereas the 0.8 kb fragment was only detected by the R1 probe (Figure 13).

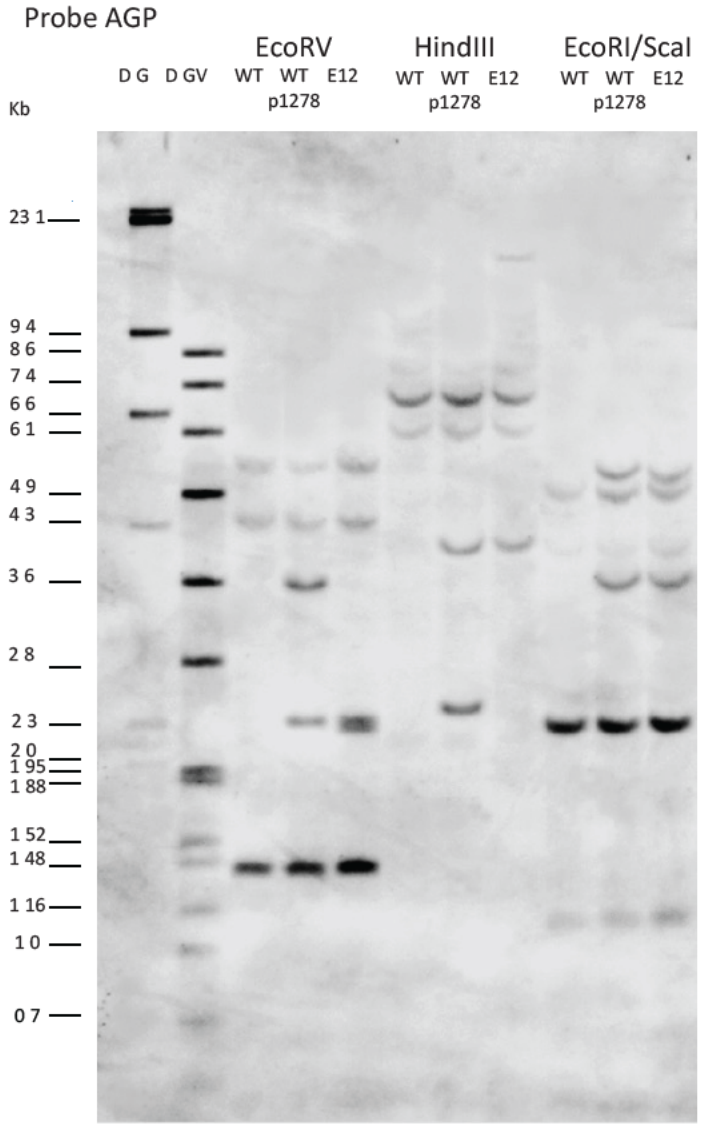
All of the internal bands migrated equivalently with similar intensities to the plasmid DNA controls spiked into the wild-type samples. These data indicate the presence of a single, intact copy of the T-DNA in E12.

The remaining structure of the insert was assessed through analysis of the junction bands associated with these same restriction digests. There were six junction bands (blue boxes) associated with the digests as shown in Figure 9. The open-ended blue boxes denote junction bands where only one of the two restriction site locations was known and thus the exact size could not be predicted. Each of these junction bands was labelled with the expected minimum size and the observed size (shown in parentheses) from the Southern blots (Figures 10 13). Characterisation of the junction sites and flanking sequences identified the presence of an *Eco*RV site in the right flanking region as shown in Figure 9 (Study 15-75-SPS-MOL). Identification of this site predicted the *Eco*RV right junction band should be 4.5 kb and this was represented by a closed box in Figure 9.

Each restriction digest resulted in two junction bands: >2.1 kb and 4.5 kb for *Eco*RV, >3.4 kb and >3.2 kb for *Hind*III, and >4.7 kb and >2.9 kb for *Eco*RI/*Sca*I. As summarised in Figure 9 and shown in the Southern blots in Figures 10 13, all of the expected junction bands were observed with the expected sizes. As expected for the structure presented in Figure 9, the AGP probe detected a junction fragment with each of the digests: 2.2 kb *Eco*RV, 16 kb *Hind*III, and 5.4 kb *Eco*RI/*Sca*I bands (Figure 10). The 5.4 kb band was also detected by the ASN probe with higher intensity because of the multiple binding sites (Figure 11). Similarly, the GBS1 and R1 probes detected the three expected junction fragments associated with the right side of the structure: 4.5 kb *Eco*RV, 5.5 kb *Hind*III, and 3.0 kb *Eco*RI/*Sca*I bands (Figures 12 and 13). The *Eco*RV junction band had higher intensity confirming the presence of an entire inverted repeat on the right side of the insert (Figure 13).

The overlapping restriction fragments covered the entire insert and confirmed the presence of a simple insert that is structurally equivalent to the T-DNA from pSIM1278. The left and right borders were shown to consist of small deletions of non-functional DNA during the characterisation of the flanking regions (Study 15-75-SPS-MOL).

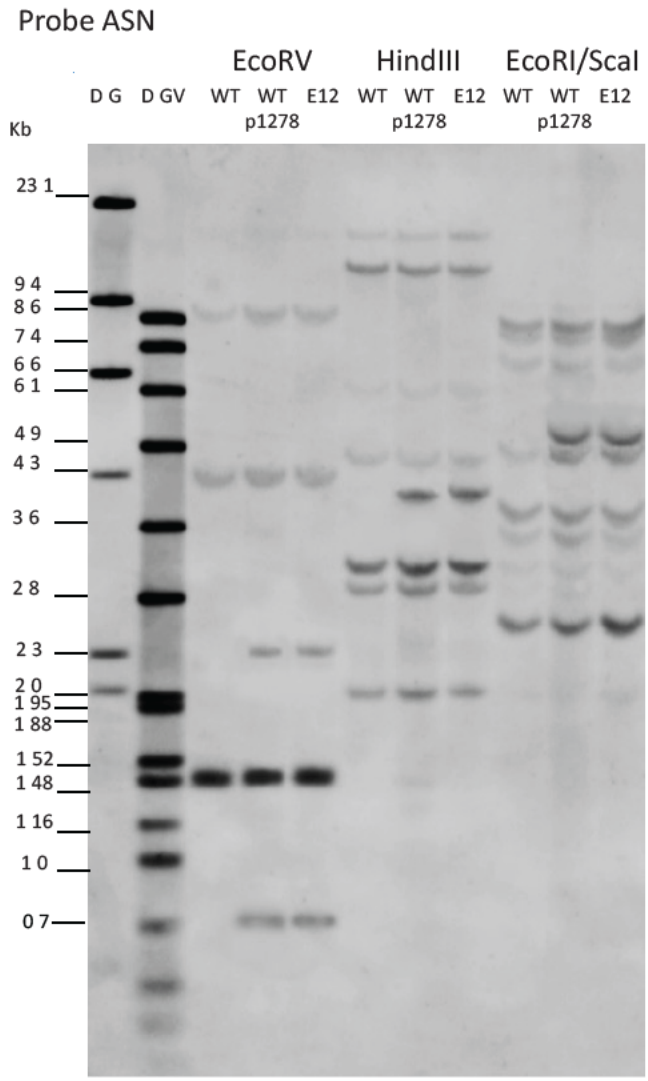
In summary, the molecular characterisation of the E12 insert identified a single, intact integration of the pSIM1278 T-DNA.



EcoRV	HindIII	EcoRI/ScaI
	JB 16	
		PB 5.6 JB 5.4
	PB 4.2 IB 4.2	
PB 3.8		PB 3.8 IB 3.8
	PB 2.4	
PB 2.3 IB 2.3 JB 2.2		

Figure 10. Southern Blot with E12 and the AGP Probe

Genomic DNA of Russet Burbank control (WT), Russet Burbank control + pSIM1278 (WT p1278), and Event E12 were digested with *Eco*RV, *Hind*III, and *Eco*RI/*Sca*I and hybridized with the AGP probe. The estimated sizes of bands are summarised in the table and classified into three groups based their position in the T-DNA: T-DNA insert fragments for both IBs and JBs are in red. Plasmid bands (PB) associated with digested pSIM1278 (WT p1278) are in black. Size of the DIGII and DIGVII molecular weight markers are indicated adjacent to the blot image. All molecular weights are presented in kilobases (kb).



EcoRV	HindIII	EcoRI/ScaI
PB 2.3 IB 2.3	PB 4.2 IB 4.2	PB 5.6 JB 5.4
PB 0.7 IB 0.7		

Figure 11. Southern Blot with E12 the ASN Probe

Genomic DNA of Russet Burbank control (WT), Russet Burbank control + pSIM1278 (WT p1278), and E12 were digested with *Eco*RV, *Hind*III, and *Eco*RI/*Sca*I and hybridized with the ASN probe. The estimated sizes of bands are summarised in the table and classified into groups based on their position in the T-DNA: T-DNA insert fragments for both IBs and JBs are in red. Plasmid bands (PB) associated with digested pSIM1278 (WT p1278) are in black. Size of the DIGII and DIGVII molecular weight markers are indicated adjacent to the blot image. All molecular weights are presented in kilobases (kb).

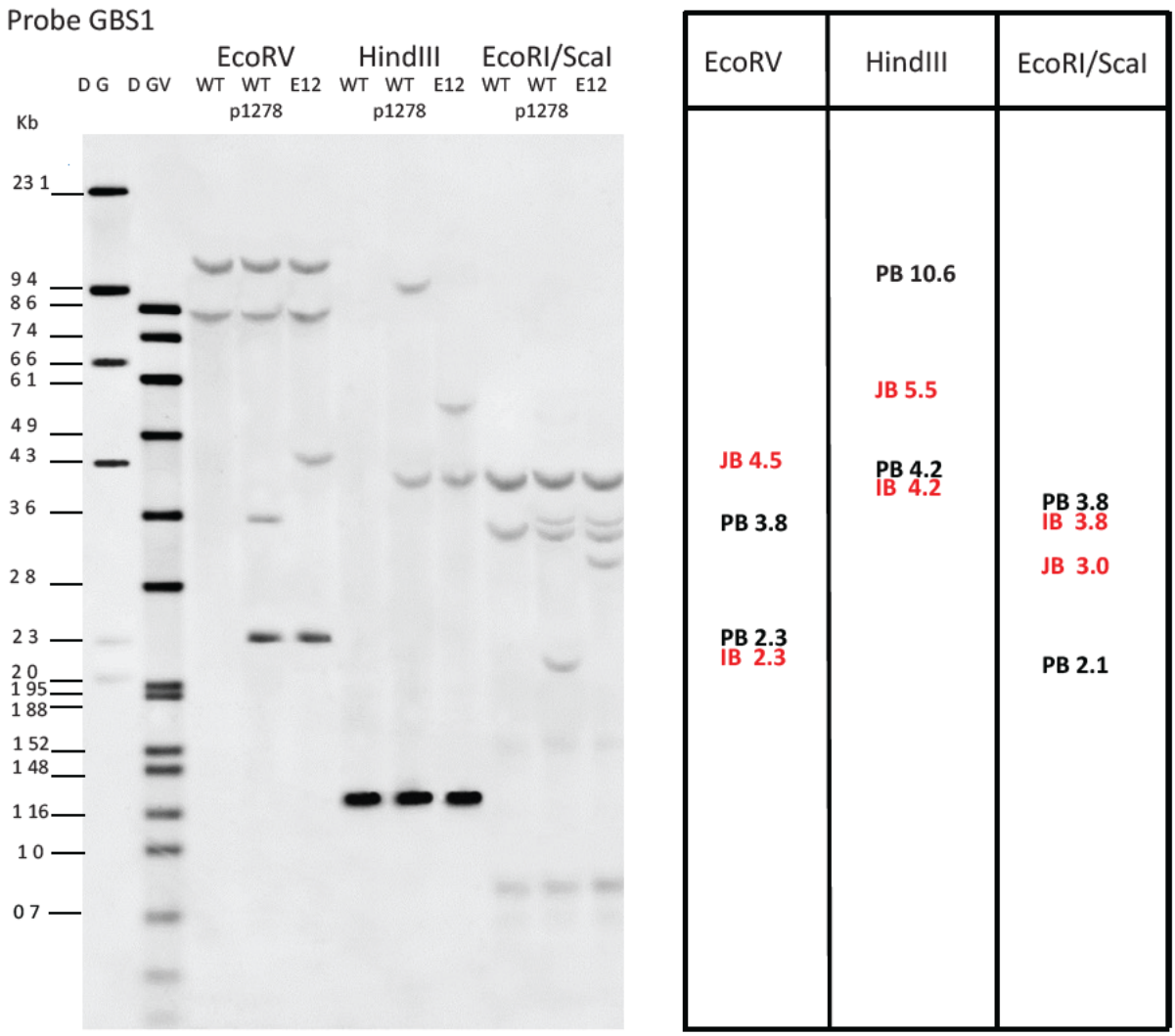


Figure 12. Southern Blot with E12 and the GBS1 Probe

Genomic DNA of Russet Burbank control (WT), Russet Burbank control + pSIM1278 (WT p1278), and E12 were digested with *Eco*RV, *Hind*III, and *Eco*RI/*Sca*I and hybridized with the GBS1 probe. The estimated sizes of bands are summarised in the table and classified into groups based on their position in the T-DNA: T-DNA insert fragments for both IBs and JBs are in red. Plasmid bands (PB) associated with digested pSIM1278 (WT p1278) are in black. Size of the DIGII and DIGVII molecular weight markers are indicated adjacent to the blot image. All molecular weights are presented in kilobases (kb).

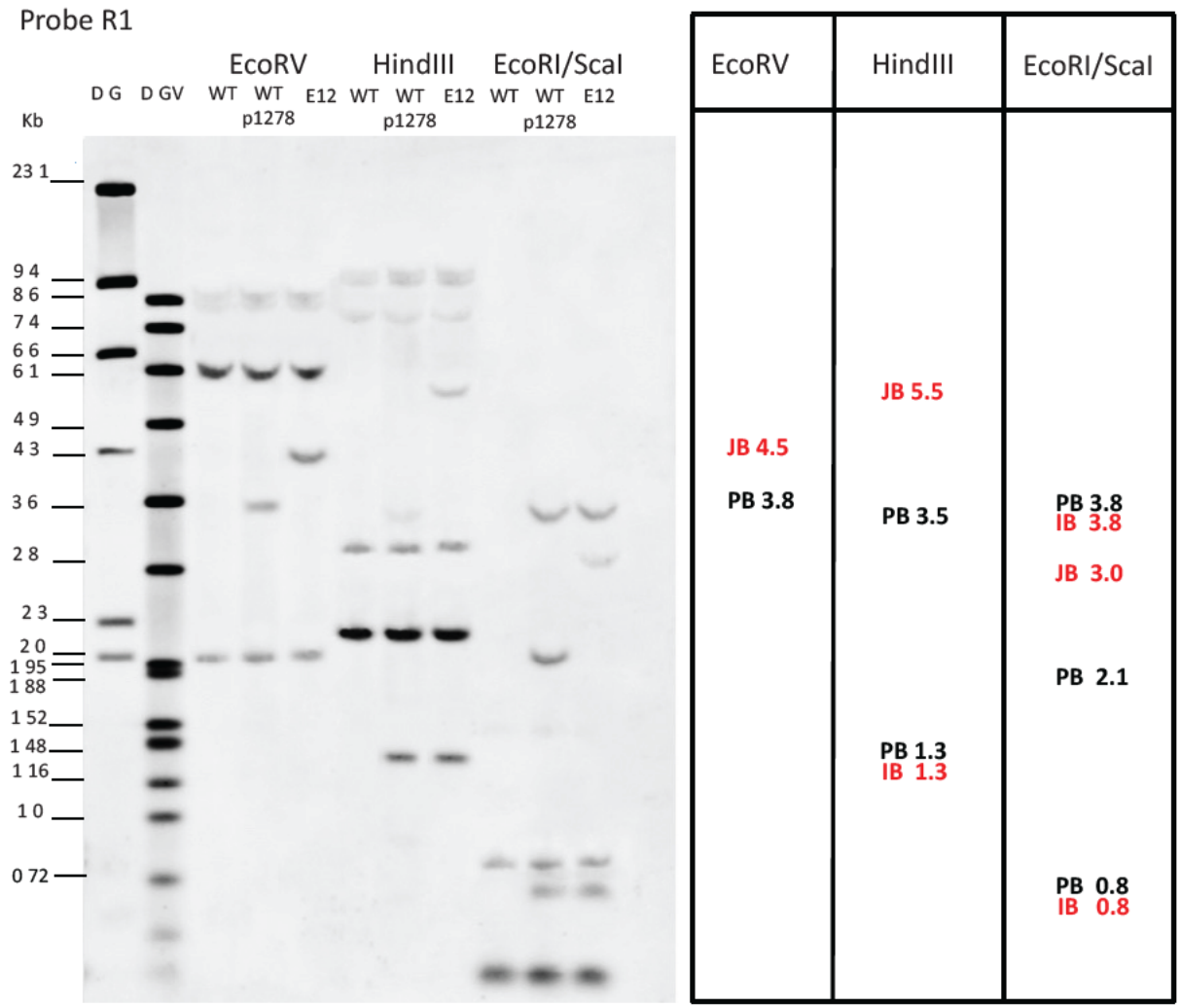


Figure 13. Southern Blot with E12 and the R1 Probe

Genomic DNA of Russet Burbank control (WT), Russet Burbank control + pSIM1278 (WT p1278), and E12 were digested with *Eco*RV, *Hind*III, and *Eco*RI/*Sca*I and hybridised with the R1 probe. The estimated sizes of bands are summarised in the table and classified into groups based on their position in the T-DNA: T-DNA insert fragments for both IBs and JBs are in red. Plasmid bands (PB) associated with digested pSIM1278 (WT p1278) are in black. Size of the DIGII and DIGVII molecular weight markers are indicated adjacent to the blot image. All molecular weights are presented in kilobases (kb).

E12 insert evaluation conclusion

A series of overlapping probes spanning the T-DNA sequence were used to demonstrate that transformation with pSIM1278 led to introduction of a single insert within the Russet Burbank genome. Using probes that hybridise to genetic elements within the T-DNA, the insert was shown to be structurally equivalent to the T-DNA from pSIM1278 with small deletions of the left and right border sequences that are not necessary for any of the desired traits.

Thus, E12 has a single, intact copy of the T-DNA with short deletions at the LB and RB. The size of these deletions was determined by sequencing the junction regions of the insert.

A schematic diagram of the E12 insert, including the two down-regulation cassettes, is shown in Figure 14.

Figure 14. Structure of the Insert in E12

Event E12 insert consists of a single, nearly full-length T-DNA from pSIM1278 with small deletions in the LB and RB. The Asn1/Ppo5 down-regulation cassette contains an inverted repeat flanked by converging *Agp* and *Gbss* promoters. Similarly, the PhL/R1 down-regulation cassette contains an inverted repeat flanked by the same set of promoters. The LB and RB regions are not functional components of the insert.

Evaluation of E12 for the Presence of pSIM1278 Plasmid Backbone

To detect backbone pSIM1278 DNA that may be present in E12, genomic DNA was isolated from Russet Burbank and E12 plants and analysed by Southern blotting following digestion with *Eco*RI or *Eco*RI/*Sca*I. The pSIM1278 plasmid served as a positive control and was spiked into Russet Burbank genomic DNA at a targeted concentration of one copy per genome equivalent (23.4 pg) prior to digestion. Detection of backbone at this concentration showed the probes are sensitive enough to detect a single copy of backbone DNA in the genome. Eight probes were used that span the backbone sequence. A linear representation of the plasmid backbone, including probe binding sites is shown in Figure 15A along with the expected fragment sizes.

Digestion of pSIM1278 with *Eco*RI produced three bands (12.5 kb, 1.57 kb, and 5.6 kb) detectable by the probe set (Figure 15A). Samples were digested with *Eco*RI for the Southern blots hybridised with probes BB1-BB6. A double digest (*Eco*RI/*Sca*I) was used for Southern blots hybridised with probes BB7 and BB8 due to cross reactivity of probe BB8 with the pSIM1278 T-DNA. The sequence of the spacer 2 element within the PhL/R1 inverted repeat is derived from the *Ubi7* promoter and is detected by the probe BB8 in the pSIM1278 plasmid control and the E12 insert (Figure 15B). The *Eco*RI/*Sca*I digest of the plasmid control and E12 produced a 0.8 kb band. The structure of the E12 insert is shown in Figure 15B along with the expected *Sca*I digestion product.

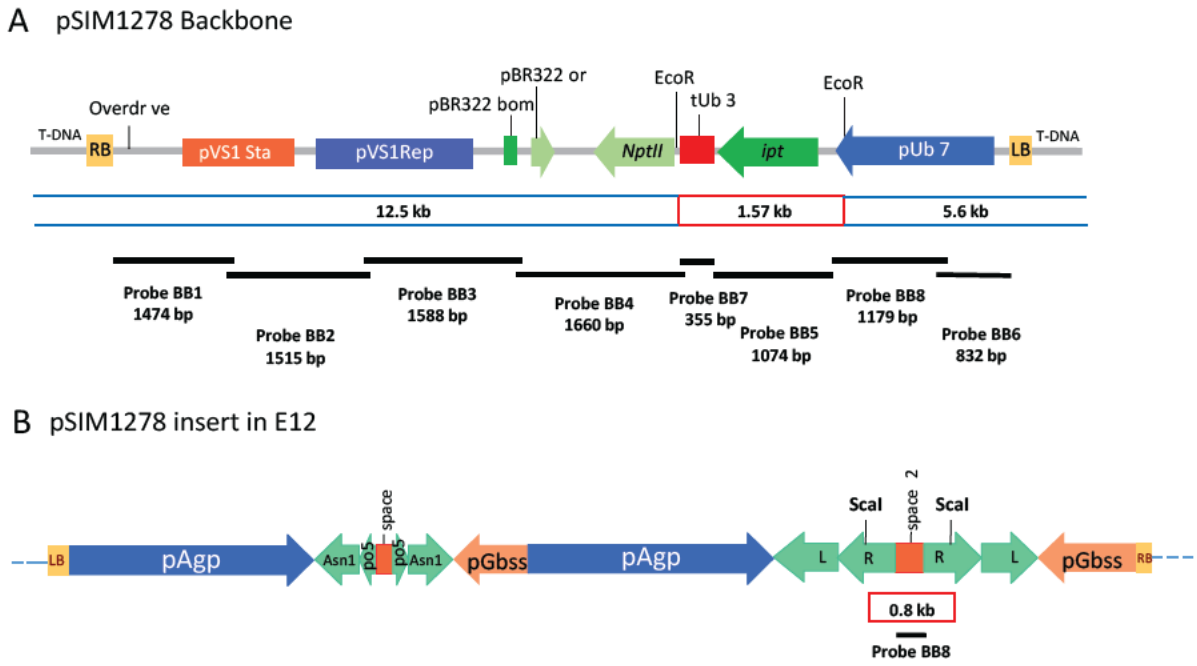


Figure 15. Linear Structures of the pSIM1278 Backbone and E12 Insert

(**A**) Linear map of the vector backbone for pSIM1278. Eight probes (BB1-BB8) used to detect the backbone are indicated with probe size in base pairs (bp). The *Eco*RI sites and expected band sizes for the plasmid control are indicated as coloured boxes. (**B**) Structure of the E12 insert is shown. The BB8 probe detects the spacer 2 region of the insert and produces a 0.8 kb band when digested by *Sca*I.

No hybridisation signal was detected in Southern blots hybridised with probes BB1-BB4 corresponding to backbone DNA in the Event E12 genome (Figure 16). The expected 12.5 kb fragment was readily detected for the positive control samples in each of these blots. Endogenous bands were detected in all samples of the blot hybridized with probe BB3, which were not related to the transformation.

There were no backbone fragments detected in the genome of E12 when hybridised with probes BB5-BB8 (Figure 17). The expected 1.57 kb plasmid fragment described in Figure 15A was detected in the positive control samples for blots hybridised with probes BB5, BB7, and BB8. Similarly, the 5.6 kb plasmid fragment (Figure 15A) was detected specifically in positive control samples by probes BB6 and BB8 (Figure 17). Lastly, the expected 0.8 kb band was detected in E12 and the pSIM1278 control sample in the blot probed with BB8. This band corresponds to the spacer 2 element of the pSIM1278 insert flanked by *Sca*I restriction sites (Figure 15B). Endogenous bands not related to the transformations were observed in all samples in the blots hybridised with probes BB6 BB8.

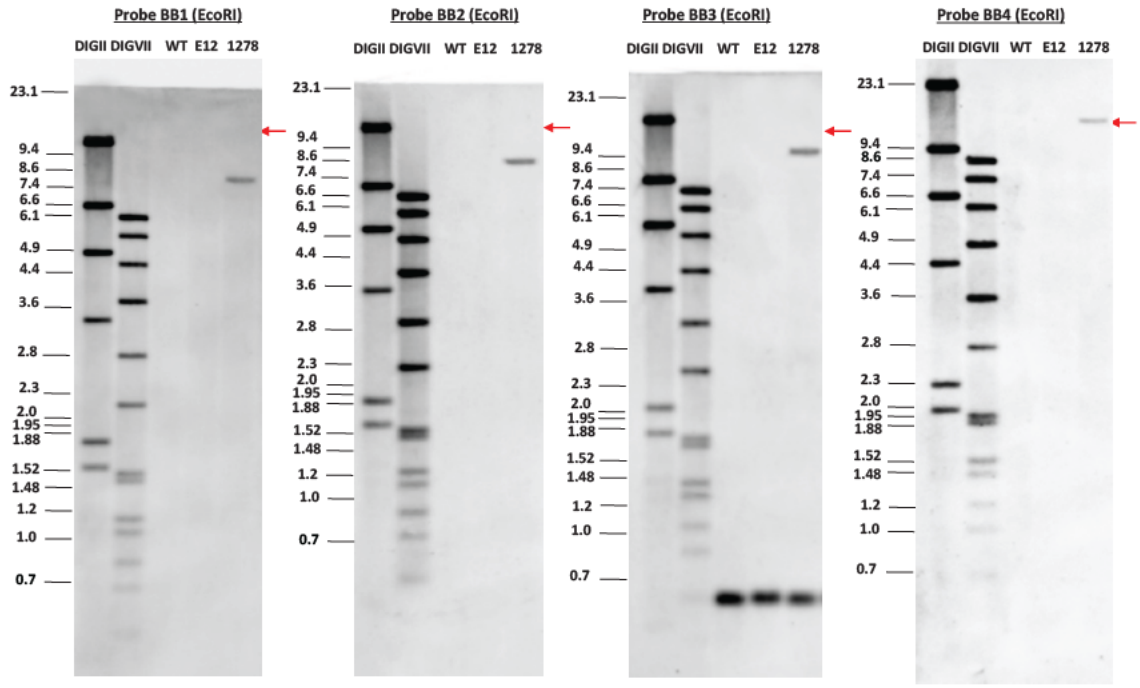


Figure 16. Southern Blots Probed with BB1–4 for E12 Backbone Analysis

Southern blots of genomic DNA isolated from Russet Burbank (WT), Event E12, and WT spiked with pSIM1278 (1278). Molecular weight markers, DIGII and DIGVII, are labelled in kilobases (kb). Red arrows indicate expected 12.5 kb bands unique to the plasmid control (1278).

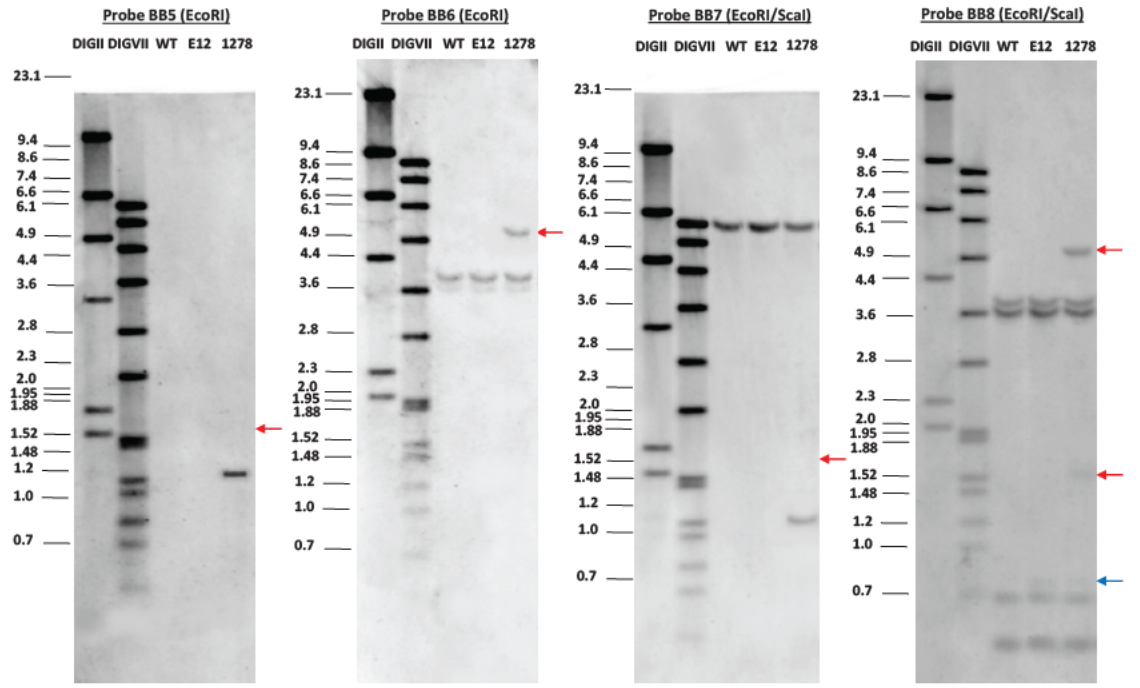


Figure 17. E12 Backbone DNA Analysis with Probes BB5-BB8

Southern blots of genomic DNA isolated from Russet Burbank (WT), Event E12, and WT spiked with pSIM1278 (1278). Molecular weight markers, DIGII and DIGVII, are labelled in kilobases (kb). Red arrows indicate expected bands (1.57 kb and 5.6 kb) unique to the plasmid control (1278). Blue arrow indicates 0.8 kb band associated with the spacer 2 element of the T-DNA.

Conclusion of backbone DNA assessment

Event E12 was evaluated for the presence of backbone DNA by Southern Blot analysis. No hybridisation signal was detected in Southern blots using a series of eight probes that span the backbone of the plasmid, pSIM1278. This confirmed that there is no pSIM1278 backbone DNA in the E12 genome.

Characterisation of E12 Insert Junction Regions

The sequence of the unique junctions at each side of the E12 insert were determined using PCR followed by Sanger sequencing. The left and right junctions were PCR amplified using a primer specific to the insert DNA and another specific to the adjacent chromosomal sequence (Figure 18). Details of the methods used for this analysis are provided in Study 15-57-SPS-MOL.

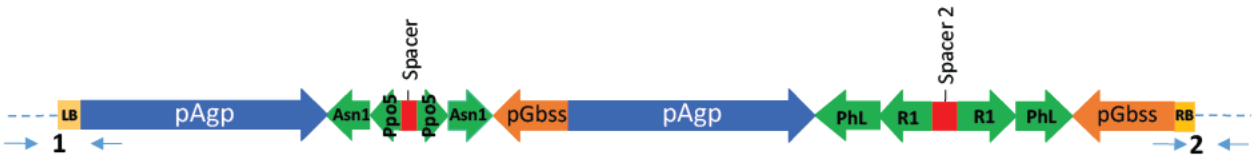


Figure 18. Structure of the Insert in Event E12

The E12 insert contains a nearly full-length copy of the pSIM1278 T-DNA, with deletions in the Left and Right border regions. Arrows denote primer pairs used to amplify the left and right junctions, labelled 1 and 2, respectively.

Sanger sequencing of the PCR amplicons from the left and right junctions (Figure 19) revealed a deletion of 24 bp from the Left Border region (LB) and a deletion of 119 bp from the Right Border region (RB). These data support Southern analysis that indicates the pSIM1278 insert consists of a nearly full-length T-DNA (Study 15-29-SPS-MOL). The predicted sequence of the E12 insert and flanking regions is provided in Study 15-57-SPS-MOL based on determined junction and flanking sequence, insert structure from Southern blot hybridisation, and the sequence of the pSIM1278 T-DNA.

It was challenging to provide a definitive sequence of the entire E12 insert using Sanger sequencing or Next-Generation Sequencing, because the insert consists entirely of potato sequence, including inverted repeat sequences. Potato is tetraploid and has multiple copies of each of the sequences in endogenous genes such as those that are the target for down-regulation. Many copies of the same sequence make it difficult to distinguish the E12 insert sequence from the endogenous sequences within the parent variety.

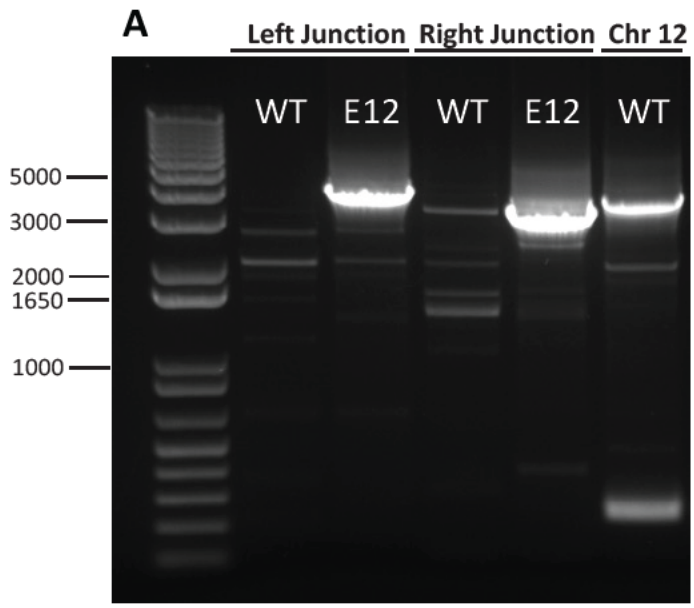


Figure 19. PCR Evaluation of the E12 Insert Junctions

PCR was used to confirm the junction between the insert and the left and right flanking regions. 4183 and 3459 bp products were amplified specific to the left and right junctions, respectively. The integration site was also amplified resulting in an amplicon of 3932 bp (Chr 12). WT denotes non-transformed Russet Burbank.

In summary, PCR followed by Sanger sequencing was used to identify the unique junctions at each side of the insert within the potato genome. Sanger sequencing the insert junction regions supported the findings that the pSIM1278 insert in E12 contains a nearly complete T-DNA with small deletions in each border region (Study 15-29-SPS-MOL). Analysis of the flanking sequences indicated no new junctions have been generated outside of those at the insert borders.

Analysis of Novel Open Reading Frames

An open reading frame (ORF) is a contiguous sequence located between a canonical start codon and the next in-frame downstream stop codon. A proprietary Python script was used to identify all start-to-stop ORFs contained in the event inserts and the adjacent potato flanking sequence. This analysis examined each of three possible reading frames in both orientations (i.e., six possible reading frames in total) for potential ORFs encoding sequences of 30 or more amino acids (i.e., equal or greater than ca. 3,300 Da).

Analyses were completed to assess novel ORFs in E12 (Study 15-79-SPS-MOL). Novel ORFs were evaluated using standard bioinformatic amino acid sequence comparison techniques to assess similarity to known toxins or allergens. Expression of toxins or allergens is unlikely, particularly in potatoes transformed with potato DNA. Most of the ORFs contained in the event inserts exist naturally elsewhere in the potato genome. The left and right junctions of the insert have potential to express ORFs initiated by regulatory sequences located in the potato genome.

Well-established techniques in bioinformatics were used to evaluate the inserts in event E12 (Goodman et al., 2008; Ladics et al., 2007; Terrat and Ducancel, 2013).

A web-based tool (http://www.allergenonline.org/databasefasta.shtml) provided by the Food Allergy Research and Resource Program (FARRP) was used to evaluate the allergenic potential of the ORFs

associated with the E12 insert. This tool allowed comparison of ORFs to known allergens using multiple sequence identity searches:

- full-length alignments;
- 80 amino acid (80-mer) high local-identity alignments; and
- 8 amino acid (8-mer) exact matches.

Evaluation of the 27 ORFs for similarity or identity to known allergens did not identify any significant matches. There are no allergen-based safety concerns associated with the E12 insert, including ORFs extending into flanking sequences.

The most recent UniProt database (49,293,307 entries) was used to extract proteins containing the keyword, "toxin", in the annotation for protein function. The toxin-related proteins were downloaded from the UniProt website (www.uniprot.org/downloads) on June 19, 2015 into a local, FASTA-formatted file (referred to as *toxin15*), which contained 10,555 protein sequences. In addition to actual toxins, this database includes proteins that have roles related to toxin production, exposure, or protection.

>fasta36 -p -E 0.0001 *V12_start_to_stop.fasta* toxin15.fasta > *E12_start_toxin15*.txt

The FASTA36 software package (http://faculty.virginia.edu/wrpearson/fasta/fasta36/) was installed locally and used to perform protein homology searches (Pearson, 2000, 2004; Pearson and Lipmant, 1988). All ORFs were queried against the toxin15 database with an E-value cut off of $1x10^{-4}$:

There are no well-curated toxin-specific databases. However, the UniProt/Swissprot database is an expansive and well-annotated protein database that can be queried for protein homology. A query against the entire database, much like a query against the NCBI database, will produce many non-specific hits that must be inspected individually to determine whether the identified proteins present toxicity concerns.

In an effort to develop an approach that more closely parallels the allergenicity analysis performed using FARPP, a more selective database was generated by SPSII through guidance from bioinformatics experts at UniProt. The database was limited to proteins annotated to be toxins or associated with toxin production or exposure. A toxin-related database (toxin15.fasta) was generated by extracting all protein sequences from the UniProt database containing the keyword, "toxin".

The ORFs associated with the E12 insert were queried against this database using search parameters similar to the full-length allergen search at FARPP (FASTA36; E-value cutoff, 10^{-4}). Evaluation of the ORFs for homology to known toxins did not identify any matches, and indicated that there are no toxicity-based safety concerns associated with the E12 insert.

It is concluded that, in the unlikely event that the potential ORFs were expressed in event E12, there is no significant similarity between the encoded sequences and any known protein toxins or allergens.

Summary of the Molecular Characterisation of Event E12

In summary, the molecular analyses described in this submission provide evidence that:

1. E12 contains a single copy of the insertion;
2. E12 does not contain residual pSIM1278 plasmid backbone sequences;
3. The E12 insert is shorter than the full distance between the Left and Right Border sequences. The LB is 24 bp shorter than the T-DNA and the RB is 119 bp shorter than the T-DNA. Such

deletions are associated with T-DNA integration and hypothesised to result from double-strand break repair (Gheysen et al., 1987);

4. The layout of elements in the T-DNA is conserved in the E12 insert structure, as confirmed by Southern blotting and sequencing;
5. The flanking sequence was characterised and is provided in the insertion site report;
6. Bioinformatic analyses of start-to-stop ORFs within the DNA insert and genomic junction regions demonstrated no putative polypeptides with relevant homology to proteins that are known to be toxic, allergenic, or to have other biologically adverse properties.

(e) A description of how the line or strain from which food is derived was obtained from the original transformant (i.e. provide a family tree or describe the breeding process).

The E12 original transformants is maintained in tissue culture and generated for seed production through asexual clonal propagation cycles as shown in Figure 3.

(f) Evidence of the stability of the genetic changes

DNA Insert Stability of Event E12

T-DNA inserts are sometimes unstable after insertion into a plant. The estimated instability rate of between 0.5-5.9×10^{-4} is reportedly associated with meiosis (Conner et al., 1998; Müller et al., 1987). Because potato is a clonally propagated crop and reproduction does not involve meiosis, sexual recombination or segregation, the E12 insert is expected to be stable. Nevertheless, the stability of the insert was confirmed in G0, G1, G2, and G3 tubers. In addition, G2 tubers from E12 were tested with a colorimetric activity assay for polyphenol oxidase activity. Data for the E12 event are provided below, with full materials and methods, results and further experimental details included in Study 15-64-SPS-MOL and Study 15-84-SPS-MOL. Although instability is undesired for commercial reasons and may result in a phenotypic reversion back to wild-type, this would not trigger biosafety issues since wild-type poses no safety risk.

The stability of the E12 insert was evaluated in the original transformant and in clonally propagated plant material using both Southern blot hybridisation and a PPO trait evaluation assay. Because potatoes are clonally propagated, standard assessments for sexually propagated crops are not directly applicable. Tubers rather than seeds were used to define subsequent clonal cycles since tubers are what is commercially planted.

Abbreviations used for these studies are as follows:

G0	Initial clonal cycle of tubers obtained from tissue culture
G1	First clonal cycle field grown plant
G2	Second clonal cycle field grown plant
G3	Third clonal cycle field grown plant
WT	The Russet Burbank wild type tuber
DIGII	Molecular size marker used in lane 1 of each gel (approximate range 2.0-23.1 kb)
DIGVII	Molecular size marker used in lane 2 of each gel (approximate range 1.2-7.7 kb).

The probes used in the Southern blot analysis are represented in Figure 20.

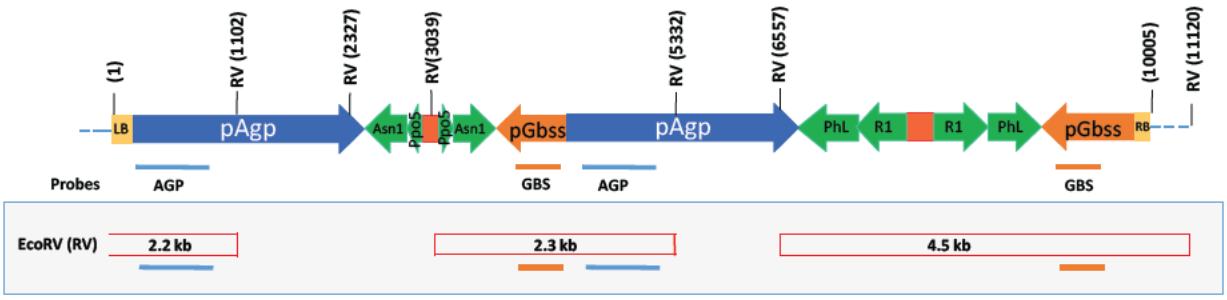


Figure 20. Probes for Southern Blot Analysis of E12 Insert Stability.

A coloured indicator notes the binding site for each probe. Expected bands following *Eco*RV digestion are indicated as red boxes with their observed size. GBS=GBS1 probe.

The stability of the E12insert was assessed at G0 and G3 clonal cycles using field grown tubers, Southern blot analysis, and the GBS1 and AGP probes (see Figure 20). Evidence of genetic stability is consistency of bands on Southern blots across clonal cycles.

Genomic DNA from Russet Burbank (WT) and E12 was digested with the restriction enzyme, *Eco*RV. The *Eco*RV restriction enzyme digests the DNA into multiple bands. The probes used in this study bind to potato genes, detecting the E12 insert and endogenous genes. Only bands unique to Event E12 are associated with the E12 insert. Bands specific to the E12 insert were distinguished by size and probe specificity (Figure 20).

The red boxes in Figure 20 show that the *Eco*RV digest is expected to produce three bands (2.2 kb, 2.3 kb, and 4.5 kb) associated with the pSIM1278 T-DNA. Southern blots hybridised separately with the GBS1 or AGP probes confirmed the presence and size these bands (Figure 21 and Figure 22).

The AGP probe hybridised to 2.2 kb and 2.3 kb bands associated with the E12 insert (Figure 20). These bands were detected consistently in the E12 plants across clonal cycles (Figure 21). The GBS1 probe hybridised to 2.3 kb and 4.5 kb bands associated with the E12 insert. These bands were observed in each of the E12 samples across clonal cycles (Figure 22) indicating the stability of the insert. The banding pattern of the WT remained constant across the clonal cycles (Figure 21 and Figure 22). These endogenous bands are observed in WT and event E12 and are not related to the insert. Details are given in Study 15-64-SPS-MOL.

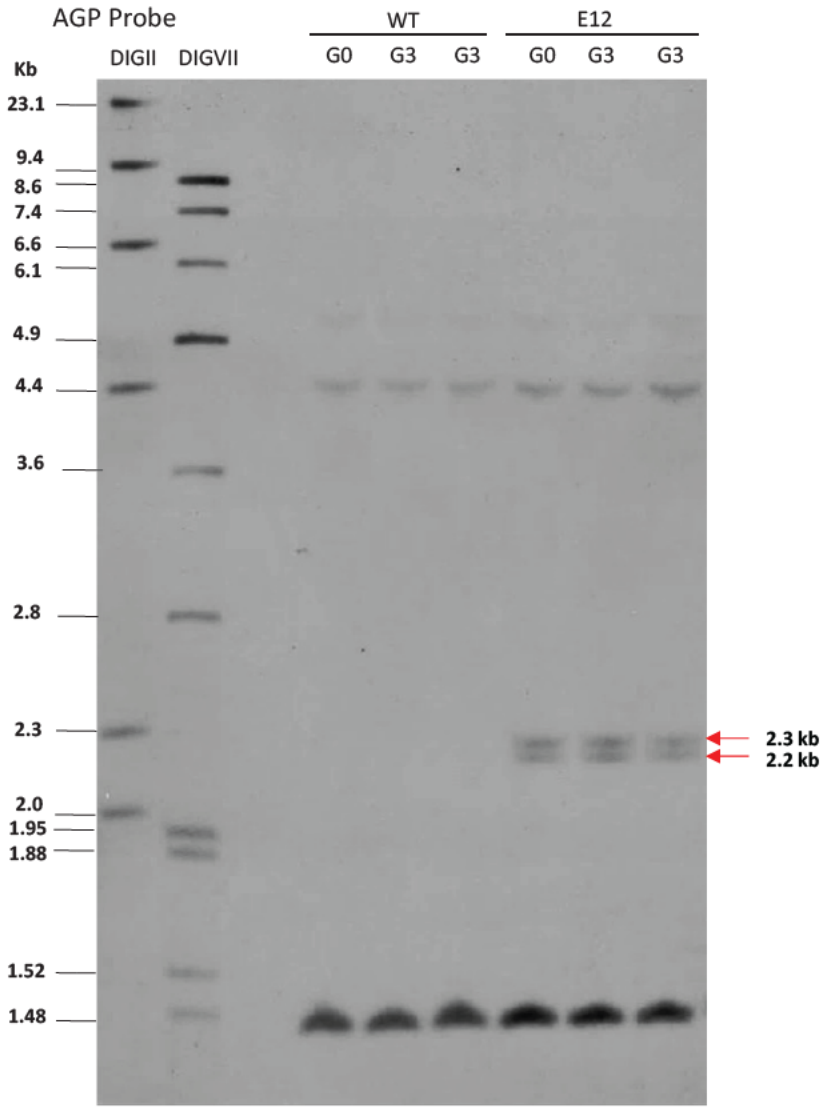


Figure 21. E12 Insert Stability Visualised with the AGP Probe

Southern blots of 4 µg genomic DNA isolated from G0 leaves and G3 tubers of Russet Burbank control (WT) and E12 hybridised with the AGP probe. Red arrows indicate E12 insert bands with the indicated sizes. Molecular weight markers (DIGII and DIGVII) were included with band sizes indicated in kilobase pairs (kb).

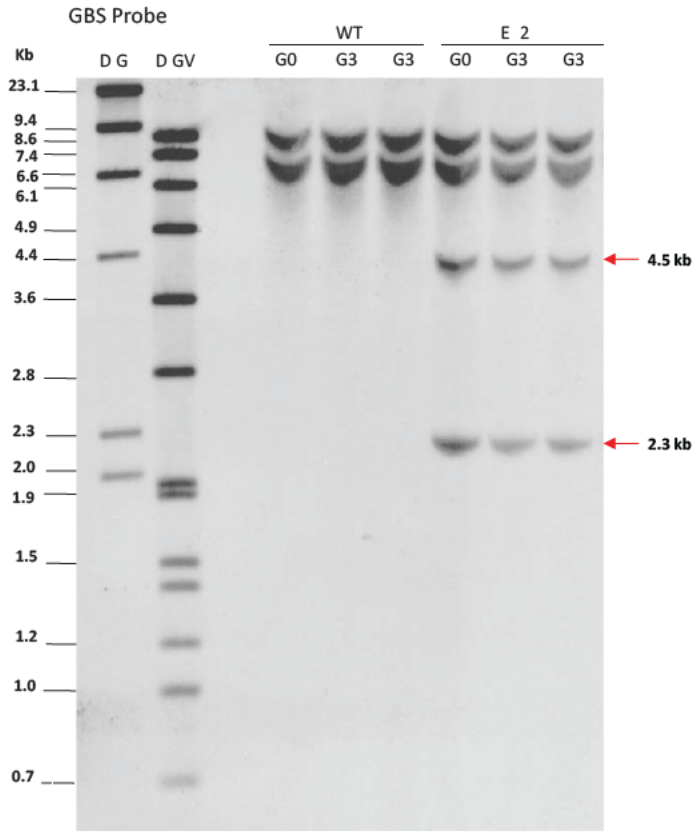
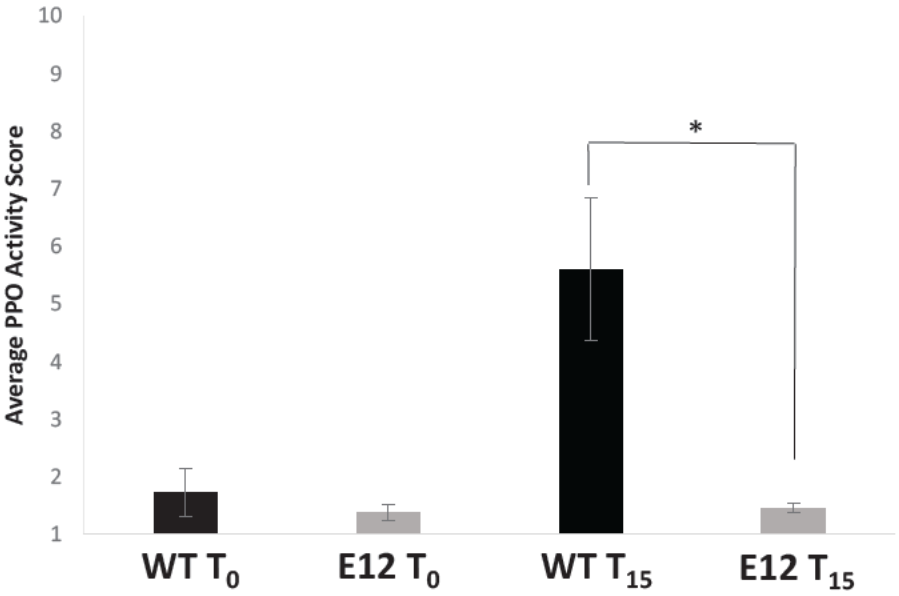


Figure 22. E12 Insert Stability Visualised with the GBS Probe
Southern blots of . 4 µg genomic DNA isolated from G0 leaves and G3 tubers of Russet Burbank control (WT) and E12 digested with EcoRV and hybridized with the GBS probe. Red arrows indicate E12 insert bands and sizes. Molecular weight markers (DIGII and DIGVII) were included with band sizes indicated in kilobase pairs (kb).

The Southern blot results showed consistent banding patterns between E12 G0 and G3 clonal cycles. This demonstrated that the E12 insert is stable across clonal cycles, as expected. All potatoes produced from an individual plant using clonal propagation are considered genetically identical. Consequently, evaluating insert stability by examining inheritance across clonal cycles using Mendelian segregation analysis is not applicable for potatoes.

Evidence for trait expression stability was obtained in G2 E12 field grown tubers using a catechol assay for polyphenol oxidase activity (Study 15-84-SPS-MOL). Preparation of the field trial material took the events through repeated tissue culture and clonal propagation cycles without affecting the stability of the inserted genetic material.

Using the PPO colorimetric activity assay, PPO activity in E12 G2 tubers samples was significantly lower than activity in untransformed control material (Figure 23). This demonstrated that E12 retained its ability to down-regulate the *Ppo5* gene in tubers after 3 clonal cycles: G0 to G2.



	WT T_0	E12 T_0	WT T_{15}	E12 T_{15}
Mean	1.733	1.383	5.600	1.450
Std Dev	0.417	0.137	1.240	0.081

Figure 23. Reduced PPO Activity in E12 Compared with Control

PPO activity scored using the PPO Activity Colourimetric Scoring Scale for each of the 10 biological samples. The mean and standard deviation (Std Dev) values are listed above (n=10). The data compares activity scores between E12 and WT Control (Russet Burbank) at T0 and T15 in the presence of catechol. The average PPO activity score at 15 minutes catechol exposure is significantly lower in E12 when compared with the wild type control (* denotes $p<0.01$).

Conclusions: Stability of Inserted DNA in Event E12

A combination of Southern blots and the catechol colourimetric activity assay demonstrated that the genetic insertion in E12 remains genotypically and phenotypically stable across clonal cycles. The stability of the E12 insert does not alter the safety characteristics compared to conventional potato varieties.

A4. Analytical Method for Detection

A quantitative event-specific polymerase chain reaction (PCR) amplification method was developed for potato event E12 (Study 16-10-SPS-MOL). The detection method specifically amplifies a DNA fragment spanning the junction between the potato genome and the 5′ region of the E12 insert. Since the insert site, and thus the junction sequences, are unique in E12, PCR amplification using junction specific primers can be used to detect the event unambiguously.

B. Information Related to the Safety of the Genetically-Modified Food

B1. Equivalence Studies

Not applicable to this submission as there is no novel protein produced in E12 food products.

B2. Information on Antibiotic Resistance Marker Genes (if used)

The event described in this application does not contain any selectable marker genes including those for antibiotic resistance. The RNAi sequences inserted into the potato variety Russet Burbank are derived from potato (see Table 2). The inserted DNA sequences down-regulate the native *Ppo5*, *Asn1*, *R1* and *PhL* genes, therefore no new proteins are produced.

Analysis for the presence of plasmid backbone sequences (Section A3) demonstrated that no plasmid backbone was incorporated into the potato genome during transformation. Thus the *neomycin phosphotransferase* II (*nptII*) gene, which was used as a bacterial selectable marker gene, or the *isopentenyl transferase* (*ipt*) gene, a screenable marker, are not present in event E12.

B3. The Characterisation of Novel Proteins or Other Novel Substances

The sequences introduced into the potato event are derived from endogenous genes already present in potato and therefore their presence in the plant is not novel. They were designed to give rise to a non-coding dsRNA. Translation of this dsRNA is considered unlikely because the hairpin secondary structure prevents engagement of the 40S ribosomal subunit necessary to initiate translation at the 5' end of the RNA, and/or it prevents unwinding of the duplex such that the 40S subunit is unable to advance along it (Kozak, 1989). Such dsRNA is cleaved into small RNA which have limited potential for translation. Therefore, no novel proteins are produced as a consequence of the genetic modification.

Small RNA produced in E12 do not present a safety concern. Small RNA are abundantly present in the human diet from both plant and animal sources (Carthew and Sontheimer, 2009; Ivashuta et al., 2009).

E12 was assessed for down-regulation of the target genes and for a reduction in PPO activity, free asparagine and reducing sugars.

RNA Expression of Down-Regulated Genes

The inserted cassettes were designed to down-regulate expression of four genes: *Asn1*, *Ppo5, PhL*, and *R1*. The RNAi cassettes are driven by promoters primarily active in tubers to facilitate tissue-specific down-regulation. The effectiveness of target gene down-regulation and tissue-specificity was evaluated by northern blot analysis in potato tuber, leaf, stem, root, and flower.

The results demonstrate reduced expression of the *Asn1* and *Ppo5* genes in tubers (Figure 24). Gene expression in other tissues was unaffected, except for a small reduction of *Asn1* RNA levels in flowers (Figures 27 30). These experiments are details in Study 15-71-SPS-MOL.

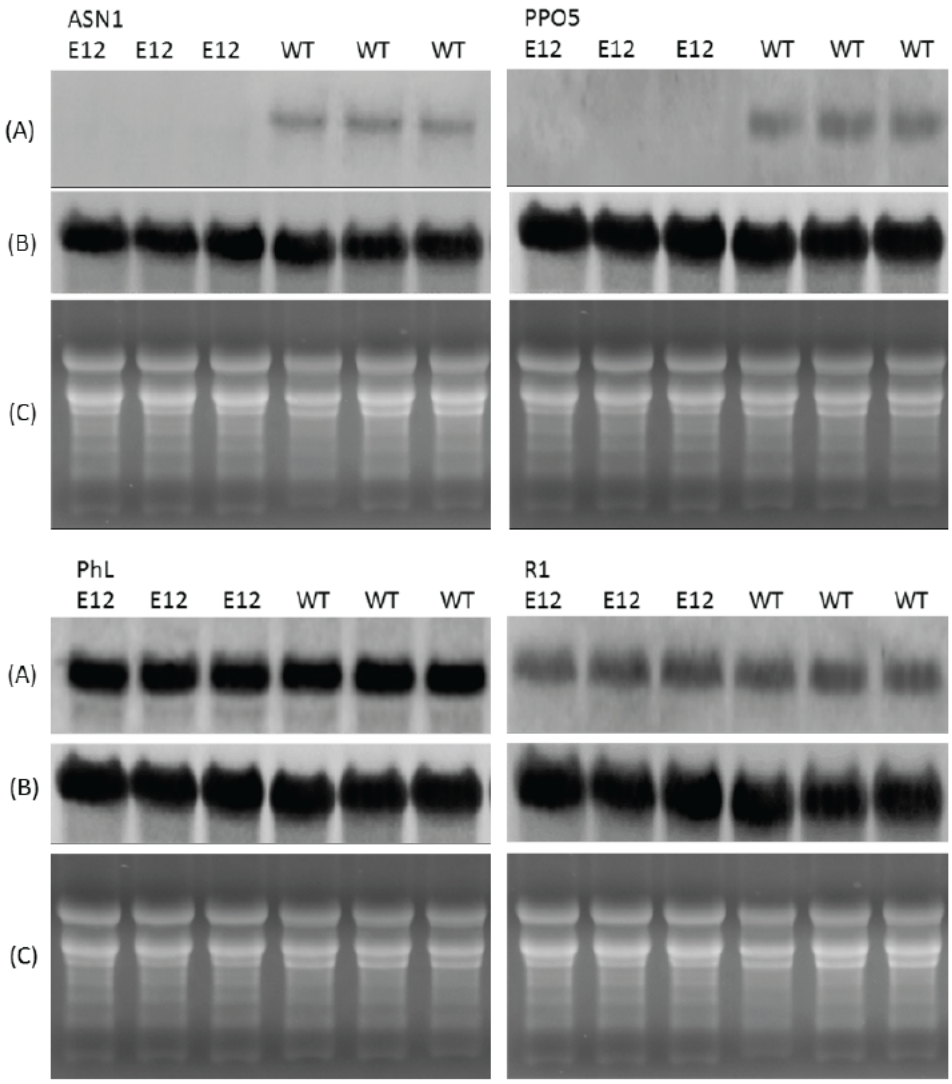


Figure 24. Changes in *Asn1* and *Ppo5* Gene Expression in E12 Tubers

Northern blots were probed to detect expression from each of the RNAi target genes: *Asn1*, *Ppo5*, *PhL*, and *R1*. (A) Samples were analysed with the indicated probe. (B) 18S RNA provides a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

In tubers, Northern blots indicated down-regulation of *Asn1* and *Ppo5* gene expression in E12 tubers but not in the Russet Burbank controls (WT) (Figure 24). The down-regulation was particularly strong with nearly complete absence of the target RNA for *Asn1* and *Ppo5*. *PhL* and *R1* expression levels were unchanged between E12 and WT tuber samples (Figure 24).

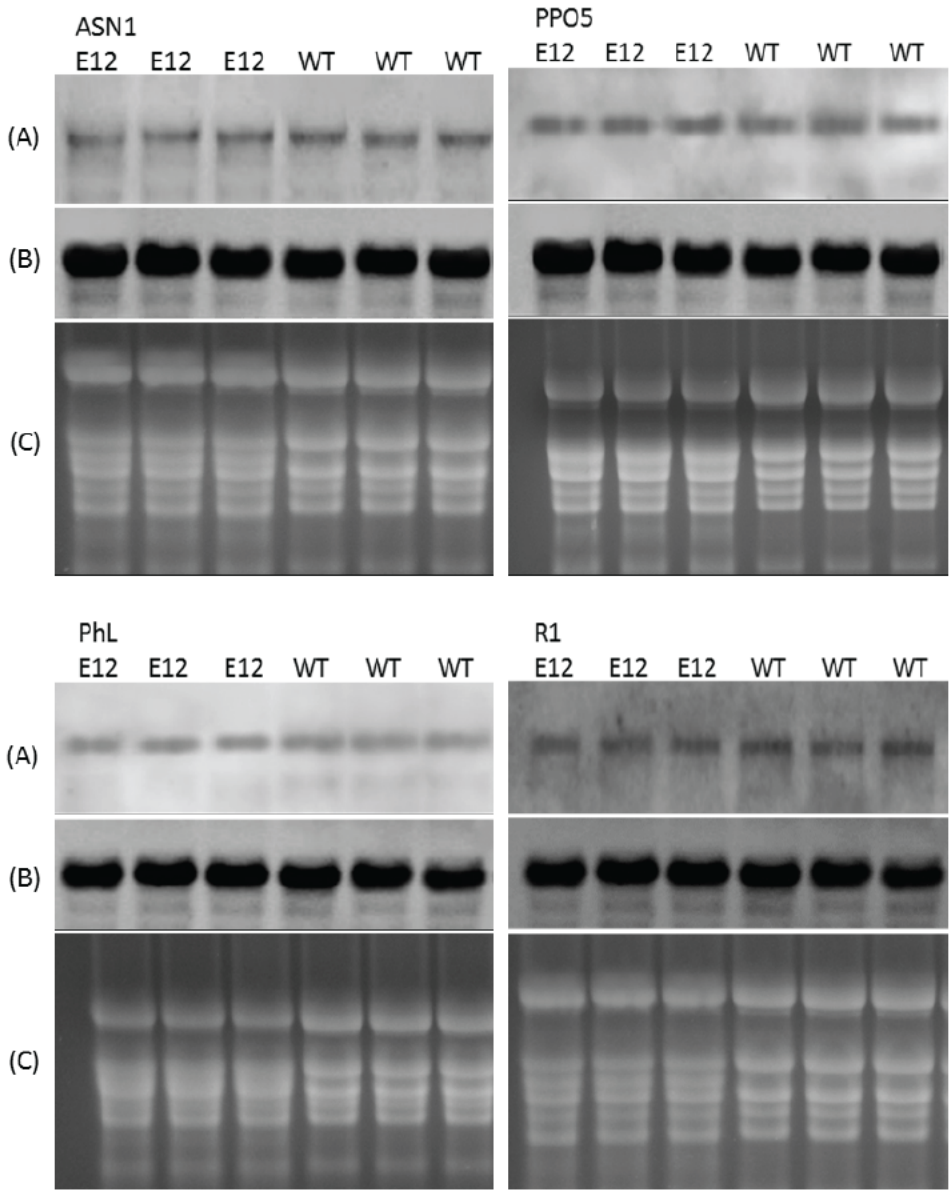


Figure 25. No Changes in RNAi Target Gene Expression in E12 Leaves

Northern blots were probed to detect each of the RNAi targets: *Asn1*, *Ppo5*, *PhL*, and *R1*. (A) Samples were analysed with the indicated probe. (B) 18S RNA provides a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

Down-regulation was not observed for any of the four target genes in E12 leaf samples compared to the Russet Burbank control (WT). This was consistent with tuber-specific down-regulation (Figure 25).

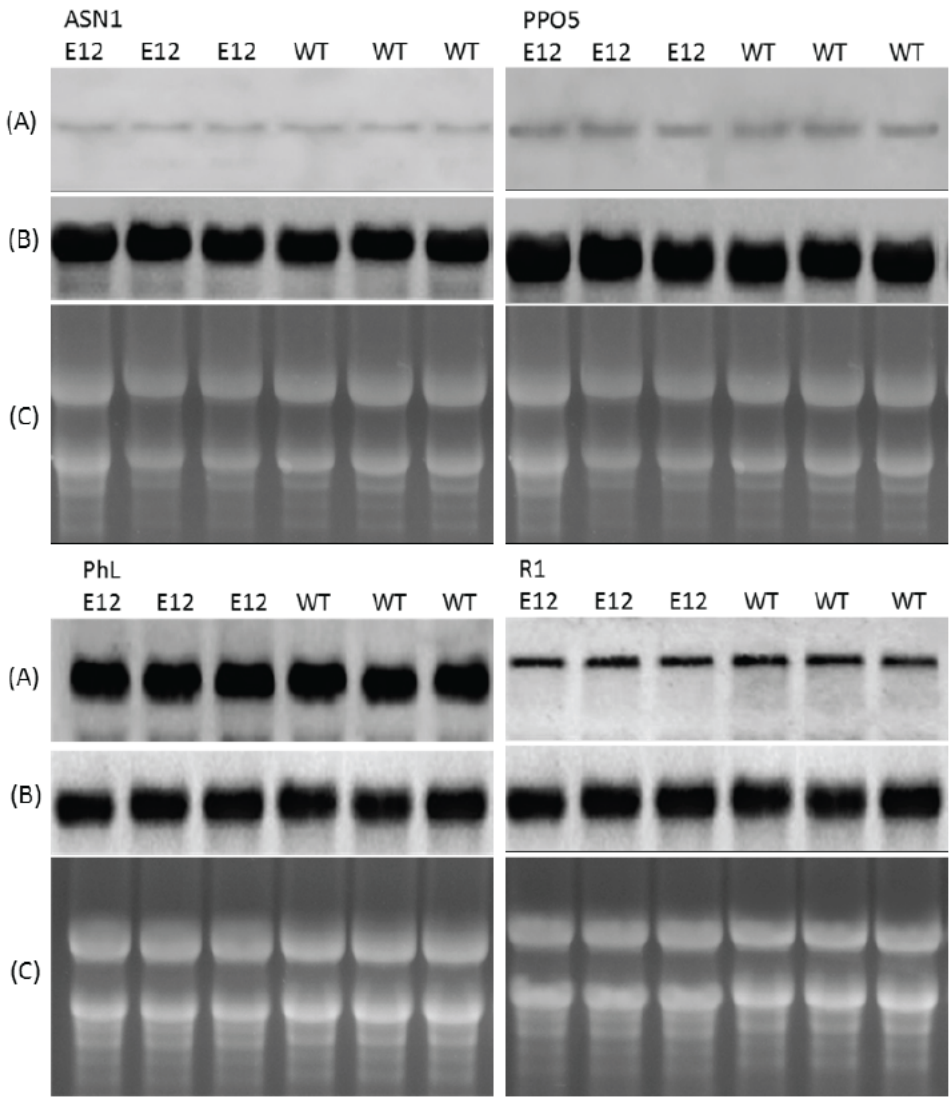


Figure 26. No Changes in RNAi Target Gene Expression in E12 Stems

Northern blots were probed to detect each of the RNAi targets: *Asn1*, *Ppo5*, *PhL*, and *R1*. (A) Samples were analysed with the indicated probe. (B) 18S RNA provides a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

Down-regulation was not observed for the four target genes in E12 stem samples compared to the Russet Burbank control (WT). This was consistent with tuber-specific down-regulation (Figure 26).

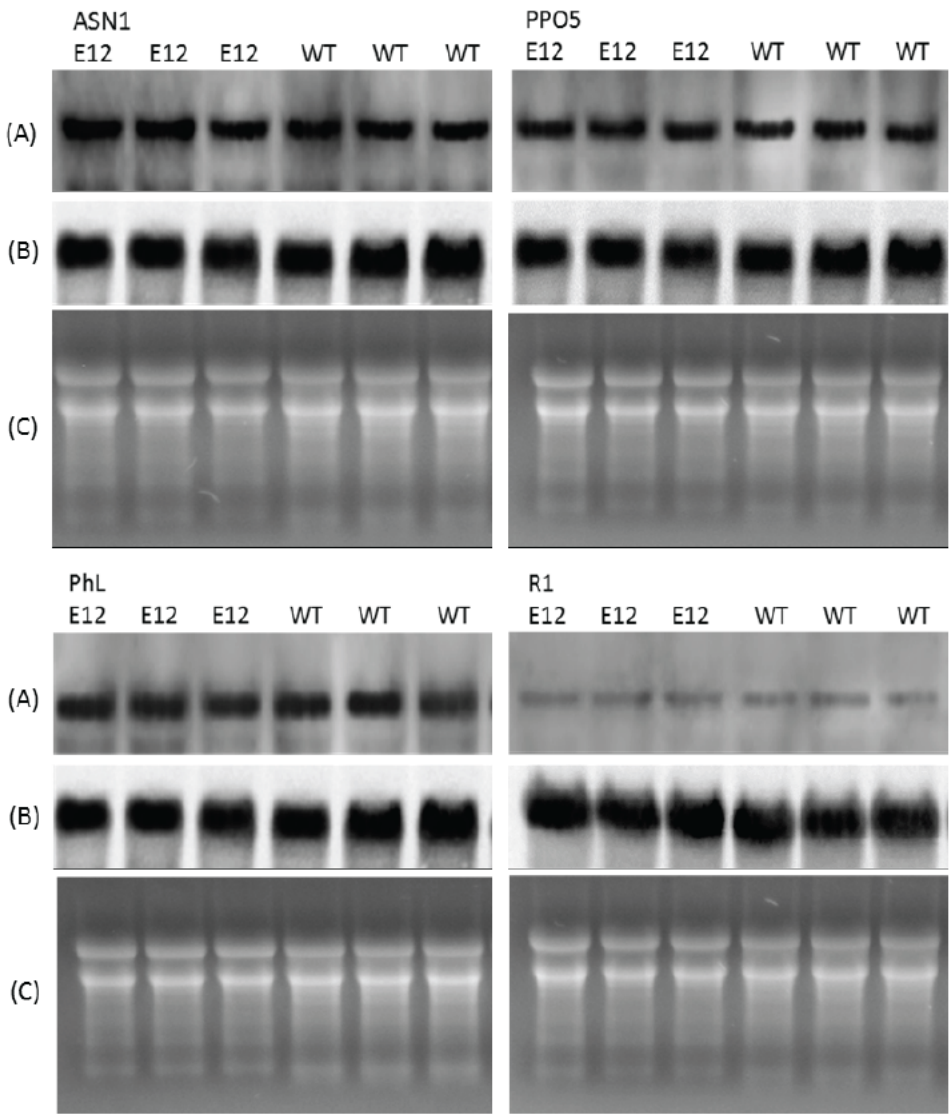


Figure 27. No Changes in RNAi Target Gene Expression in E12 Roots

Northern blots were probed to detect each of the RNAi targets: *Asn1*, *Ppo5*, *PhL*, and *R1*. (A) Samples were analysed with the indicated probe. (B) 18S RNA provides a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

Down-regulation was not observed for the four target genes in E12 root samples compared to the Russet Burbank control (WT). This was consistent with tuber-specific down-regulation (Figure 27).

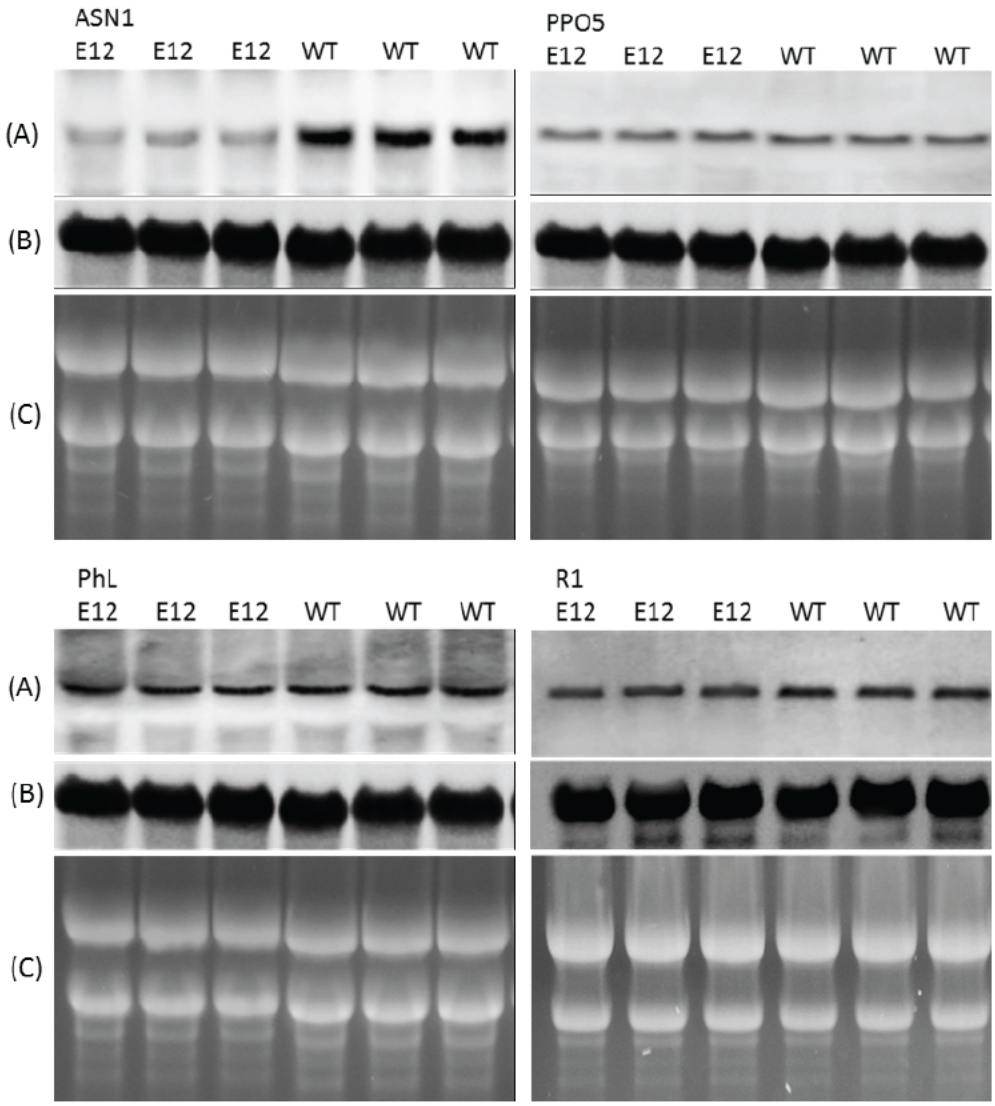


Figure 28. Partial *Asn1* Down-regulation in E12 Flowers

Northern blots were probed to detect each of the RNAi targets: *Asn1*, *Ppo5*, *PhL*, and *R1*. (A) Samples were analysed with the indicated probe. (B) 18S RNA provides a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

A minor decrease in *Asn1* gene expression was observed in E12 flower samples (Figure 28). There was no observable down-regulation of other genes in E12 flower samples, which was consistent with tuber-specific down-regulation (Figure 28).

The expression studies confirmed down-regulation of *Asn1* and *Ppo5* in tubers, partial down-regulation of *Asn1* in flowers, and no other down-regulation of the four target genes in other parts of the potato plant.

B4. The Potential Toxicity of Novel Proteins

The pSIM1278 T-DNA inserted into E12 does not produce a translatable mRNA molecule and so does not result in the expression of any polypeptides. Consequently, there are no new proteins expressed in this event requiring toxicology or allergenicity assessment.

The E12 insert has introduced only non-coding DNA (Table 2). The insert includes an inverted repeat of fragments of asparagine synthetase-1 gene and the polyphenol oxidase-5 gene between an *Agp* promoter and *Gbss* promoter, and fragments of the water dikinase gene (*R1*) and the phosphorylase-L gene (*PhL*) also positioned between the *Agp* and *Gbss* promoters. The pool of mRNA produced by these two cassettes, forms double stranded RNA that is unlikely to be processed into amino acids or proteins. Instead, all dsRNA produced by the DNA insert is processed using RNAi machinery in plant cells. The small RNA are effective at down-regulating the target genes.

Based on this discussion and the information on Novel Open Reading Frame analysis (Section A3), there are no new polypeptides expressed and no proteins for which toxicological analysis is relevant in this event. With respect to the levels of endogenous toxins or anti-nutritional compounds, there are a number of compounds in potatoes which can be toxic at certain levels and these have all been tested as part of the compositional analysis and shown to be no different from the Russet Burbank control.

Similarly, there are no new polypeptides which require an analysis of potential allergenicity in this event.

B5. The Potential Allergenicity of Novel Proteins

Potatoes are not among the 'Big Eight' group of foods that account for approximately 90% of all food allergies in the United States (FARRP, 2014). Potato contains only one allergen (patatin) referenced in the literature (OECD, 2002). Eleven entries for potato are listed in the Allergen Online database (http://www.allergenonline.org/ Version 16, Released January 27, 2016) based on their protein sequence similarity to known allergens in other species.

There are a few reports of allergies to cooked potato in children (De Swert et al., 2007, 2002). Patatin (Sol t 1) has been identified as the major allergen involved in this reaction (Astwood et al., 2000). Patatin is a storage glycoprotein that displays lipase activity and makes up about 40% of the soluble protein in potato tubers (Mignery et al., 1988). Because potato protein naturally contains a relatively large proportion of patatin, people allergic to potatoes will likely avoid them whether or not there are changes in the patatin levels of potatoes.

Not only is patatin already a highly expressed protein, but there are multiple expressed copies of the gene in the potato genome (Stupar et al., 2006). The potential for the insertion in event E12 to increase the level of patatin can be considered negligible because it would require multiple, unlikely scenarios to occur together. Further, it is extremely unlikely that the insertion was near a patatin gene, given the size of the potato genome.

Based on the discussions above, there is no expectation of increased endogenous allergenicity in the E12 potato event that is the subject of this submission.

The lack of any novel polypeptide expression in E12 precludes any direct toxicity or allergenicity testing. The potential for increased levels of endogenous allergens has been discounted based on the extremely unlikely series of events which would need to occur in order to increase the level of the one known allergen in potato significantly above its existing levels. This is supported by the observation that insertions in transformed crops are no more likely to trigger unintended effects than natural mutations, transitional breeding or somaclonal variation (Schnell et al., 2015).

An intended benefit of the integration of the DNA insert in this potato event is the reduction in levels of acrylamide in cooked potato products, through a reduction in free asparagine and reducing sugars. Acrylamide has been identified as having potential negative impact on human health, such that efforts to reduce exposure are being considered. The reduction in acrylamide potential which would result from the adoption of these events could have a possible impact on human health.

B6. Toxicity of Novel Herbicide Metabolites in GM Herbicide-Tolerant Plants

Not applicable to this submission. The potato event does not contain herbicide tolerance modifications.

B7. Compositional Analyses of the GM food

A safety assessment for novel foods and animal feeds is based on the concept that a novel food or feed retains the properties of the original recipient organism. A comparative approach focusing on the analysis of the novel food or feed and its conventional counterpart aids in the identification of potential safety and nutritional issues. The concept of substantial equivalence was developed as a practical approach to the safety assessment of genetically modified foods. It is seen as a key step in the safety assessment process.

Compositional comparison is an important element in the determination of substantial equivalence. This analysis starts with identifying key nutrients, key toxicants, and anti-nutrients for the novel food or feed in question. The critical components of a novel food or feed are compared to a conventional counterpart with a history of safe use. The data for the conventional counterpart can be measured levels or published values in the literature for all edible and commercial varieties or parental controls.

Compositional assessment has been undertaken for event E12. Full assessment details are provided in Study 15-60-SPS-COMP.

Analytes were selected by considering the important nutritional components of potatoes (OECD, 2002), the analytes expected to be altered based on the inserted DNA, and those analytes considered important in the potato industry. A summary of analytes tested are presented in Table 4. The studies were conducted to:

- Show equivalence to the untransformed controls;
- Compare the novel potato events to literature ranges and tolerance intervals for the component under investigation; and
- Show that there were no changes in glycoalkaloids.

Table 4. Tuber Composition Analytes Measured

Proximates and Fiber (7)		
Protein	Fat	Ash
Crude Fiber	Carbohydrates	Calories
Moisture		
Vitamins (3)		
Vitamin B3	Vitamin B6	Vitamin C
Minerals (3)		
Copper	Magnesium	Potassium
Total Amino Acids (18)		
Alanine	Histidine	Proline
Arginine	Isoleucine	Serine
Aspartic Acid + Asparagine	Leucine	Threonine
Cystine (including cysteine)	Lysine	Tryptophan
Glutamic Acid + Glutamine	Methionine	Tyrosine
Glycine	Phenylalanine	Valine
Free Amino Acids (4)		
Asparagine	Aspartic Acid	Glutamic Acid
Glutamine		
Sugars (2)		
Fructose + Glucose	Sucrose	
Anti-Nutrients (1)		
Glycoalkaloids		
Cooked Potato Analyte (1)		
Acrylamide[2]		

[1]Analyzed in both fresh tissue and at various monthly intervals.
[2]Analyzed in cooked materials at harvest and at various storage intervals.

The nutritional composition and trait efficacy of the potato event was compared with the conventional control, Russet Burbank. Commercially available reference varieties with a history of safe use for food and feed were included as comparators. Field trials for event E12 were conducted at eleven sites during the 2009, 2010, and 2011 growing seasons. Plots of the test, control, and reference varieties were harvested, and tubers were assessed for those analytes important to potato nutrition as well as those related specifically to trait efficacy.

The nutritional assessment, evaluating proximates, vitamins, minerals, amino acids, and glycoalkaloids demonstrated that the E12 potato event is compositionally equivalent to the control. In addition, the efficacy assessment evaluating free amino acids and reducing sugars as well as acrylamide concentrations in fries demonstrated that the event has lower levels of free asparagine, slightly lower levels of reducing sugars, and lower acrylamide potential in fries compared with the control.

Nutritional Analysis of Event E12 and the Russet Burbank Control

Analyses were conducted to confirm that the composition of event E12 remained within the range for commercial potato varieties and has equivalent food quality, feed quality, and safety when compared to its

control, Russet Burbank, and other conventional potatoes. The compositional assessments determined the concentrations of:

1. Proximates, vitamins, and minerals (Table 5);
2. Total amino acids (Table 6); and
3. Glycoalkaloids (Table 7).

Proximates, Vitamins and Minerals

Table 5. Proximates, Vitamins, and Minerals in E12 and Russet Burbank

Variable	Variety	Mean	P-Value[1]	Standard Deviation	N	Range		Tolerance Interval		Combined Literature Range[2]	
						Min	Max	Min	Max	Min	Max
Prote n (%)	E12	2.30	0.8796	0.443	33	1.47	3.02	1.26	3.59	0.7	4.6
	Contro	2.32	.	0.431	33	1.64	3.10				
Fat (%)	E12	0.100	0.9772	0.0323	33	0.0500	0.200	0	0.341	0.02	0.2
	Contro	0.100	.	0.0365	32	0.0300	0.200				
Ash (%)	E12	1.05	0.5871	0.294	32	0	1.46	0.391	1.89	0.44	1.9
	Contro	1.08	.	0.307	33	0.150	2.00				
Crude F ber (%)	E12	0.470	0.8625	0.107	33	0.330	0.700	0.142	0.690	0.17	3.5
	Contro	0.470	.	0.0979	33	0.320	0.640				
Carbohydrates (%)	E12	18.5	0.9866	1.78	33	14.2	22.2	12.3	25.9	13.3	30.53
	Contro	18.5	.	1.55	33	16.1	21.1				
Ca or es (kca /100 g)	E12	83.9	0.9537	6.49	33	64.9	96.3	59.1	115	70	110.2
	Contro	84.0	.	5.54	33	73.1	93.3				
Mo sture (%)	E12	78.1	0.8815	1.52	33	75.5	82.5	70.3	83.9	63.2	86.9
	Contro	78.0	.	1.36	33	75.5	80.4				
V tam n B3 (mg/100 g)	E12	1.90	0.7264	0.277	33	1.43	2.53	0.922	3.12	0.09	3.1
	Contro	1.87	.	0.268	33	1.44	2.38				
V tam n B6 (mg/100 g)	E12	0.140	0.9380	0.0317	33	0.110	0.230	0.0590	0.192	0.13	0.41
	Contro	0.14	.	0.0241	33	0.110	0.200				
V tam n C (mg/100 g)	E12	18.3	0.3510	5.50	33	11.8	32.9	0	129	1	54
	Contro	16.8	.	8.20	33	8.88	42.9				
Copper (ppm)	E12	1	0.6422	0.227	33	0.540	1.52	0.111	2.24	0.15	7
	Contro	0.960	.	0.242	33	0.500	1.34				
Magnes um (ppm)	E12	246	0.7238	49.4	33	188	405	102	372	112.5	550
	Contro	242	.	45.9	33	165	350				
Potass um (ppm)	E12	4,777	0.6940	1,042	33	3,550	6,537	2,711	6,882	3,500	6,250
	Contro	4,681	.	951	33	3,040	6,339				

[1]P-va ues nd cat ng s gn f cant d fferences w th contro s are under ned.
[2]Comb ned terature ranges are from, Horton and Anderson, 1992; L s nska and Leszczynsk , 1989; Rogan et a ., 2000; Ta burt et a ., 1987.

No statistical differences were found between E12 and Russet Burbank for analysed proximates, vitamins and minerals (Table 5). All mean values for E12 were within the tolerance interval and/or the combined literature range.

Total Amino Acids

Table 6. Total Amino Acids in E12 and Russet Burbank

Variable	Variety	Mean (ppm)	P Value[1]	Standard Deviation	N	Range		Tolerance Interval		Combined Literature Range[2]	
						Min	Max	Min	Max	Min	Max
A an ne	E12	718	0 8299	145	33	430	1,053	100	1,335	392	952
	Contro	704		245	33	323	1,258				
Arg n ne	E12	1,080	**0.0314**	454	33	490	2,159	368	1,922	700	1,383
	Contro	945		296	33	508	1,731				
ASP+ASN	E12	2,846	**<.0001**	947	33	1,874	5,114	100	13,340	3,385	7,380
	Contro	5,391		1,492	33	2,090	8,228				
Cyst ne[3]	E12	219	**0.0455**	39 8	12	162	282	0	471	286	12,500
	Contro	186		38 8	12	118	251				
GLU+GLN	E12	4,843	**<.0001**	1,010	33	3,517	7,376	568	7,011	2,915	6,035
	Contro	3,051		549	33	1,840	3,982				
G yc ne	E12	1,329	0 4886	760	33	489	2,885	100	2,865	500	1,990
	Contro	1,459		937	33	307	3,548				
H st d ne	E12	426	0 2571	216	33	170	991	100	761	133	469
	Contro	373		157	33	100	762				
so euc ne	E12	705	0 7803	197	33	406	1,266	145	1,346	525	953
	Contro	687		128	33	372	912				
Leuc ne	E12	1,266	0 6831	329	33	804	2,024	477	2,174	685	1,383
	Contro	1,228		295	33	530	1,783				
Lys ne	E12	1,111	0 2049	248	33	763	1,795	100	3,363	687	1,368
	Contro	894		176	33	560	1,304				
Meth on ne	E12	418	**0.0410**	152	33	228	826	100	593	300	500
	Contro	368		104	33	186	617				
Pheny a an ne	E12	799	0 7459	201	33	542	1,247	397	1,395	552	1087
	Contro	787		178	33	417	1,150				
Pro ne	E12	768	0 4801	239	33	487	1,425	100	1,492	355	1,464
	Contro	718		229	33	321	1,415				
Ser ne	E12	575	0 5840	209	33	220	985	100	1,362	500	1,022
	Contro	548		169	33	214	817				
Threon ne	E12	659	0 7744	286	33	299	1,347	226	1,315	500	1,022
	Contro	646		243	33	220	1,331				
Tryptophan[3]	E12	204	0 5724	24 9	12	163	237	115	435	436	855
	Contro	200		14 9	12	182	224				
Tyros ne	E12	620	0 3040	203	33	266	989	100	1,523	457	942
	Contro	562		127	33	328	792				
Va ne	E12	1,076	0 2361	196	33	612	1,519	100	1,860	752	1,450
	Contro	1,176		324	33	564	2,012				

[1]P-va ues nd cat ng s gn f cant d fferences w th contro s are under ned.
[2]Comb ned terature ranges are from L s nska and Leszczynsk , 1989; OECD, 2002; Rogan et a ., 2000; Ta ey et a ., 1984.
[3]Cyst ne and tryptophan measured n 2011 on y.

Overall, for all total amino acids, the mean values for E12 were within the tolerance interval and/or the combined literature range for potato.

As expected, significant differences were observed between event E12 and the Russet Burbank control for the amino acids ASP+ASN and GLU+GLN (Table 6). Amino acids ASP+ASN were expected to be lower and amino acids GLU+GLN were expected to be higher in E12 than in the Russet Burbank control. This is due to the down-regulation of the *Asn1* gene. In each case, the mean for E12 was within the tolerance interval and/or the combined literature range. As such, E12 is considered equivalent with respect to amino acid composition to conventional potatoes.

A significant difference between E12 and the control was also noted for the amino acids arginine, cystine (including cysteine) and methionine (Table 6). In all cases, the mean values for E12 were within the tolerance interval and/or the combined literature range.

Glycoalkaloids

The mean concentration of glycoalkaloids in tubers from E12 was not statistically different from the Russet Burbank control, was lower than the generally accepted safety limit, and fell within the tolerance interval and/or the combined literature range (Table 7).

Table 7. Glycoalkaloids in E12 and Control Russet Burbank

Variable	Variety	Mean (mg/100 g)	P-Value[1]	Standard Deviation	N	Range		Tolerance Interval		Combined Literature Range[2]	
						Min	Max	Min	Max	Min	Max
G ycoa ka o ds	E12	5.68	0.0524	2.06	33	1.69	10.5	0	33.1	3.20	210.4
	Contro	7.40	.	3.49	33	2.74	18.6				

[1]P-va ues nd cat ng s gn f cant d fferences w th contro s are under ned.
[2]Comb ned terature ranges from Kozukue et a ., 2008.

Efficacy Analysis of E12 and the Russet Burbank Control

An assessment of trait efficacy of E12 for low acrylamide potential and lowered reducing sugars consisted of the following analyses:

1. Free amino acids in tubers (Table 8);
2. Reducing sugars in tubers (**Error! Reference source not found.**); and
3. Acrylamide in tubers (Table 10).

Free Amino Acids

Free amino acid analysis demonstrated that down-regulation of the *Asn1* gene was effective in reducing free asparagine in tubers. The results show that E12 tubers contained statistically less free asparagine and statistically more free glutamine than Russet Burbank tubers (Table 8). Although less than the control, the mean concentrations of free asparagine, aspartic acid, free glutamine, for event E12 were still within the tolerance intervals and/or the combined literature range and therefore are considered within the normal range for potatoes.

Table 8. Free Amino Acids in Tubers of E12 and Control Russet Burbank

Variable	Variety	Mean (ppm)	P-Value[1]	Standard Deviation	N	Range		Tolerance Interval		Combined Literature Range[2]	
						Min	Max	Min	Max	Min	Max
Asparagine	E12	618	<.0001	173	33	425	1,250	500	4,952	312	6,890
Asparagine	Control	2,421	.	901	33	822	4,030				
Aspartic Acid	E12	448	0.9073	130	33	293	824	100	1,411	64	752
Aspartic Acid	Control	439	.	137	33	280	760				
Glutamine	E12	1997	<.0001	663	33	865	3,490	100	3,449	440	5,396[3]
Glutamine	Control	1111	.	292	33	605	1,700				
Glutamic Acid	E12	460	0.9008	133	33	227	741	100	1,192	450	742
Glutamic Acid	Control	466	.	133	33	221	689				

[1]P-values indicating significant differences with controls are underlined.
[2]Combined literature ranges are from Davies, 1977; Lisinska and Leszczynski, 1989; Shepherd et al., 2010.
[3]For glutamine, the maximum value from the CLR high level is reported as 5,396 ppm from the mean of four sites (Davies, 1977). A value of 18,244 ppm from a single site was not included because it appeared to be an outlier.

Reducing Sugars

Event E12 contains a cassette designed to lower levels of the reducing sugars, fructose and glucose, in tubers. This cassette contains sequences from starch-associated (*R1*) and phosphorylase-L (*PhL*) regulatory elements. Down-regulation of *R1* and *PhL* should function by slowing the conversion of starch to the reducing sugars, glucose and fructose.

Mean results for E12 fructose plus glucose and sucrose levels were within the range of the tolerance interval values for fresh, one, three and five months storage. Partial down-regulation of R1 and PhL resulted in lower levels of reducing sugars at harvest and after one month storage with statistical differences found only at 1 month of storage (Table 9). Values for three and five months were similar for E12 and the control.

Table 9. Russet Burbank and Event E12 Sugars in Tubers at Harvest and After Storage

Variable	Variety	Mean	P-Value[1]	Standard Deviation	N	Range		Tolerance Interval		CLR[2]	
						Min	Max	Min	Max	Min	Max
Fructose and Glucose (%)											
Fresh[3]	E12	0.160	0.1101	0.0760	12	0.0690	0.339	0	0.307	0.018	0.803
	Contro	0.195	.	0.0943	12	0.0530	0.357				
Month 1[4]	E12	0.107	**0.0051**	0.0612	21	0.0340	0.251	0	0.307	0.018	0.803
	Contro	0.162	.	0.0946	21	0.0530	0.393				
Month 3[5]	E12	0.249	0.2001	0.151	25	0.0810	0.580	0	0.307	0.018	0.803
	Contro	0.290	.	0.155	27	0.117	0.660				
Month 5[6]	E12	0.145	0.2885	0.0909	18	0.0850	0.489	0	0.307	0.018	0.803
	Contro	0.190	.	0.151	20	0.0920	0.630				
Sucrose (%)											
Fresh[3]	E12	0.213	0.2115	0.0653	12	0.134	0.299	0	0.315	0.0397	1.39
	Contro	0.179	.	0.0608	11	0.113	0.288				
Month 1[4]	E12	0.132	**0.0123**	0.0583	21	0.0600	0.258	0	0.315	0.0397	1.39
	Contro	0.168	.	0.0601	21	0.103	0.252				
Month 3[5]	E12	0.0734	0.5109	0.0246	25	0.0340	0.126	0	0.315	0.0397	1.39
	Contro	0.0793	.	0.0203	27	0.0470	0.121				
Month 5[6]	E12	0.0882	0.5554	0.0259	18	0.0510	0.141	0	0.315	0.0397	1.39
	Contro	0.0792	.	0.0288	21	0.0350	0.127				

[1] P-va ues nd cat ng s gn f cant d fferences w th contro s are under ned.
[2] Comb ned L terature Ranges from Amre n et a ., 2003a; V vant et a ., 2006.
[3] Tubers from the fresh t me po nt were ana ysed from a s tes n 2011.
[4] Tubers from the 1-month t me po nt were ana ysed from a s tes n 2009 and 2010.
[5] Tubers from the 3-month t me po nt were ana ysed from a s tes n 2009 and 2010 and from B ngham and Adams count es n 2011.
[6] Tubers from the 5-month t me po nt were ana ysed from a s tes n 2009 and 2010.

Acrylamide in Tubers

To demonstrate the downstream benefit of lower acrylamide potential, field-grown tubers of E12 and Russet Burbank at harvest and after 2, 3, 5, 6, and 7 months of storage were processed into cooked fries, and the acrylamide concentration was measured (Table 10).

At the time of harvest, fries made with E12 tubers contained 67.2 % less acrylamide than fries made with Russet Burbank (Table 10). When potatoes were stored for 2 months, acrylamide concentrations in E12 were 69.6 % lower than the control. Acrylamide concentrations in E12 fries were statistically lower than Russet Burbank fries after tuber storage for 3, 5, and 6 months. The significantly lower acrylamide levels after storage were expected from down-regulation of the *Asn1*, *R1* and *PhL* genes, thus reducing the reactants free asparagine and reducing sugars. Similar reductions in reducing sugars and acrylamide were reported by Zhu et al., 2014.

Mean results for acrylamide levels were within range of the tolerance interval.

Table 10. Acrylamide in Fries from Russet Burbank and Event E12 at Harvest and After Storage

Variable	Variety	Mean (ppb)	P-Value[1]	Standard Deviation	N	Percent Reduction	Range		Tolerance Interval	
							Min	Max	Min	Max
Fresh[2]	E12	162	**0.0340**	56	24	67.2	95.0	298	0	3,198
	Control	494	.	130	24		286	770		
Month 2[3]	E12	237	0.1700	13	9	69.6	209	251	0	3,198
	Control	779	.	125	9		619	950		
Month 3[4]	E12	315	**<.0001**	97	24	61.3	169	254	0	3,198
	Control	814	.	230	24		502	1,240		
Month 5[5]	E12	82.0	**<.0001**	10	9	64.3	67.0	97.0	0	3,198
	Control	230	.	20	9		207	260		
Month 6[6]	E12	192	**0.0019**	66	12	52.1	105	272	0	3,198
	Control	401	.	175	12		234	776		
Month 7[7]	E12	192	0.8835	26	3	71.0	165	217	0	3,198
	Control	661	.	55	3		598	693		

[1]P-va ues nd cat ng s gn f cant d fferences w th contro s are under ned.
[2]Tubers from the fresh t me po nt were ana ysed from a s tes n 2010 and 2011.
[3]Tubers from the 2-month t me po nt were ana ysed from a s tes n 2009.
[4]Tubers from the 3-month t me po nt were ana ysed from a s tes n 2010 and 2011.
[5]Tubers from the 5-month t me po nt were ana ysed from a s tes n 2009.
[6]Tubers from the 6-month t me po nt were ana ysed from a s tes n 2010.
[7]Tubers from the 7-month t me po nt were ana ysed on y from the Canyon County s te n 2009.

Conclusions: Compositional Assessment

The intentional lowering in ASN and reducing sugars in E12 resulted in lower acrylamide levels in fries. These modifications did not alter the quality of potato as food because:

1. Altered ASN and GLN levels are still within the normal range for potato; and
2. Any impact on nutritional quality would be minimal because ASN, ASP, GLU and GLN are non-essential amino acids and can be synthesised by the body.

A significant difference was noted between E12 and the control for the amino acids arginine, cystine (including cysteine) and methionine. However, the mean values for E12 were still within the tolerance interval and/or the combined literature range.

The efficacy assessment, evaluating free amino acids, reducing sugars, and acrylamide concentrations in fries, demonstrated that E12 has lower levels of free asparagine and lower acrylamide potential in fries than Russet Burbank.

The nutritional assessment, evaluating proximates, vitamins, minerals, amino acids, and glycoalkaloids, demonstrated that E12 is compositionally equivalent to Russet Burbank and is as safe and nutritious for food and feed as conventional potatoes that have a long history of safe consumption.

C. Information related to the nutritional impact of the genetically-modified food

Potato has a long history of safe use. Global production in 2013[5] was in excess of 374 million tonnes. Two thirds was consumed directly by humans and the remaining fed to animals or used to produce starch. The E12 event in this submission has been transformed with T-DNA designed to down-regulate endogenous potato genes. The introduction of the RNAi sequences has no nutritional impact on the potato event. This is supported by the fact that:

- Molecular characterisation demonstrated stability of the insertion across clonal propagation cycles;
- No new or novel proteins were formed through the introduction of the insert; and
- Compositional analysis did not indicate biologically significant changes to the levels of nutrients in E12 compared to the conventional counterpart. Event E12 composition is within the normal variation of potato cultivars and varieties and is substantially equivalent to conventional potato varieties.

C1. Data to Allow the Nutritional Impact of Compositional Changes in the Food to be Assessed

The most important nutritional changes between E12 potatoes and the untransformed control, Russet Burbank, relate to reduction of the amino acid, asparagine (ASN), and reducing sugars. Processing is unlikely to alter the compositional components of tubers other than the changes already observed for conventional varieties. Thus, food products derived from E12 are anticipated to be nutritionally equivalent to food products derived from other commercially available potatoes, except that E12 potatoes cooked at high temperatures are expected to have a lower acrylamide potential.

C2. Data From an Animal Feeding Study, if available

Compositional analysis is the cornerstone of the nutritional assessment of a food derived from a new plant variety. When compositional equivalence between the new food and its conventional counterpart has been established nutritional equivalence may be assumed (EFSA, 2006; OECD, 2003).

No new polypeptides are produced by the single inserts in potato event E12. Considering the compositional equivalence between the potato event and conventional potato and the lack of any observed phenotypic characteristics indicative of unexpected unintended effects arising from the genetic modification process, there was no plausible risk hypotheses that would indicate the need for animal feeding studies.

United States and Japan have not required feeding studies E12.

[5] Food and Agr cu ture Organ zat on of the Un ted Nat ons, http://faostat3.fao.org; data retr eved 15th January 2016.

List of Supporting Studies

Study 15-29-SPS-MOL E12 Structure Characterization
Study 15-52-SPS-MOL-01 Evidence for the Absence of Backbone in Russet Burbank E12
Study 15-60-SPS-COMP-01 Compositional Assessment of E12 Compared to Russet Burbank
Study 15-64-SPS-MOL-01 Stability of DNA Insert in Russet Burbank E12
Study 15-71-SPS-MOL RNA Expression of Down-Regulated Genes in Russet Burbank
Study 15-75-SPS-MOL-01 Construction of pSIM1278
Study 15-79-SPS-MOL E12 start to stop ORF
Study 15-84-SPS-MOL- PPO Efficacy of E12

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