Application to FSANZ for the Inclusion of BG25 Potatoes with late blight protection, *Potato virus Y* protection, lower reducing sugars, and reduced polyphenol oxidase in Standard 1.5.2 Food Produced Using Gene Technology

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SPS International, Inc.

OECD Unique identifier: OECD unique identifier: **SPS-ØBG25-7**

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Part 1 General Requirements (3.1.1)

A. Executive Summary

SPS International, Inc. (SPSII) has developed the SPS-ØBG25-7 (BG25) potato event as a new transformation of the Russet Burbank potato variety, hereafter referred to as event BG25. This is a Gen3 event with a single insertion that has late blight protection, *Potato virus Y* (PVY) protection, lower reducing sugars, and reduced polyphenol oxidase (PPO).

The BG25 event follows Simplot's Gen1 and Gen2 potatoes (Richael, 2021) by improving the variety Russet Burbank, one of the most grown potato varieties across Australasia and North America. The Russet Burbank is a premier storage variety that is excellent for the fresh market and for processing. The traits in BG25 potatoes were introduced through *Agrobacterium*-mediated transformation of Russet Burbank with the plasmid pSIM4363 and include:

- Durable foliar and tuber protection against late blight infection from three potato resistance genes (R-genes) that are cis-genes
- Protection against PVY infection by inhibition of viral replication using viral coat protein (CP) sequences to trigger the plant's RNA interference (RNAi) pathway
- Lower reducing sugars (fructose and glucose) from down regulation of vacuolar invertase (VINV) using RNAi; and
- Reduced black spot from the down regulation of polyphenol oxidase (PPO) using RNAi.

A modified potato (*Solanum tuberosum*) acetolactate synthase gene (*StmAls*) was also used as a marker for selecting transformed events *in vitro*. The StmALS protein is expressed in BG25 but is not a commercial trait.

FSANZ has previously reviewed a single late blight R-gene (*Rpi-vnt1*) in four SPSII Gen2 events (SPS-ØØØ**W8**-4, SPS-ØØ**X17**-5, SPS-ØØØ**Y9**-7, and SPS-ØØØ**Z6**-5; A1139). In comparison to these potato events previously reviewed by FSANZ, BG25 has two additional late blight R-genes, PVY protection, and a modified potato ALS marker gene. This event still has down regulated VINV and PPO but does not have a reduction in asparagine. The late blight R-genes are cis-genes from wild Solanum species.

Late blight is a serious potato disease managed through frequent sprays of preventive fungicides. The introduction of late blight protection into commercial potato varieties enables reduced fungicide applications (Ghislain et al., 2019), which further reduces both the costs and the release of fungicide into the environment. Combining three resistance proteins (R-proteins) that recognise different pathogen effector proteins reduces the chance that the pathogen can overcome the late blight protection, and this is expected to improve durability of the late blight protection trait.

The addition of PVY protection in BG25 addresses a long-standing issue in the potato industry: the reduction of viruses in certified virus-free potato seed tubers. *Potato virus Y* protection benefits the potato seed producer and protects the grower from potential yield loss due to PVY infection.

FSANZ has previously reviewed lower reducing sugars and reduced black spot in four SPSII Gen1 events (SPS-ØØE12-8 in A1128; SPS-ØØF10-7 and SPS-ØØØJ3-4 in A1139; SPS-ØØV11-6 in A1199) and four Gen2 events noted above (A1139). Reducing sugars increase during tuber storage and lead to dark colours in fries and lower the processing quality of tubers. Black spot develops in fresh potato tubers from handling during harvest, transport, storage, and processing. This discoloration of tubers is undesirable for both consumers and processors and results in food waste.

This submission contains the following information and data demonstrating the food safety of BG25 compared to Russet Burbank and other conventional potato varieties:

- Information on the development of event BG25
- Molecular characterisation of the insert in BG25, evaluating the insert structure, flanking sequence, insert copy number, integration site and demonstrating absence of vector backbone sequences
- Stability of the insert over several generations
- Characterisation and safety assessments for the four expressed proteins
- Small interfering RNA (siRNA) safety assessment
- Compositional assessment evaluating levels of key nutrients (proximates, vitamins, minerals, starch, fibre), and glycoalkaloids; and
- A comparative analysis for similarity between open reading frames (ORFs) introduced into BG25 and known allergens or toxins.

Based on these analyses, SPSII concludes that the foods derived from BG25 potatoes are as safe as those from conventional varieties. Except for the intended changes, the BG25 potatoes are not different in composition, safety, or any relevant food safety parameter to comparable potato varieties that are grown, marketed, and consumed in Australia or New Zealand.

Collectively, results support this application for amendment to the *Australia New Zealand Food Standards Code* to allow inclusion of the potato event BG25 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

B. Applicant Details

(a)	Applicant's name/s	<CCI>
(b)	Company/organisation name	SPS International, Inc.
(c)	Address (street and postal)	1099 West Front Street, Boise, ID 83702, USA
(d)	Telephone number	<CCI>
(e)	Email address	<CCI>
(f)	Nature of the applicant's business	SPS International, Inc. primarily files applications for approval of Simplot biotech events in international markets.
(g)	Details of other individuals, companies or organisations associated with the application	PTM Solutions Australia Pty Ltd <CCI><CCI> <CCI>

C. Purpose of the Application

This application seeks to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of potato event BG25 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

SPSII has developed and tested a potato event that has durable late blight protection, PVY protection, lower reducing sugars and reduced black spot. The potato event described in this application has the unique OECD code: SPS-ØBG25-7 and is referred to as event BG25 in this submission.

The potato industry is a substantial and important industry across both Australia and New Zealand. Potatoes are the highest value horticultural crop grown for consumption in Australia and are grown in all states of Australia except the Northern Territory. In New Zealand, potatoes are grown in Pukekohe, Waikato, Hawkes Bay, Manawatu, Canterbury, and Southland. SPSII currently does not intend to import the potato event into Australia or New Zealand. The primary aim of this application is to obtain a food safety approval to protect international trade. 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 in production and import markets.

D. Justification for the Application

SPS International, Inc. has developed a new potato event, BG25. The new potato event was created using inserts containing potato DNA sequences that confer lower levels of reducing sugars and lower levels of polyphenol oxidase which reduces black spot. The event also contains wild potato genes that confer durable late blight protection and *Potato virus Y* DNA sequences that protect the plant from infection by this plant virus.

Potatoes are highly heterozygous and subject to inbreeding depression, so many years are required to develop new varieties through conventional breeding. Due to these challenges, potato varieties do not have a high frequency of introduction and discontinuation. Because it is difficult to breed and backcross traits into potatoes, biotech offers a mechanism to modify traits while maintaining desired traits in a variety.

Late Blight Protection: Late blight caused by the oomycete *Phytophthora infestans*, is a devastating disease among cultivated *Solanaceae* species. If left untreated, late blight affects potato foliage and tubers causing rapid necrosis and crop loss. The Irish potato famine was the result of late blight and illustrates the destructive nature of the disease. Combining three resistance proteins (R-proteins) that recognise different pathogen effector proteins reduces the chance that the pathogen can overcome the late blight protection, and this is expected to improve durability of the late blight protection trait.

BG25 has foliar protection against the *P. infestans* strains US-8, US-22, US-23, and US-24 because of VNT1. The addition of two different potato R-genes gives BG25 both foliar and tuber resistance to late blight. This provides valuable protection to stored potatoes.

Studies have shown that reduced use of fungicides lowers the selection pressure for metalaxyl-resistant late blight strains and may slow the development of resistance to metalaxyl-based fungicides (Hwang et al., 2014).Combining R-gene-mediated late blight protection in BG25 with fungicide applications that use a different mode of action is part of an integrated pest management (IPM) strategy to control late blight and to delay the development of resistance to fungicides and the R-proteins in BG25.

An indirect benefit of late blight protected potato is lower disease pressure in the environment. Disease pressure describes the prevalence of a pathogen at a given location during a particular growing season. Disease pressure can be high or low depending on the susceptibility of host plants, pathogen presence, and environmental conditions (Agrios, 2004). In a country with intensive potato production, the best way to reduce disease pressure is by growing protected varieties (Forbes et al., 2020). The planting of protected

potato varieties can limit the dispersal of *P. infestans* to susceptible potato varieties by providing a physical barrier to pathogen growth that reduces spore and sporangium production and dispersal. This lowers the amount of pathogen inoculum in the environment (Pilet et al., 2006).

Late blight sporangia may spread in wind or water over several kilometers, demonstrating that distance from the disease outbreak is not a guarantee against infection (Fry, 2008; Schumann and D'Arcy, 2000). Foliar protection against late blight in potato lowers the overall disease pressure for immediate and surrounding fields by limiting pest sporangia and spores (Vlaams Instituut voor Biotechnologie, 2010). Tuber late blight protection reduces tuber damage in storage facilities that hold harvested potatoes for months.

PVY Protection: The addition of PVY protection in BG25 addresses the need for certified virus-free potato seed tubers, which is a long-standing issue in the potato industry. *Potato virus Y* protection benefits potato seed producers and protects growers from potential yield loss due to PVY infection.

Potato virus Y (PVY) is the most economically important virus affecting potato production worldwide (Kreuze et al., 2020). PVY is the type species of the genus potyvirus, in the family *Potyviridae*. Spread of the virus by aphid transmission occurs rapidly when an insect probes PVY-infected potato leaf tissue. The virus has a positive-sense, single stranded RNA genome (Baebler et al., 2020). The RNA is enclosed in the viral capsid, made up of approximately 2,000 copies of a single polypeptide Coat Protein (CP). PVY protection in BG25 potatoes results from RNAi that targets the CP region and inhibits PVY replication.

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 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.

Lower Reducing Sugars: Potato composition can change over the course of harvest to consumption with most changes occurring during storage and processing (Burton et al., 1992). Low temperature storage is an important practice as it reduces loss and inhibits sprouting. However, at low temperatures, starch is rapidly converted to reducing sugars (Smith 1975). Maintaining low reducing sugar levels in stored BG25 tubers ensures that processed products retain their light colour and preferred flavour.

E. Information to Support the Application

This application consists of 2 parts containing information in accordance with the following checklists:

- Part 1: General requirements (3.1.1)
- Part 2: Foods produced using gene technology (3.5.1) main document, Part 2 information. Supplement form molecular analysis.

F. 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.

G. Confidential Commercial Information (CCI)

Confidential Commercial Information (CCI) has been included in this submission document. A separate expurgated copy of this application is also provided.

Specific information that is considered confidential is highlighted in this submission.

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 this 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.

H. Other Confidential Information

No additional confidential material is included in this submission document.

I. 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*.

Costs and Benefits

Most potatoes consumed in Australia and New Zealand are grown domestically. Domestic production of potato in Australia (2021/2022 – approximately 1.4 million metric tonnes over 27,000 ha)[1] is supplemented by imports of processed potato, predominantly from the United States. Australian fresh potato exports were 40,523 tonnes for the year ending June 2022. Of the total domestic production in 2021/2022, 67% was sent to processing, 30% was sold fresh and 3% was sent for fresh export[2].

Potatoes enjoy consistent market penetration year-round at 52-58%, confirming their role as a staple Australian product on both summer and winter menus. In Australia, for the year ending June 2022, per capita consumption for fresh potatoes based on the volume supplied was 17.01 kg. Family households typically buy more potatoes at each purchase than smaller households, while households with lower discretionary income

[1] The Australian Horticulture Statistics Handbook 2021/22 (AH15001): retrieved November 2023 from https://www.horticulture.com.au/growers/help-your-business-grow/research-reports-publications-fact-sheets-and-more/australian-horticulture-statistics-handbook/
[2] *IBID*

are more likely to purchase lower priced pre-packed products compared to those with higher discretionary income (Brunton, 2015).

Domestic production in New Zealand (2021/2022– 527,190 metric tonnes over 8,424 ha)[3] with a farm gate value of more than $NZ 174 million per annum. New Zealand exports were 84,074 metric tonnes for the year ending 2022.

In both Australia and New Zealand, 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 and New Zealand diets, 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.

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, predicted to reach 9 billion by 2050 (Alexandratos and Bruinsma, 2012). Current 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.

In the meantime, while food insecurity remains unacceptably high, each year, massive quantities of food are lost worldwide 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 storage, processing, marketing, and food preparation, to the final decision by the consumer to eat or discard the food.

Post-harvest loss reduction interventions are a critical component of efforts to reduce food insecurity, 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, cause 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

[3] Potatoes New Zealand Inc. AGM Papers and Annual Report 2023: retrieved November 2023 from https://potatoesnz.co.nz/administration/annual-reports/

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.

The BG25 event uses RNAi gene silencing technology to regulate the expression of the genes responsible for the enzymatic darkening process. As a result, BG25 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.

Potatoes with black spot typically are trimmed or discarded before processing, resulting in both quality control challenges and economic loss. One study suggests that 1.9 million metric tons of bruise losses occurred at the grower, packer, retailer and foodservice levels of the market chain in the United States in 2013 (Halterman et al., 2016). Another study estimates that 35% of fresh potatoes were lost as food waste at the retail and consumer levels of the market chain in the United States in 2008 (Buzby et al., 2011); a significant portion of these losses would be associated with black spot. Reducing PPO levels in potatoes decreases the occurrence of black spot, resulting in increased tuber quality and less food waste (Halterman et al., 2016). In BG25, reduced black spot was achieved by down regulating PPO in tubers.

In addition to reduced black spot, the BG25 event has lower levels of 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. The United States FDA and the European Food Safety Authority have classified acrylamide as a probable carcinogen. Many international and national regulatory agencies advise limiting dietary intake of acrylamide.

The importance of potato and the impact of black spot darkening in the fresh potato market was highlighted in Brunton 2015 that reported - "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'."

The event BG25 contains three R-genes, *Rpi-vnt1* gene from *S. venturii, Rpi-amr3* from *S. americanum,* and *Rpi-blb2* from *S. bulbocastanum* that confers durable foliar and tuber late blight protection caused by the potato pathogen, *P. infestans*(Foster et al., 2009). Late blight is a devastating disease among cultivated *Solanaceae* species. In potato, late blight affects foliage and tubers causing rapid necrosis and crop loss once established (Haverkort et al., 2008). The Irish potato famine was the result of late blight and demonstrates the destructiveness of the disease (FAO, 2008). Symptoms first appear as water-soaked spots on leaves. Inside the infected leaves, sporangiophores emerge through the stomata of the stems and leaves and produce sporangia. The sporangia, when ripe, become detached and are easily spread by wind and rain causing new infections (Agrios, 2004). During irrigation or wet weather conditions, sporangia are washed down from the leaves and into the soil where they subsequently penetrate and infect the tubers (Agrios, 2004). Infected tubers will likely rot in storage, contaminate equipment, and spread infection to non-infected tubers (Miller et al., 2006). Under favorable conditions for late blight (i.e., humidity, temperature, and host susceptibility), asexual reproduction of the pathogen occurs in as few as four days leading to disease epidemics (Agrios, 2004).

Nine of the top ten registered seed varieties grown in Canada are susceptible to late blight, comprising approximately 66% of planted acreage (CFIA, 2015; NPC, 2015). Therefore, IPM practices to control late blight rely primarily on chemical fungicides, crop rotation, and other cultural controls to minimise risk of infection (Canadian Horticulture Council, 2011; Miller et al., 2006). The Pest Management Centre of

Agriculture and Agri-Food Canada (2020) recommend both a prophylactic spray fungicide program and field rotations with crops such as cereals and forages in order to minimize the occurrence of disease and improve soil and crop productivity (Agriculture and Agri-Food Canada, 2005). The planting of late blight resistant varieties allows the use of a balanced disease control strategy that employs IPM programs while maintaining durability of the trait.

R-genes in wild *Solanum* species provide natural protection to late blight (Sedlák et al., 2005). These genes can be bred or introduced into new varieties that then become resistant to late blight. Potato breeding programs from the mid-twentieth century used *Solanum demissum*, a wild potato species found in central Mexico and a good source of protection against certain strains of late blight (Pel, 2010; Vleeshouwers et al., 2011). *Solanum demissum* R-genes designated *R1-R11* have been identified, some of which have been widely used for introgression in European breeding programs to help control late blight (Malcolmson and Black, 1966).

Numerous research studies have been carried out worldwide to develop late blight resistant potato cultivars and improve their durability. The introduction of new cultivars containing these R-genes was initially successful, but rapidly evolving populations of *P. infestans* reduced their efficacy (Fry et al., 2015). Durability in the field of a particular R-gene is variable (Leach et al., 2001), and additional novel R-genes against *P. infestans* are being discovered from other wild Solanum species. Genes from wild Solanum species have been integrated into many edible potato cultivars through breeding with wild species such as *S. bulbocastanum* (Park et al., 2005), *S. stoloniferum* (Haverkort et al., 2008; Hutten et al., 2013), *S. microdontum* (Tan et al., 2008), and *S. phureja* (Śliwka et al., 2013).

Food approvals in influential countries like Australia increase the likelihood that these potatoes can become available in developing countries to help alleviate hunger in resource-scarce parts of the world. Simplot is working to help ensure this can happen by partnering with Michigan State University to develop late blight protected varieties for Bangladesh and Indonesian farmers. In 2019 Simplot provided late blight protected events to Michigan State University for field efficacy testing in these two countries. These events have three R-genes for durable late blight protection and are in two farmer preferred varieties chosen by Bangladesh and Indonesia. This initiative is part of the United States Government's Feed the Future initiative.

The potato industry is a substantial and important industry across both Australia and New Zealand. Potatoes are the highest value horticultural crop grown for consumption in Australia and are grown in all states of Australia except the Northern Territory. In New Zealand, potatoes are grown in Pukekohe, Waikato, Hawkes Bay, Manawatu, Canterbury, and Southland.

The Biosecurity Plan for the potato Industry (Plant Health Australia 2019) was developed by Plant Health Australia (PHA) in collaboration with industry and government stakeholders and was launched in May 2007 and updated in 2013 and 2019. The Potato Biosecurity Plan notes that Australia's geographic isolation and lack of shared land borders have, in the past, provided a degree of natural protection from exotic threats.

The Potato Biosecurity Plan lists Late blight – A2 mating type – *Phytophthora infestans*, as one of the top-ranked pest threats to the Australian potato industry. The event BG25 is currently not intended for the Australian market, however, such technology represents future opportunities for Australian growers and consumers.

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

- Innate® potatoes reduce bruise and black spot up to 44% compared to 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 Innate® potatoes show less 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 – it is estimated that up to 35% of fresh potatoes are wasted in the United States alone, representing 1.67 billion kg per year which has been estimated to cost upwards of $1.7 billion annually (Buzby et al., 2011).

J. International and Other National Standards

Applications for approval of SPS-ØBG25-7 have been submitted to other jurisdictions (Table 1).

Responsible environmental stewardship and deployment of biotechnology-derived products are important to SPS International, Inc., to its parent company the J.R. Simplot Company, and to Simplot Plant Sciences (SPS), the biotechnology group within the J.R. Simplot Company. 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, 2022) 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-ØBG25-7

Country	Competent National Authority	Type of Authorisation	Approval Status
			BG25
United States	United States Department of Agriculture (USDA)	Determination of non-regulated status	Non-regulated
	Food and Drug Administration (FDA)	Food and feed safety assessment	Under Review
	Environmental Protection Agency (EPA)	Permanent exemption from Tolerance to VNT1; Event Registration	Registration Under Review. Have permanent tolerance for VNT1. StmALS and PVY are exempt from tolerance.
Canada	Canadian Food Inspection Agency (CFIA)	Unconfined environmental release	Will submit in January 2024
		Use in livestock feed	Will submit in January 2024
	Health Canada	Food approval	Submitted at same time as FSANZ for joint review programme

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-ØBG25-7 (BG25) in Standard 1.5.2–Food Produced Using Gene Technology.

Simplot notes: that the *Codex Guideline* does not make allowances for familiarity with risk, such as occurs with events resulting from the retransformation of different varieties of a vegetatively reproduced crop.

K. Statutory Declaration – Australia

<CCI>

L. Checklists Provided With Application

General Requirements

General requirements (3.1.1)		
Check	**Page No.**	**Mandatory requirements**
☒	2	A Form of application ☒ Application in English ☒ Executive Summary (separated from main application electronically) ☒ Relevant sections of Part 3 clearly identified ☒ Pages sequentially numbered ☒ Electronic copy (searchable) ☒ All references provided
☒	3	B Applicant details
☒	4	C Purpose of the application
☒	4	D Justification for the application ☒ Regulatory impact information ☒ Impact on international trade
☒	5	E Information to support the application ☒ Data requirements
☒	5	F Assessment procedure ☒ General ☐ Major ☐ Minor ☐ High level health claim variation
☒	5	G Confidential commercial information ☒ CCI material separated from other application material ☒ Formal request including reasons ☒ Non-confidential summary provided
☒	6	H Other confidential information ☐ Confidential material separated from other application material ☐ Formal request including reasons
☒	6	I Exclusive Capturable Commercial Benefit ☒ Justification provided
☒	10	J International and other national standards ☒ International standards ☒ Other national standards
☒	12	K Statutory Declaration
☒	13	L Checklist/s provided with application ☒ 3.1.1 Checklist ☒ All page number references from application included ☒ Any other relevant checklists for Chapters 3.2–3.7

Foods Produced Using Gene Technology

Foods produced using gene technology (3.5.1)		
Check	**Page No.**	**Mandatory requirements**
☒	23	A.1 Nature and identity
☒	24	A.2 History of use of host and donor organisms
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☒	92	B.2 New proteins
☒	97	B.3 Other (non-protein) new substances
☐	N/A	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants
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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 July 2019.

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Abbreviations, Acronyms and Definitions[4]

Abbreviation	Definition
ADF	Acid Detergent Fibre
A. tumefaciens	*Agrobacterium tumefaciens*
ALS	Acetolactate synthase and probe used to detect *StmAls*
AMR3	AMR3 protein
APRT	*Adenine phosphoribosyltransferase gene*
APRT	Adenine phosphoribosyltransferase protein
Backbone DNA	DNA associated with construct backbone
BLAST	Basic Local Alignment Search Tool
BWA	Burrows-Wheeler Aligner
BLB2	BLB2 protein or probe used to detect *Rpi-blb2*
CC-NBS-LRR	Coiled coil-Nucleotide Binding Site- Leucine-rich Repeat
CFIA	Canadian Food Inspection Agency
COMPARE	COMprehensive Protein Allergen REsource
CP	Coat protein
DAP	Days after planting
ddPCR	Droplet digital PCR
DEEM	Dietary Exposure Evaluation Model
dsRNA	Double-stranded RNA
DW	Dry Weight
DM	Dry Matter
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FARRP	Food Allergy Research & Resource Program
FASTA	Text-based format representing nucleotides or amino acid sequences
FCID	Food Commodity Intake Database
FDA	Food and Drug Administration
FSANZ	Food Safety Australia New Zealand
FW	Fresh Weight
Gbss	Granule-bound starch synthase gene
GRAS	Generally recognized as safe
GST	Glutathione transferase
GUS	β-glucuronidase
VINV/PPO	Probe used to detect VInv/Ppo inverted repeat sequence
IPM	Integrated Pest Management
ipt	Isopentenyl transferase gene
KmR	*Kanamycin resistance gene*
LOD	Limit of Detection
LOQ	Limit of Quantitation
MOE	Margins of Exposure
NBY	Nutrient broth yeast extract
NDF	Neutral Detergent Fibre
NGS	Next Generation Sequencing
NOAEL	No Observed Adverse Limit Effect
OECD	Organization for Economic Cooperation and Development

[4] NOTE: Abbreviations of units of measure and of physical and chemical quantities are used according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/).

Abbreviation	Definition
ORF	Open Reading Frame
P. infestans	*Phytophthora infestans*
PAMP	Pathogen associated molecular pattern
pGap	ADP glucose pyrphophorylase gene promotor
pGbss	Granule-bound starch synthase gene promoter
PGSC	Potato Genome Sequence Consortium
Ppo	Polyphenol oxidase gene
PPO	Polyphenol oxidase enzyme
PTI	Pathogen triggered immunity
pUbi7	Potato Polyubiquitin Promoter
PVY	*Potato virus Y* or probe used to detect PVY-CP
PVY-CP	*Potato virus Y* coat protein
RBD	Randomized Block Design
RISC	RNA-induced silencing complex
RNAi	RNA interference
Rpi-amr3	Resistance gene from *S. americanum* for protection against *P. infestans*
Rpi-blb2	Resistance gene from *S. bulbocastanum* for protection against *P. infestans*
Rpi-vnt1	Resistance gene from *S. venturii* for protection against *P. infestans*
RT	Room Temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
R-Gene	Resistance gene
R-Protein	Resistance protein
siRNA	Small interfering RNA
SPS-ØBG25-7	OECD identifier for BG25
StALS	*Solanum tuberosum* ALS protein
StALS	*Solanum tuberosum Als gene*
StmAls	Modified *Solanum tuberosum* acetolactate synthase gene
StmALS	Modified *Solanum tuberosum* acetolactate synthase protein
T-DNA	Transfer DNA
tUbi3	Potato Terminator
Ubi7	*Potato polyubiquitin 7 gene*
UniProtKB	Universal Protein Resource Knowledgebase
USDA	United States Department of Agriculture
VInv	Vacuolar invertase gene
VINV	Vacuolar invertase protein
VNT1	VNT1 protein

A. Technical Information on the Food Produced Using Gene Technology

A.1. Nature and Identity of the Genetically Modified Food

A.1(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 event BG25 was developed by transforming the potato variety Russet Burbank, with the *Agrobacterium* vector pSIM4363. The event was developed to confer late blight protection, *Potato virus Y* protection, lower levels of reducing sugars, and reduced polyphenol oxidase, which contribute to improved storage potential and reduced black spot (Table 2).

The vector pSIM4363 contains six different cassettes. Three of the cassettes are designed to introduce durable late blight protection through expression of three different R-proteins. Two of the cassettes are designed as inverted repeats for the down regulation of gene expression of PPO/VInv for reduced PPO and lower reducing sugars and the PVY-CP for PVY protection through the RNAi pathway. The sixth cassette in the pSIM4363 T-DNA was designed to introduce a modified *StmAls* for use as a selection marker during transformation.

Table 2. Summary of genes, intended traits, and benefits in event BG25

Gene	Expression / Down Regulation	Trait	Benefit
Rpi-amr3	Protein expression	Protection against *Phytophthora infestans*	Late blight protection
Rpi-blb2	Protein expression	Protection against *P. infestans*	Late blight protection
Rpi-vnt1[1]	Protein expression	Protection against *P. infestans*	Late blight protection
PVY-CP	RNAi down regulation	Protection against PVY	PVY protection
VInv[1]	RNAi down regulation	Lower reducing sugars	Retains light color when fried because sugars remain low in storage
Ppo[1]	RNAi down regulation	Lower PPO	Reduces black spot, which improves potato quality and reduces waste
StmAls: modified potato acetolactate synthase gene	Protein expression	No commercial trait	Herbicide tolerance marker for transformation

[1] Traits have previously been evaluated by FSANZ see A1128, A1139 and A1199.

A.1(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 is SPS-ØBG25-7.

A.1(c) The name the food will be marketed under (if known).

The potato containing the Innate® technology will be marketed on the Canadian and United States fresh potato market as:

- Event BG25–Brigade

This potato will be marketed under a variety of labels as fresh, fresh cut or processed, depending on the licenced user of the event.

A.2. History of use of the host and donor organisms

A.2(a) For the donor organism(s) from which the genetic elements are derived:

A.2(a)(i) Any known pathogenicity, toxicity or allergenicity of relevance to the food

Genetic elements from donor organisms

Potato event BG25 expresses three R-proteins to provide potato blight protection and a modified *StmALS* gene for selection in tissue culture.

R-proteins provide disease protection

R-genes and their expressed resistance proteins (R-proteins) are found throughout the plant kingdom, including in many edible crops. R-proteins, however, are intractable proteins, expressed at exceptionally low levels. Purification of biologically active R-proteins from the host plant or heterologous expression systems is problematic (Habig et al., 2018). As a result, generating a standard protein safety data package for R-proteins is impractical. However, R-protein safety can be comprehensively and adequately assessed without isolation of functional proteins by considering the long history of safe use, bioinformatic analysis, and known modes of action (Roper et al., 2021).

R-proteins are expressed from dominant genes in plant genomes that confer disease protection against pathogens. R-proteins directly or indirectly recognize intracellular avirulence (Avr) effectors from pathogens and result in effector-triggered immunity in the host plant (Nimchuk et al., 2003). This resistance response is a form of programmed cell death that prevents disease spread by restricting cell-to-cell movement of the pathogen (Gururani et al., 2012).

Most R-proteins consist of a nucleotide binding site (NBS) and a leucine rich repeat (LRR) domain and are often referred to as NBS-LRR R-proteins. The LRR domain is the least conserved portion of the protein and is involved in effector recognition as well as ligand binding, while the NBS domain binds either adenosine triphosphate/adenosine diphosphate or guanosine triphosphate/guanosine diphosphate activating the plant's signaling pathway resulting in plant immunity. NBS-LRR R-proteins can be further subdivided into Toll/interleukin-1 receptor (TIR-NBS-LRR) and coiled-coil (CC-NBS-LRR) subfamilies (Figure 1; McHale et al., 2016).

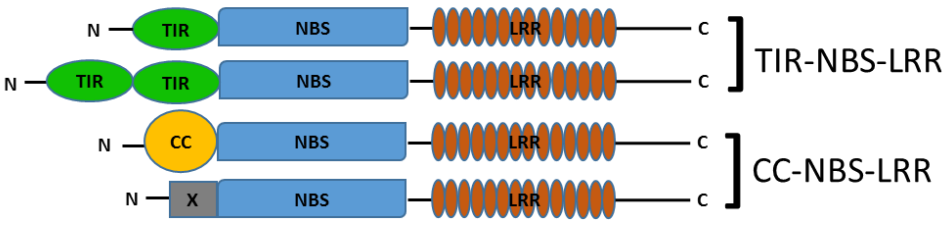


Figure 1. Subfamilies of NBS-LRR R-proteins: TIR-NBS-LRR and CC-NBS-LRR

N, amino terminus; TIR, Toll/interleukin-1 receptor-like domain; CC, coiled-coil domain; X, domain without obvious CC motif; NBS, nucleotide binding site; LRR, leucine-rich repeat domain; C, carboxyl terminus. Modified from (McHale et al., 2006).

R-genes and R-proteins for late blight disease protection in potatoes

Late blight, caused by *P. infestans*, is a devastating disease among cultivated Solanaceae species (e.g., potato and tomato). In potato, late blight affects foliage and tubers, causing rapid necrosis and crop loss if left unprotected (Haverkort et al., 2008).

R-genes in wild and some cultivated Solanum species provide natural protection against late blight (Ballvora et al., 2002; Sedlák et al., 2005). These genes can be bred into commercial potato varieties to confer late blight protection (Table 3). Potato breeding programs from the mid-twentieth century used the Mexican wild species, *S. demissum,* as a donor for protection against late blight (Pel, 2010; Vleeshouwers et al., 2011). To date, R-genes designated *R1* to *R11* have been identified in *S. demissum*, some of which have been widely used for introgression into European breeding programs to control late blight (Malcolmson and Black, 1966). Commercial potato varieties such as Sarpo Mira, Jacqueline Lee, Missaukee, PB-06, Toluca, and Bionica, possess R-proteins from wild Solanum species that provide protection against late blight (Vleeshouwers et al., 2011; Vossen et al., 2016). In addition, R-genes have been integrated into potato cultivars through breeding with wild species such as *S. bulbocastanum* (Park et al., 2005), *S. stoloniferum* (Haverkort et al., 2008; Hutten et al., 2013), *S. microdontum* (Tan et al., 2008), and *S. phureja* (Śliwka et al., 2013).

In addition, Simplot's GM Acclimate (X17) potato, which expresses the VNT1 R-protein from a wild *Solanum* species, has been on the market in the United States since 2017. Between 2017 and 2022 this has resulted in approximately 121,044,000 pounds of X17 potatoes consumed with no reported safety issues. FSANZ has assessed the safety of VNT1 and included potato events containing VNT1 in Schedule 26 of the Food Standards Code (see FSANZ A1139 and A1199).

The safe consumption of these conventional and GM commercial potatoes demonstrates that R-proteins from wild potato species that provide protection against late blight have a history of safe use in food.

Table 3. Examples of commercial potato varieties with R-genes for late blight protection

Growing Region	Commercial Potato Variety (R-gene if known)	Reference
North America	Dakota Trailblazer	(NDSU, 2017)
	Defender	(Novy et al., 2006)
	Jacqueline Lee (*R8*)	(Vossen et al., 2016)
	Kennebec (*R1*)	(PAA, 2013)
	Missaukee (*R8*)	(Vossen et al., 2016)
	Payette Russet	(Karki et al., 2021; Novy et al., 2017)
	Palisade Russet	(Novy et al., 2012)
	Acclimate X17 (*Rpi-vnt1*)	(Richael, 2021)
	Hibernate Y9 (*Rpi-vnt1*)	(Richael, 2021)
Europe	Alouette (*Rpi-vnt1.3*)	(Armstrong et al., 2019)
	Bionica (*Rpi-blb2*, *Rpi-blb3*)	(Haverkort et al., 2008; VIB, 2017)
	Carolus (*Rpi-chc1*)	(Agrico, 2017; Haverkort et al., 2016)
	Sárpo Mira (*R3a, R3b, R4, R8, Rpi-Smira1*)	(Rietman et al., 2012; Vossen et al., 2016)
	Stirling (*R2* homolog)	(The James Hutton Institute, 2017)
	Toluca (*Rpi-blb2, Rpi-blb3*)	(Haverkort et al., 2009; VIB, 2017)
China	C-88, PB-06, S-60 (*R8*)	(Vossen et al., 2016)
Japan	Toyoshiro (*R1*); Konafubuki (*R1, R3*)	(JRTA, 2012)

The three R-proteins in BG25 are AMR3, BLB2, and VNT1 are cis-genes from Solanum species. Protein expression for each gene is regulated by the native promoter and terminator sequences and all three proteins share the common domain structure found in other R-proteins.

The R-proteins in BG25 have a non-toxic mode of action consisting of recognition of pathogen effectors followed by activation of the plant hypersensitive response (summarised in Report G). This results in plant cell death at the site of the infection, which prevents the spread of the pathogen in the plant. In event BG25, the proteins AMR3, BLB2, and VNT1, recognise specific *P. infestans* effectors and activate a conserved signalling pathway already present and active in the cell. Importantly, unlike pesticidal proteins, R-proteins do not confer protection by acting on the invading pathogen, but instead, they activate the plant's hypersensitive response to prevent spread of the pathogen (Panstruga et al., 2009).

StmALS for tissue culture selection

BG25 contains a modified version of the *Solanum tuberosum* acetolactate synthase gene (*StmAls*), which is used as a selection marker during transformation. The StmALS protein is resistant to ALS-inhibiting herbicides. However, herbicide tolerance is not a commercial trait in BG25, and Simplot will not claim resistance to ALS-inhibiting herbicides.

Acetolactate synthase, which is also known as acetohydroxyacid synthase (AHAS), is an enzyme expressed in bacteria, fungi, algae, and all plant species, but not in animals. ALS catalyses the first common step in the biosynthesis pathway of the branched-chain amino acids isoleucine, leucine, and valine in plants. Humans do not have this pathway and must obtain these amino acids from their diet.

The native potato *StAls* gene, which includes a chloroplast transit sequence, encodes a 659 amino acid polypeptide that interacts with a second ALS polypeptide to form a homodimer. This homodimer is the active form of the enzyme. ALS converts two pyruvate molecules to 2-acetolactate (a precursor of leucine and valine), or pyruvate and 2-ketobutyrate to 2-aceto-2-hydroxy-butyrate (a precursor of isoleucine; Figure 2).

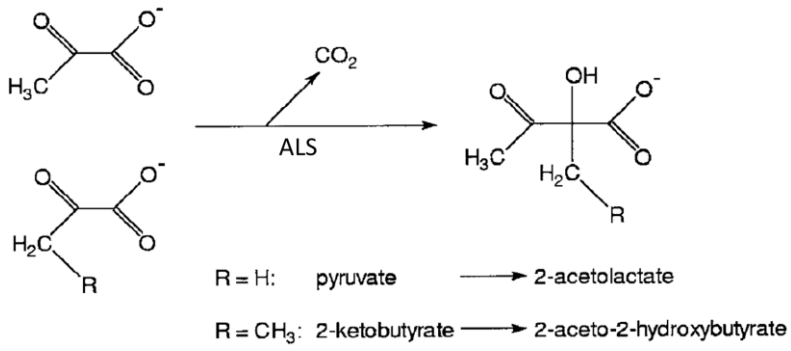


Figure 2. Acetolactate synthase enzymatic reaction

Acetolactate synthase (ALS) converts two molecules of pyruvate to 2-acetolactate or converts pyruvate and 2-ketobutyrate to 2-aceto-2-hydroxy-butyrate (Duggleby and Pang, 2000).

The *Als* gene in event BG25 was sourced from conventional potato and altered to change two amino acids in the protein sequence, giving the modified *StmAls* gene. Plants resistant to ALS inhibiting herbicides through modifications to the *Als* gene are commonly consumed (Table 4). The safety of StmALS is detailed in Report K.

Table 4. Examples of amino acid substitutions conferring ALS herbicide tolerance

Product/Crop	Line/Event	Amino Acid	Substitution Method	Reference
Potato	BG25	W563L S642I	Biotechnology	This submission
Clearfield®[2] Corn	XI12	S653N[1]	Tissue culture	(Tan et al., 2005a)
	XA17	W574L[1]	Pollen mutagenesis	
	Mutant 1	A122T		
	ICI 8532 IT	A155T		
Clearfield® Canola	PM1	S653N[1]	Microspore mutagenesis	(Tan et al., 2005a)
	PM2	W574L[1]		
Clearfield® Rice	Several	G654E[1] S653N[1]	Seed mutagenesis	(Tan et al., 2005a)
Clearfield® Wheat	TealIMI 11A; et al.	S653N[1]	Seed mutagenesis	(Tan et al., 2005a)
Clearfield® Sunflower	Several	A205V[1]	Natural mutant	(Tan et al., 2005a)
Tobacco	KS-43, et al.	Multiple	Cell culture	(Chaleff and Bascomb, 1987; Le et al., 2010; Subramanian et al., 1990)
Cotton	DO-2; PS-3	Not determined	Cell culture	(Subramanian et al., 1990; Tan et al., 2005b)
Sugar beet	Sir-13	A113T[1]	Somatic cell	(Tan et al., 2005a; Wright et al., 1998)
	Sur	P188S[1]	Somatic cell	
Lettuce	ID-BR	P197H	Natural mutant	(Eberlein et al., 1999; Thill, 1997)
Rice		P171S	Biotechnology	(Fartyal et al., 2018)
Soybean	GM-HRA	P196A W573L	Biotechnology	(Mathesius et al., 2009a; FSANZ A1006)
	CV127 (*csr1-2* gene from Arabidopsis)		Biotechnology	(FSANZ A1064)
Plenish® soybean	305423	P183A and W560L	Biotechnology	(Pioneer Hi-Bred International, 2007)
Pink pineapple	Ef2-114	Same as GM-HRA	Biotechnology	(Health Canada, 2021)

[1] Amino acid residue in reference to Arabidopsis ALS

Additional information on R-proteins and StmALS

Bioinformatic analyses confirm that the AMR3, BLB2, VNT1 and StmALS proteins lack significant homology to known toxins and allergens (see Section B.2; Report G and Report K). Additionally, bioinformatic analyses demonstrate that proteins highly like these proteins are present in the human diet, supporting a history of safe use.

A.2(a)(ii) 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 three R-proteins in BG25 are AMR3, BLB2, and VNT1 and are from Solanum species. Protein expression for each gene is regulated by the native promoter and terminator sequences and all three proteins share the common domain structure found in other R-proteins (Table 5). The StmALS protein is from *Solanum tuberosum*.

Table 5. Source of the R-proteins in event BG25

R-protein	Donor	Amino Acid Number	R-protein Domain Structure
AMR3	*S. americanum*	887	CC-NBS-LRR[1]
BLB2	*S. bulbocastanum*	1267	
VNT1	*S. venturii*	891	

[1] CC = coiled-coil, NBS = nucleotide-binding site, LRR = leucine-rich repeat see Figure 1

AMR3 from *Solanum americanum*

The source of AMR3 in BG25 is *S. americanum.* This is a wild Solanum species from the Morelloid clade, which is commonly referred to as the black nightshades. The native range for this plant includes North, Central, and South America, Melanesia (Tonga, Fiji, Vanuatu, the Solomon Islands, Papua New Guinea), New Guinea, and Australia (NBN Atlas, 2021).

Black nightshades, including *S. americanum*, are found naturally growing on disturbed ground, but are also cultivated by humans for medicinal and food use (Särkinen et al., 2018). *Solanum americanum* does not produce tubers, but both leaves and berries of *S. americanum* are consumed by humans.

Solanum americanum is a source of R-genes for late blight protection and has been shown to be naturally resistant to *P. infestans* (Witek et al., 2016).

BLB2 from *Solanum bulbocastanum*

The source of the BLB2 protein is the wild Solanum species *S. bulbocastanum*. The first formal description of *S. bulbocastanum* was published in 1814 by Dunal, and the species is distributed from northern Mexico to Honduras (Spooner et al., 2004).

Solanum bulbocastanum is not typically cultivated or grown for commercial use, but it does produce tubers (Cultivariable, 2023).

Solanum bulbocastanum is highly resistant to late blight infection and has been a primary source of late blight protection in breeding efforts to transfer this trait to cultivated *S. tuberosum* potato varieties. However, because *S. bulbocastanum* is a diploid species and has an endosperm balance number of 1 (OECD, 1997a), interspecific crosses with *S. tuberosum* are difficult. Using a series of bridge crosses (Hermsen, 1966; Hermsen and Ramanna, 1973), as well as somatic hybridizations (Helgeson et al., 1998), several R-genes from *S. bulbocastanum* were successfully bred into *S. tuberosum* varieties. Due to challenges with crossing *S. bulbocastanum* and *S. tuberosum*, it took almost 50 years to produce potato varieties with *S. bulbocastanum*-sourced late blight protection. Two conventionally bred varieties, Toluca and Bionica, contain the BLB2 R-protein and are protected against late blight. These varieties are cultivated on a small scale, primarily in Europe (Haverkort et al., 2009).

VNT1 from *Solanum venturii*

The source of VNT1 in BG25 is *S. venturi*, a diploid (2n=24) wild potato species native to north-west Argentina. *Solanum venturii* produces very small tubers and so is rarely used for food. It can be crossed with *S. tuberosum* (Jackson and Hanneman, 1999) and forms part of several breeding programs in Europe and South America to introgress *Rpi-vnt1* into commercial potatoes (Coca-Morante and Tolín-Tordoya, 2013; Gabriel et al., 2013).

The *Rpi-vnt1* gene has been introgressed into *S. tuberosum* from an interspecific cross between a *S. phureja*-derived clone and the potato cultivar Sárpo Mira (Tomczyńska et al., 2014). The *Rpi-vnt1* gene is found in both *S. venturii* and *S. phureja*, so the introduction of *Rpi-vnt1* into BG25 is expected to have similar safe use. Further, *Rpi-vnt1* and the VNT1 protein have been previously assessed by FSANZ (see A1139 and A1199).

A.2(b) A description of the host organism into which the genes were transferred:

A.2(b)(i) Its history of safe use for food

Potato is the host organism

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, 2021).

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, summarised from several sources[5].

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

Global production in 2021 was more than 376 million tonnes, grown in over 160 countries over more than 20 million hectares (FAO, 2023). The largest producers were in the regions of Asia and Europe.

Potatoes were introduced into Australia and New Zealand with the early European settlers in the late 18th century. In 1797, Governor Hunter reported that 11 acres (4.5 ha) were under potato crop in the Parramatta district west of Sydney. A decade later, this area had increased to 301 acres (122 ha); and nearly a century later in 1906, 119,000 acres (48,000 ha) of potatoes were under crop in Australia.

Today, potato production occurs around Australia except for the far northern areas where temperatures exceed the optimal growing conditions for this cool-season crop. All states grow significant quantities of potatoes with predominant production in the cooler states of South Australia, Tasmania, and Victoria.

New Zealand uses more land for growing potatoes than any other crop, with over 8,000 hectares grown by commercial potato growers in 2022 (Potatoes New Zealand, 2023). Most of New Zealand's potatoes are grown in Canterbury, Auckland, Waikato, Manawatu-Wanganui, and Hawke's Bay.

[5] *Sources:* International Year of the Potato (FAO, 2008); Feature Article: Potatoes – The world's favourite vegetable (Year Book Australia 2008, Australian Bureau of Statistics, 2008)

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 (Camire et al., 2009). Potatoes for direct consumption are typically cooked before eating because of the indigestibility of non-gelatinised starch and the presence of anti-nutritional proteins in raw, unheated potatoes (Camire et al., 2009). Potatoes are baked, boiled, or fried and used in a range of recipes: mashed potatoes, potato pancakes, potato dumplings, potato soup, potato salad, potato au gratin, jacket potatoes, potato wedges, fries/chips, and hashed browns/rösti (AHDB, 2019).

All potatoes contain natural toxins called glycoalkaloids, the most prevalent of which are α-solanine and α-chaconine. Solanine is also found in other plants in the family Solanaceae, which includes plants such as the edible crops eggplant and tomato.

The European Commission asked EFSA for a scientific opinion on the risks for animal and human health related to the presence of glycoalkaloids (GAs) in feed and food with a focus on potatoes and potato derived products (EFSA 2020). Their assessment concluded that, in humans, there is no identifiable evidence of health problems associated with repeated long-term intake of GAs from potatoes.

Data on potato GAs in feed were insufficient to perform an exposure assessment for animals.

Potatoes are not among the 'Big Eight' group of foods that account for ~90% of all food allergies in the U.S. (FARRP, 2023). There are a few reports of allergies to cooked potato in children (DeSwert et al., 2002, 2007). However, most children with potato allergy develop tolerance at an average age of four years (De Swert et al., 2007). Patatin (Sol t 1) has been identified as the primary 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 tubers (Mignery et al., 1988). There is no mechanistic reason to suggest that the level of patatin would be changed in event BG25. Because potato protein naturally contains a relatively large proportion of patatin, any unexpected change in patatin levels would be unlikely to affect allergenicity enough to alter consumption patterns for people allergic to potatoes.

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

ORDER:	Solanales
FAMILY:	Solanaceae
GENUS:	*Solanum*
SPECIES:	*S. tuberosum*, *S. verrucosum* Schltdl and *S. venturii*
COMMON NAME:	Potato, Wild Potato

A.2(b)(ii) The part of the organism typically used as food

Potato tubers are the only part consumed as food.

A.2(b)(iii) The types of products likely to include the food or food ingredient

In Australia and New Zealand, 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 and New Zealand 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
- Snacks – potato chips / straws.

A.2(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 (Camire et al., 2009).

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
- 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.

A.3. The nature of the genetic modification

A.3(a) A description of the method used to transform the host organism

The BG25 event was developed by transforming the Russet Burbank potato variety with pSIM4363 using *Agrobacterium tumefaciens* AGL1 (Lazo et al., 1991a; De Saeger et al., 2021). The *StmAls* gene was used as a selectable marker for transformation (Figure 3).

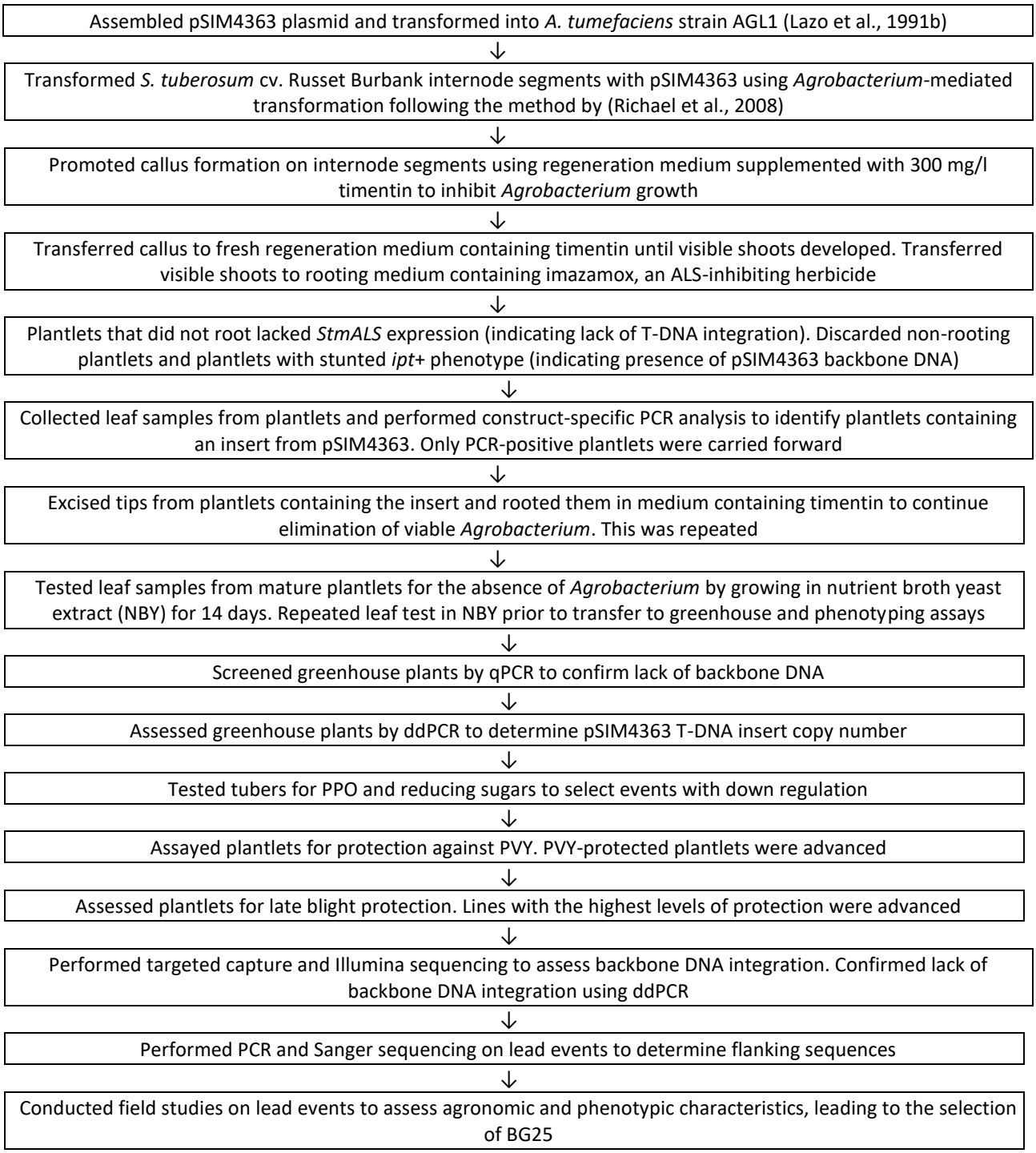


Figure 3. Transformation and event selection of BG25

Agrobacterium Transformation

Potato 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 were inoculated with *A. tumefaciens* AGL1 strain carrying pSIM4363. Inoculated 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. Callus tissue was transferred every four weeks to fresh regeneration medium until visible shoots developed. Shoots were transferred to magenta boxes with half-strength hormone-free medium containing timentin to generate roots.

Screening of pSIM4363 Transformed Events

Imazamox, and ALS-inhibiting herbicide, was added to rooting medium to inhibit root development. Plantlets that did not root with imazamox lacked StmALS expression and were likely lacking T-DNA integration. These non-rooting plantlets were discarded. In addition, plantlets with stunted ipt+ phenotype were likely to contain pSIM4363 backbone DNA and were discarded. The function of *ipt* was reviewed by FSANZ in all previous Gen1 and Gen2 applications.

Once roots developed, leaf samples from the plantlets were collected and PCR analysis was performed to identify plantlets containing an insert from the pSIM4363 T-DNA. Only PCR-positive plantlets were carried forward for further evaluation.

Obtaining *Agrobacterium*-Free Plants

Obtaining plants without *Agrobacterium* was achieved by adding timentin in the regeneration and rooting media. Tips from insert-positive plantlets were excised and transferred to fresh magenta boxes containing half-strength medium and timentin to root plantlets and select against *Agrobacterium*. Leaves were removed from plantlets and incubated in nutrient broth-yeast extract (NBY) for 14 days to confirm the absence of *Agrobacterium*. Plants showing no bacterial growth in NBY after 14 days were tested a second time in NBY to confirm absence of *Agrobacterium*.

Maintenance and Identification of Lead Events

Agrobacterium-free plantlets were maintained in tissue culture as a source of planting material for each event. Plantlets propagated from each event were transferred to soil, and after acclimation were transferred to greenhouse facilities. These events were tested for the absence of backbone DNA by qPCR. The pSIM4363 T-DNA insert copy number was assessed by droplet digital PCR (ddPCR). Tubers from these plants were tested using a PPO activity assay to confirm presence of a functional insert and were analysed for reducing sugar levels.

PVY Screen

Plantlets were assayed in the greenhouse to assess protection against three strains of PVY: $PVY^{N\text{-}Wi}$, PVY^{O}, and PVY^{NTN}. Three plantlets of each line were evaluated for each strain of PVY. Plantlets were rub-inoculated with symptomatic infected tissue macerated with inoculation buffer in a ratio of 1: 3 to 1: 4. Greenhouse conditions were 21 to 27 °C and 16/8 hours day/night. Three weeks post inoculation, the youngest, fully developed compound leaves were harvested and analyzed for PVY infection with enzyme-linked immunosorbent assay (ELISA), following the manufacturer's protocol. This triple antibody sandwich ELISA detection kit can detect all PVY strains.

Plants that tested negative for PVY were advanced. Protection against PVY was confirmed by visually comparing PVY symptoms in transformed plantlets to susceptible wild-type control plantlets and tobacco at the time of sampling. Plantlets protected against PVY were advanced to late blight screening.

Late Blight Screen

Plantlets were spray-inoculated with five different strains of *P. infestans* (NL14278, NL14538, NL14518, NL14277, and EC1), to assess the functionality of the late blight R-genes *Rpi-amr3*, *Rpi-blb2*, and *Rpi-vnt1*. For each late blight strain, three or more cuttings from each plantlet were rooted in tissue culture medium and transplanted into individual pots in growth chambers. Ten days after planting (DAP), the plant material was inoculated with 3×10^5 sporangia/ml of the late blight pathogen. Inoculated plantlets were incubated at 100% relative humidity and 18 °C for three days then transferred to 90% humidity for the remainder of the assay. Levels of protection against *P. infestans* were confirmed based on a visual assessment of the foliar symptoms and defoliation of the plants at ten days post-inoculation. Lines with the highest levels of protection were advanced.

Early Molecular Screen

Illumina Next Generation Sequencing (NGS) libraries were made from greenhouse plants for targeted capture and Illumina sequencing to assess presence of integrated vector backbone DNA. The sequencing results were confirmed using ddPCR.

PCR and Sanger sequencing were performed on the remaining events to check identity of flanking sequences.

Events with single inserts from pSIM4363, the intended phenotypes, and without backbone DNA were considered lead events and were evaluated further under commercial field conditions.

A.3(b) A description of the construct and the transformation vectors used

Event BG25 was developed by transforming the potato variety Russet Burbank with pSIM4363. The pSIM4363 plasmid contains two parts: 1) the backbone and 2) the transfer DNA (T-DNA; Figure 4).

T-DNA of pSIM4363

All sequences in event BG25 are derived from Solanum species except for an inverted repeat from PVY-CP. Donor DNA in the insert consists of both coding and non-coding genetic elements from plasmid pSIM4363 as described in Table 6.

Three of the six pSIM4363 cassettes introduce durable late blight protection through expression of three different cis-genic potato R-genes using their native promoter and terminator sequences:

- *Rpi-amr3* (Elements 15-17 in Table 6) from *S. americanum*
- *Rpi-blb2* (Elements 19-21 in Table 6) from *S. bulbocastanum;* and
- *Rpi-vnt1* (Elements 11-13 in Table 6) from *S. venturii.*

One cassette in the pSIM4363 T-DNA introduces a modified potato *StmAls* gene for use as a selection marker after transformation (Elements 3-9 in Table 6). The *StmAls* gene is a modified form of the potato *Als* gene (*StAls*). Both the promoter (pUbi7) and terminator (tUbi3) sequences are from potato.

Two of the cassettes in the pSIM4363 T-DNA are inverted repeats designed for the down regulation of gene expression through the RNAi pathway. One cassette targets the PVY-CP for conferring PVY protection to the

plant (Elements 39-41 in Table 6). Transcription of the PVY-CP inverted repeat is regulated by the potato pUbi7 (Elements 35-37 in Table 6) and potato terminator (tUbi3; Element 43 in Table 6).

The other cassette targets down regulation of two potato proteins, VINV for lower reducing sugars and PPO for reduced black spot (Elements 23-33 in Table 6). These inverted repeat sequences are from potato. Transcription of the VInv/Ppo inverted repeat is regulated by two inward facing potato promoters, the granule-bound starch synthase gene promoter (pGbss) and the ADP glucose pyrophosphorylase gene promoter (pAgp) (Elements 23 and 33 in Table 6, respectively).

Each of the cassettes also contain regulatory sequences (six promoters and four polyadenylation termination sequences). The function and source of each of the regulatory sequences is provided in Table 7.

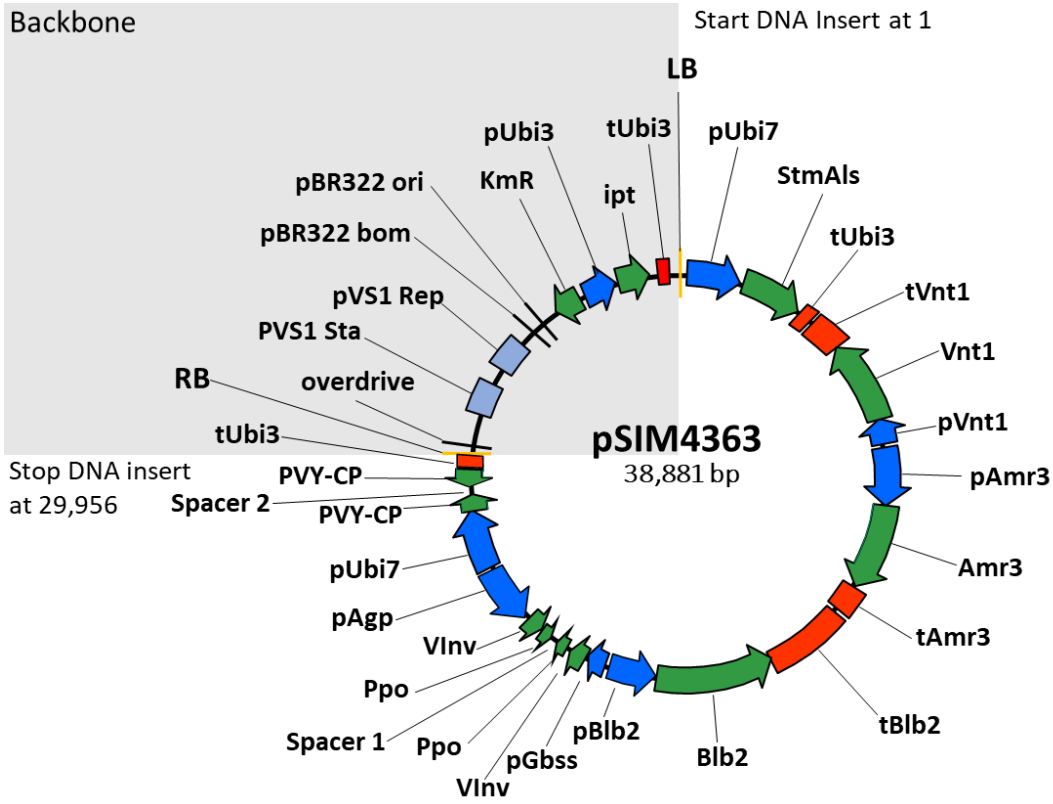


Figure 4. Plasmid map of pSIM4363

Map showing the backbone (shaded) and T-DNA elements of the pSIM4363 construct.

Table 6. Genetic Elements of pSIM4363, from Left Border to Right Border

Genetic Element	Origin	Accession Number	Position	Size (bp)	Intended Function
1. Left border (LB) region					
A. LB site	Synthetic DNA	AY566555	1-25	25	Secondary cleavage site releases ssDNA inserts from pSIM4363
B. LB buffer	*S. tuberosum* var. Ranger Russet.	AY566555	26-187	162	Buffer for truncations during insertion
2. Intervening sequence	*S. tuberosum*	AF393847	188-192	5	Sequence used for DNA cloning
3. Polyubiquitin promoter (pUbi7)	*S. tuberosum* var. Ranger Russet	U26831	193-1,131	939	Drives expression of the *StmAls* gene
4. *StUbi7* gene intron	*S. tuberosum*	U26831	1,132-1,700	569	Improves expression of StmALS; is spliced out before translation
5.*Ubiquitin* gene monomer	*S. tuberosum*	U26831	1,701-1,928	228	Expressed as a fusion with StmALS, then cleaved off
6. Intervening sequence	Synthetic DNA		1,929-1,934	6	Sequence used for DNA cloning
7. Modified *Als* gene (*StmAls*)	*S. tuberosum*	HM114275	1,935-3,914	1,980	Selection marker; modified potato ALS (StmALS)
8. Intervening sequence	Synthetic DNA		3,915-3,920	6	Sequence used for DNA cloning
9. Ubiquitin-3 gene terminator (tUbi3)	*S. tuberosum*	GP755544	3,921-4,275	355	Terminates transcription of *StmAls* gene
10. Intervening sequence	Synthetic DNA		4,276-4,285	10	Sequence used for DNA cloning
11. *Rpi-vnt1* native terminator (tVnt1)	*S. venturii*	FJ423044	4,286-5,210	925	Terminates transcription of *Rpi-vnt1*

Genetic Element	Origin	Accession Number	Position	Size (bp)	Intended Function
12. *Rpi-vnt1* gene coding sequence (*Vnt1*)	*S. venturii*	FJ423044	5,211-7,886	2,676	Expresses the VNT1 protein for foliar late blight protection
13. *Rpi-vnt1* native promoter (pVnt1)	*S. venturii*	FJ423044	7,887-8,595	709	Drives expression of the *Rpi-vnt1*
14. Intervening sequence	Synthetic DNA		8,596-8,601	6	Sequence used for DNA cloning
15. *Rpi-amr3* native promoter (pAmr3)	*S. americanum*	KT373889.1	8,602-10,519	1,918	Drives expression of the *Rpi-amr3* gene
16. *Rpi-amr3* gene coding sequence (*Amr3*)	*S. americanum*	KT373889.1	10,520-13,183	2,664	Expresses the AMR3 protein for late blight protection
17. *Rpi-amr3* native terminator (tAmr3)	*S. americanum*	KT373889.1	13,184-13,953	770	Terminates transcription of *Rpi-amr3* gene
18. Intervening sequence	Synthetic DNA		13,954-13,965	12	Sequence used for DNA cloning
19. *Rpi-blb2* native terminator (tBlb2)	*S. bulbocastanum*	DQ122125	13,966-16,497	2,532	Terminates transcription of *Rpi-blb2* gene
20. *Rpi-blb2* gene coding sequence (*Blb2*)	*S. bulbocastanum*	DQ122125	16,498-20,387	3,890	Expresses the BLB2 protein for late blight protection
21. *Rpi-blb2* native promoter (pBlb2)	*S. bulbocastanum*	DQ122125	20,388-21,932	1,545	Drives expression of the *Rpi-blb2* gene
22. Intervening sequence	Synthetic DNA		21,933-21,940	8	Sequence used for DNA cloning
23. Granule-bound starch synthase promoter (pGbss)	*S. tuberosum* var. Ranger Russet	HM363755	21,941-22,626	686	Drives expression of the VInv/Ppo inverted repeat
24. Intervening sequence	Synthetic DNA		22,627-22,633	7	Sequence used for DNA cloning

Genetic Element	Origin	Accession Number	Position	Size (bp)	Intended Function
25. Vacuolar invertase gene fragment (sense orientation; *VInv*)	*S. tuberosum* var. Ranger Russet	DQ478950	22,634-23,137	504	Generates dsRNA to down regulate *VInv* transcripts
26. Intervening sequence	Synthetic DNA		23,138-23,143	6	Sequence used for DNA cloning
27. 3'-untranslated region of the polyphenol oxidase gene (antisense orientation; *Ppo*)	*S. verrucosum*	HM363754	23,144-23,287	144	Forms dsRNA to reduce expression of polyphenol oxidase to reduce black spot
28. Spacer 1	*S. tuberosum* var. Ranger Russet	HM363753	23,288-23,450	163	Sequence between the inverted repeats; forms loop in dsRNA transcript
29. 3'-untranslated region of the polyphenol oxidase gene (sense orientation; *Ppo*)	*S. verrucosum*	HM363754	23,451-23,594	144	Forms dsRNA to reduce expression of polyphenol oxidase to reduce black spot
30. Intervening sequence	Synthetic DNA		23,595-23,601	7	Sequence used for DNA cloning
31. Vacuolar invertase gene fragment (antisense orientation, *VInv*)	*S. tuberosum* var. Ranger Russet	DQ478950	23,602-24,099	498	Generates dsRNA to down regulate invertase transcripts
32. Intervening sequence	Synthetic DNA		24,100-24,105	6	Sequence used for DNA cloning
33. ADP glucose pyrophosphorylase promoter (pAgp)	*S. tuberosum* var. Ranger Russet	HM363752	24,106-26,365	2,260	Drives expression of VInv/Ppo inverted repeat
34. Intervening sequence	Synthetic DNA		26,366-26,377	12	Sequence used for DNA cloning
35. Polyubiquitin promoter (pUbi7)	*S. tuberosum* var. Ranger Russet	U26831	26,378-27,316	939	Drives expression of the PVY inverted repeats
36. StUbi7 gene intron	*S. tuberosum*	U26831	27.,317-27,885	569	Improves expression of PVY-CP

Genetic Element	Origin	Accession Number	Position	Size (bp)	Intended Function
37. *Ubiquitin* gene monomer	*S. tuberosum*	U26831	27,886-28,113	228	Expressed as a fusion with PVY-CP, then cleaved off
38. Intervening sequence	Synthetic DNA		28,114-28,119	6	Sequence used for DNA cloning
39. *Potato virus Y* coat protein gene fragment (sense orientation, PVY-CP)	*Potato virus Y* strain N	AJ890342	28,120-28,641	522	Generates dsRNA that target PVY genome for degradation
40. Spacer 2	*S. tuberosum* var. Ranger Russet	HM363755	28,642-28,878	237	*Gbss* intron sequence between PVY inverted repeat to form loop in dsRNA
41. *Potato virus Y* coat protein gene fragment (antisense orientation, PVY-CP)	*Potato virus Y* strain N	AJ890342	28,879-29,400	522	Generates dsRNA that target PVY genome for degradation
42. Intervening sequence	Synthetic DNA		29,401-29,406	6	Sequence used for DNA cloning
43. Ubiquitin-3 gene terminator (tUbi3)	*S. tuberosum*	GP755544	29,407-29,761	355	Terminator for PVY-CP dsRNA transcript
44. Intervening sequence	Synthetic DNA		29,762-29,770	9	Sequence used for DNA cloning
45. Right border (RB) region					
A. RB buffer	*S. tuberosum* var. Ranger Russet	AY566555	29,771-29,931	161	Supports primary cleavage at RB
B. RB site	Synthetic	AY566555	29,932-29,956	25	Primary cleavage site releases ssDNA insert from pSIM4363

PVY protection

Potato virus Y protection in event BG25 potatoes results from RNAi that targets the CP region and inhibits PVY replication. The transcribed inverted repeat is expected to form a double-stranded RNA (dsRNA) hairpin that is processed rapidly into siRNA in the cell. The dsRNA is not translated into protein.

Down regulation of VINV and PPO

The event BG25 insert also contains a cassette designed to reduce gene expression of *VInv*, *Ppo* genes. Down regulation of these genes is not the result of any expressed proteins. Instead, expression of these sequences produces dsRNA which is recognised by the plant's RNAi pathway, cut into short pieces, and processed to capture and degrade mRNA of the targeted genes.

The activity and function of VINV and PPO in event BG25 is identical to that of potato events W8, X17, Y9 and Z6, all previously reviewed by FSANZ (see A1139 and A1199).

Regulatory elements of event BG25

The potato blight R-genes *Rpi-amr3*, *Rpi-blb2* and *Rpi-vnt1* are expressed via their native promoters and termination sequences (Table 7). Expression of the *StmAls* gene and the PVY-CP inverted repeat are constitutively induced by wounding and stress via the potato *polyubiquitin 7* (*Ubi7*) gene promoter (Garbarino et al., 1995). The pUbi7 in pSIM4363 contains common regulatory elements known to promote transcription, followed by an intron and one monomer of ubiquitin (Garbarino et al., 1995).

The down regulation of *VInv* and *Ppo* in event BG25 tubers is facilitated by the potato granule-bound starch synthase promoter and potato ADP glucose pyrophosphorylase (pAgp) promoter (Nakata et al., 1994). These regulatory elements have been previously assessed by FSANZ (see A1139 and A1199).

The termination of cassette expression for the *StmAls* gene and the PVY-CP inverted repeat is via a native potato ubiquitin-3 (tUbi3) polyadenylation sequence.

pSIM4363 backbone

The backbone is 8,925 bp in length and consists of elements used as markers for selection in bacteria (kanamycin resistance; *KmR*), for screening out of events with backbone DNA (isopentenyl transferase; *ipt*), and other elements required for plasmid replication and maintenance in bacteria (Table 8). The T-DNA is 29,956 bp in length and consists of multiple cassettes, including a selection marker for transformants (*StmAls*) and elements that introduce new traits into the transformed variety including late blight protection, PVY protection, VINV down regulation, and PPO down regulation (Table 8).

The backbone contains two well-characterised regions required for bacterial maintenance:

- pVS1 (Sta and Rep) for replication in *A. tumefaciens* (Elements 4 and 6 in Table 8); and
- pBR322 (bom and ori) for replication in *Escherichia coli* (Elements 9 and 11 in Table 8).

Additional backbone elements include:

- *Agrobacterium* DNA overdrive sequence (Element 2 in Table 8) for more efficient cleavage at the right border (RB) site (Toro et al., 1988);
- An aminoglycoside phosphotransferase-III gene (*KmR*, Element 13 in Table 8) for use as a kanamycin selectable marker in bacteria (Gray and Fitch, 1983); and
- *Agrobacterium ipt* gene (Element 17 in Table 8) for use as a screenable marker allowing negative selection of events containing backbone. Transcription is regulated by a *S. tuberosum* polyubiquitin 3 gene promoter and terminator (Elements 15 and 19 in Table 8) (Garbarino and Belknap, 1994).

Table 7. Regulatory sequences in event BG25

Regulatory sequence	Associated expressed sequence	Characterisation	Previously reviewed in deregulated events	Reference line in Table 6
Promoters				
Potato polyubiquitin (pUbi7)	*StmAls* gene	Constitutive; inducible by wounding and stress (Garbarino et al., 1995)	No	3-5
	PVY-CP inverted repeat			35-37
Native *Rpi-vnt1* (pVnt1)	*Rpi-vnt1* gene	Inducible by *P. infestans* (Karasov et al., 2017)	Yes	13
Native *Rpi-amr3* (pAmr3)	*Rpi-amr3* gene		No	15
Native *Rpi-blb2* (pBlb2)	*Rpi-blb2* gene		No	21
Potato granule-bound starch synthase (pGbss)	VInv/Ppo inverted repeat	Active in leaves and tubers (Nakata et al., 1994)	Yes	23
Potato ADP glucose pyrophosphorylase (pAgp)		Highly active in tubers and stolons, with lower expression in leaves (Nakata et al., 1994)	Yes	33
Terminators				
Ubiquitin-3 (tUbi3)	*StmAls* gene	Native potato terminator	No	9
	PVY-CP inverted repeat			43
Rpi-vnt1 (tVnt1)	*Rpi-vnt1* gene	Native Solanum terminator	Yes	11
Rpi-amr3 (tAmr3)	*Rpi-amr3* gene	Native Solanum terminator	No	17
Rpi-blb2 (tBlb2)	*Rpi-blb2* gene	Native Solanum terminator	No	19

Table 8. Genetic elements of the pSIM4363 backbone

Genetic Element	Origin	Accession Number	Position	Size (bp)	Function
1. Intervening sequence	Synthetic DNA	-	29,957-29,962	6	Sequence used for cloning
2. Overdrive	*Agrobacterium tumefaciens* Ti-plasmid	NC_002377	29,963-29,992	30	Enhances cleavage at right border site
3. Intervening sequence	*Pseudomonas fluorescens* pVS1	AJ537514	29,993-31,074	1,082	pVS1 backbone
4. pVS1 partitioning protein StaA (PVS1 Sta)	*P. fluorescens* pVS1	AJ537514	31,075-32,075	1,001	pVS1 stability
5. Intervening sequence	*P. fluorescens* pVS1	AJ537514	32,076-32,668	593	pVS1 backbone
6. pVS1 replicon (pVS1 Rep)	*P. fluorescens* pVS1	AJ537514	32,669-33,669	1,001	pVS1 replication region in *Agrobacterium*
7. Intervening sequence	*P. fluorescens* pVS1	AJ537514	33670-33,907	238	pVS1 backbone
8. Intervening sequence	pBR322	AF234297	33,908-34,078	171	pCambia1301 backbone
9. pBR322 bom	pBR322	AF234297	34,079-34,339	261	pBR322 region for replication in *E. coli*
10. Intervening sequence	pBR322	AF234297	34,340-34,478	139	pBR322 backbone
11. Origin of replication for pBR322 (pBR322 ori)	pBR322	AF234297	34,479-34,759	281	Region for replication in *E. coli*
12. Intervening sequence	pBR322	AF234297	34,760-35,049	290	pCambia1301 backbone
13. Kanamycin resistance (*KmR*) gene	*Staphylococcus aureus*	AF234297	35,050-35,844	795	Aminoglycoside phosphotransferase-III used as selection marker for cloning in bacteria
14. Intervening sequence	Vector DNA	FJ362602	35,845-36,039	195	pCambia vector backbone
15. Promoter of the ubiquitin-3 gene (pUbi3)	*Solanum tuberosum*	L22576	36,040-37,188	1,149	Drives expression of *ipt* gene
16. Intervening sequence	Synthetic DNA	-	37,189-37,193	5	Sequence used for cloning
17. Isopentenyl transferase (*ipt*) gene	*A. tumefaciens* Ti-plasmid	NC_002377	37,194-37,916	723	Screenable marker that catalyses formation of isopentenyl-AMP, a plant cytokinin causing abnormal plant growth phenotypes
18. Intervening sequence	*A. tumefaciens* Ti-plasmid	NC_002377	37,917-38,267	351	Sequence used for DNA cloning
19. Terminator of the ubiquitin-3 gene (tUbi3)	*S. tuberosum*	GP755544	38,268-38,622	355	Terminator for *ipt* gene transcription
20. Intervening sequence	Synthetic DNA	-	38,623-38,631	9	Sequence used for DNA cloning
21. Intervening sequence	pBR322	AF234297	38,632-38,875	244	pCambia1301 backbone
22. Intervening sequence	Synthetic DNA	-	38,876-38,881	6	Sequence used for DNA cloning

A.3(c) A full molecular characterisation of the genetic modification in the new organism

This Section provides information that addresses the requirements for Part A.3(c) A full molecular characterisation of the genetic modification in the new organism, including:

(i) Identification of all transferred genetic material and whether it has undergone any rearrangements;

(ii) A determination of the number of insertion sites, and the number of copies at each insertion site;

(iii) Full DNA sequence of each insertion site, including junction regions with the host DNA;

(iv) A map depicting the organisation of the inserted genetic material at each insertion site; and

(v) Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs).

Further information is provided in:

- Report E
- Report A

Insert structure of pSIM4363 in BG25

The pSIM4363 T-DNA insert structure and flanking genomic sequences were determined using DNA from BG25 leaf material. DNA mate pair libraries were prepared from the extracted DNA using the Illumina Nextera Mate Pair Library Preparation Kit. Mate pair library sequences with homology to pSIM4363 were capture using probes of 80 nucleotides in length, synthesised every 20 nucleotides across the pSIM4363 plasmid, including backbone. Captured samples were sequenced using Illumina NGS.

Junction finding scripts using the captured sequences and the potato reference genome, Potato Genome Sequencing Consortium (PGSC) *S. tuberosum* group Phureja clone DM1-3 pseudomolecules (v4.03), indicated a single pSIM4363 T-DNA insertion site in BG25 (PGSC, 2011; (Sharma et al., 2013); http://spuddb.uga.edu/pgsc_download.shtml . Based on these data, primers were designed to amplify the junction regions between the T-DNA insert and the flanking genomic DNA in BG25. The PCR primers were designed to include at least 1 kb of flanking genomic DNA for both the left and right flanks of the T-DNA insert (Figure 5). PCR products were Sanger sequenced to confirm the left and right junctions of the T-DNA insert in BG25 (Figure 6). The sequence of the event BG25 insert including 1498bp of left and right flanking sequence is provided in Appendix A.

To determine the structure of the pSIM4363 insert, Illumina mate pair reads were aligned using CLC Genomic Workbench (Qiagen, v12.0) to pSIM4363 as well as the Russet Burbank draft genome (v1.0). The target capture enrichment yielded thorough depth of coverage (average 99x) across the entire pSIM4363 insert, including the junctions and adjacent flanking genomic DNA (Figure 6). The alignments were inspected to confirm accuracy by depth of coverage and sequence quality scores across the entire insert. Combined sequencing data showed that the pSIM4363 insert contains a nearly full-length T-DNA, with deletions of 330 bp from the annotated left border element and 34 bp from the annotated right border element (detailed below).

Copy number of pSIM4363 in event BG25

Droplet digital PCR is a quantitative endpoint assay used to measure the insert copy number in BG25. Droplet digital PCR was used to test for insertion number by quantifying eight targets across the BG25 insertion relative to a reference gene (either *VInv* or vacuolar protein sorting). Duplexed quantification was performed using a HEX-labelled probe to detect the reference gene and a FAM-labelled probe to detect the T-DNA-specific assays (Figure 7).

Targets 1 to 6 were duplexed with the reference gene *VInv* and targets 7 to 8 were duplexed with vacuolar protein sorting gene. Reference genes used in duplex reactions were selected based on efficiency of the reaction. BG25 genomic DNA was tested both uncut and digested with restriction enzymes to determine both locus number and copy number, respectively (Table 9). For example, if a portion of the T-DNA was duplicated upon insertion at a single locus, the locus number assay using undigested template DNA would result in a value of approximately 1, while after restriction digestion the copy number assay would result in a value of approximately 2. In BG25, each amplicon was found to be present singly in each assay (Figure 5). This indicated that a single insert was present in BG25.

Table 9. Locus number and copy number per target in BG25

Target	Locus Number (no restriction enzyme)	Copy Number (restriction enzyme added)
1	0.9	0.9
2	0.9	1.2
3	0.9	1.1
4	0.7	0.9
5	0.7	0.9
6	0.8	1.1
7	0.8	0.8
8	0.9	0.9

Genomic integration site of pSIM4363

Russet Burbank potatoes are tetraploid, containing four homologous sets of chromosomes. A T-DNA insert is expected to occur at a unique locus on one of the four homologues. Therefore, BG25 retains three chromosomes that each have a native locus homologous to the insertion site.

The sequence of the genomic integration site for the insert in BG25 was determined using primers that hybridise to the genomic regions flanking the insert. PCR with these primers amplified the homologous, native locus in Russet Burbank, resulting in an amplicon of approximately 1.6 kb. By comparing the Sanger-sequenced plasmid with the insert in BG25, the pSIM4363 insertion site was identified. These data indicated that 55 bp of genomic DNA were deleted because of the pSIM4363 insertion. These data also showed that the flanking sequence has identity to the DM potato reference genome (v4.03) (Potato Genome Sequencing Consortium, 2011a) chromosome 12 and is not located in any annotated gene (Figure 8).

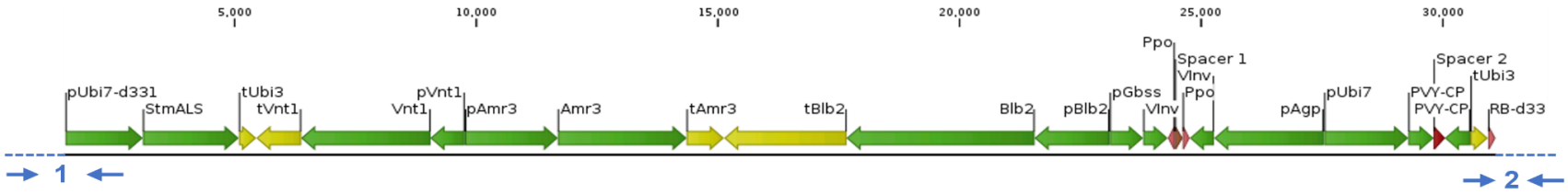


Figure 5. Insert structure in BG25 with junction amplification assays

BG25 contains a single T-DNA insert from pSIM4363. The plant genomic flanking sequence is indicated with a dashed line. Arrows denote primer pairs used to amplify the junction near the left flank (1) and the right flank (2).

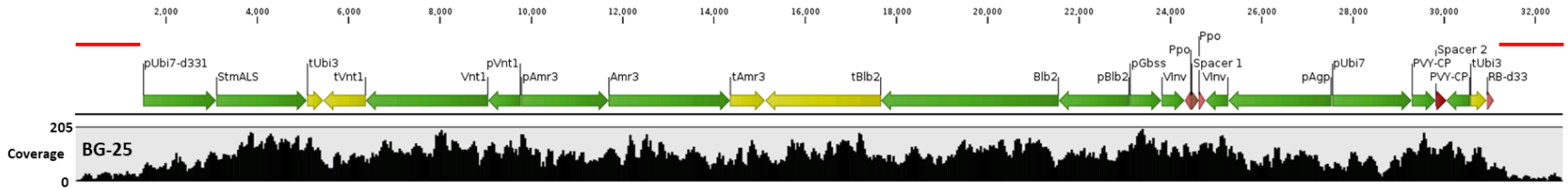


Figure 6. Summary of sequence read alignment to the pSIM4363 insert

Sequenced reads mapping to the pSIM4363 insert are summarized by plotting depth of coverage. Red lines represent Sanger sequenced regions.

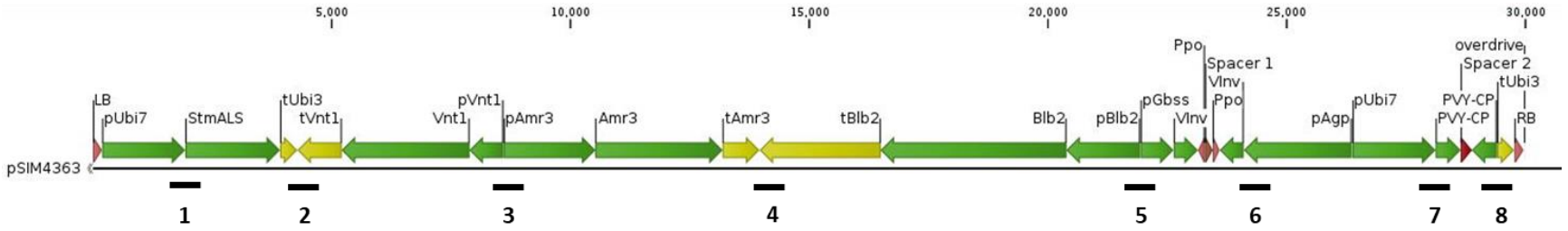


Figure 7. Regions of the pSIM4363 T-DNA assayed by ddPCR

Assays for targets labelled 1-8 were designed to amplify junction regions in pSIM4363 for quantification relative to a reference gene (*Vlnv* or vacuolar protein sorting).

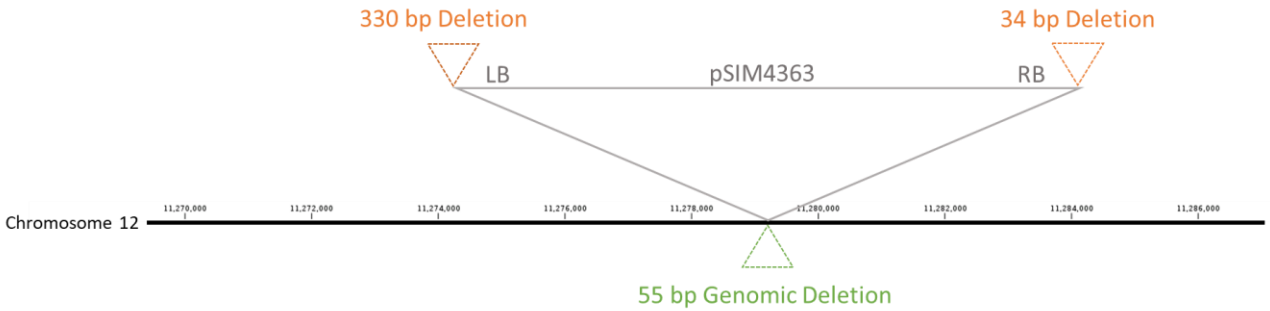


Figure 8. Characterisation of the BG25 insertion site on chromosome 12

The pSIM4363 insert contains a nearly full-length T-DNA, with deletions of 330 bp from the annotated left border element and 34 bp from the annotated right border element (orange). 55 bp of genomic DNA were deleted upon insertion of the T-DNA (green). The insertion site and the region deleted by the T-DNA insertion do not occur in a region containing an annotated gene.

Absence of vector backbone DNA

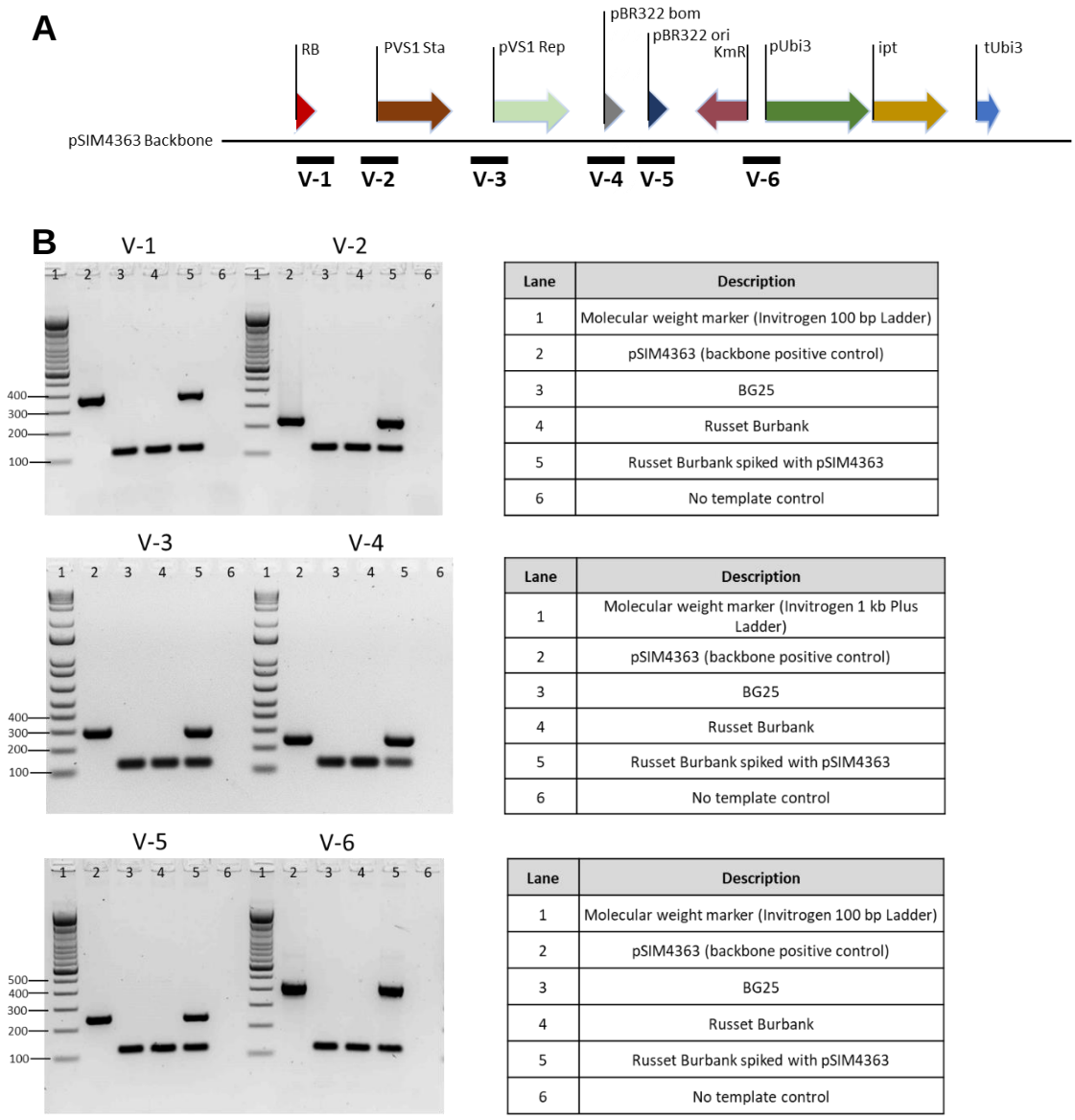
The absence of pSIM4363 backbone in BG25 was confirmed using two assays: 1) PCR analysis and 2) Targeted Sequence Capture and Illumina Sequencing.

PCR analysis for plasmid backbone

For PCR confirmation of absence of backbone DNA, six PCR assays were designed to amplify regions of the plasmid backbone (V-1 to V-6, Table 10 and Figure 9A). Each PCR reaction was duplexed with Adenine phosphoribosyltransferase gene (*APRT;* 121 bp), which served as an endogenous genomic control. As seen in Figure 9B, BG25 (lane 3), Russet Burbank (lane 4), and the template control (lane 6) all showed no amplification for backbone DNA for each of the six PCR assays. Samples containing added pSIM4363 plasmid DNA (lanes 2 and 5), showed amplification for each of the six PCR assays, as expected. All samples containing genomic potato DNA (lanes 3, 4, and 5) resulted in APRT amplification, as expected.

Table 10. PCR primers for detection of plasmid backbone

Assay	Amplicon Size (bp)	Primers (5' to 3')
V-1	<CCI>	<CCI> <CCI>
V-2	<CCI>	<CCI> <CCI>
V-3	<CCI>	<CCI> <CCI>
V-4	<CCI>	<CCI> <CCI>
V-5	<CCI>	<CCI> <CCI>
V-6	<CCI>	<CCI> <CCI>
APRT	<CCI>	<CCI> <CCI>



Lane	Description
1	Molecular weight marker (Invitrogen 100 bp Ladder)
2	pSIM4363 (backbone positive control)
3	BG25
4	Russet Burbank
5	Russet Burbank spiked with pSIM4363
6	No template control

Lane	Description
1	Molecular weight marker (Invitrogen 1 kb Plus Ladder)
2	pSIM4363 (backbone positive control)
3	BG25
4	Russet Burbank
5	Russet Burbank spiked with pSIM4363
6	No template control

Lane	Description
1	Molecular weight marker (Invitrogen 100 bp Ladder)
2	pSIM4363 (backbone positive control)
3	BG25
4	Russet Burbank
5	Russet Burbank spiked with pSIM4363
6	No template control

Figure 9. Plasmid backbone detection PCR assays

(A) Position of plasmid backbone detection products V-1 to V-6 (black bars) relative to pSIM4363 backbone and right border of the T-DNA. (B) Agarose gel electrophoresis of six plasmid detection assays V-1 to V-6 (for expected sizes see Table 10) duplexed with an assay for the potato *APRT* gene (121 bp).

Targeted Sequence Capture and Illumina Sequence Analysis

Illumina mate pair libraries were captured to enrich for sequences with identity to pSIM4363, including the backbone. The captured mate pair read depth aligned to the T-DNA averaged 99x (Figure 6), while plasmid backbone shows alignment of only a few reads (Figure 10). Similar results were observed between BG25 and Russet Burbank. The backbone contains two elements comprised of potato DNA, the *Ubi3* promoter and terminator. Reads were only observed aligning to the *Ubi3* promoter and terminator sequences, indicating that these sequences were captured due to the presence of these native sequences in the potato genome and not from pSIM4363. The captured *Ubi3* promoter and terminator mate pairs were further inspected and the sequence on the corresponding mate pair ends did not map to the pSIM4363 T-DNA or insertion site. No other backbone elements from pSIM4363 were identified from the enriched libraries. These results, in conjunction with the PCR analysis, show that no backbone from the plasmid is inserted in BG25.

Conclusion

A combination of Sanger and Illumina NGS sequencing corroborated studies using ddPCR and targeted sequence capture showed the presence of a single insert associated with transformation of BG25 using pSIM4363. The structure and sequence of the insert in BG25 is provided, with flanking DNA sequence. No backbone DNA was integrated into the Russet Burbank genome. No annotated genes were disrupted by the insertion of the T-DNA.

A.3(c)(iv) A map depicting the organisation of the inserted genetic material at each insertion site

Details of the organisation of the inserted genetic material at the integration site are described above. Specifically:

Event BG25–Detailed organisation of the genetic elements in the insert (Figure 5 and Figure 6), integration of pSIM4363 T-DNA (Figure 8).

A.3(c)(v) Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs)

The sequences of the ORFs covering the junctions between the inserts and the potato flanking regions were evaluated as part of the ORF analysis (see Section B1(d) and Report A). None of the junction ORFs were identified as homologs of known toxins or allergens (see Section B1(d)).

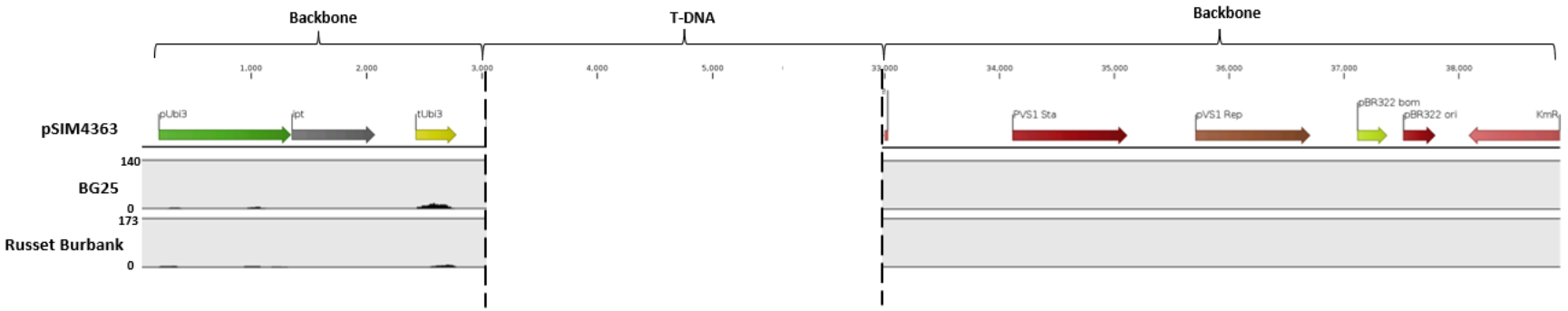


Figure 10. Summary of sequence read alignment to pSIM4363 backbone regions

Sequenced reads aligning to pSIM4363 are summarized by plotting depth of coverage. To better visualize backbone regions, the T-DNA region is masked (dotted lines). The mapped reads in BG25 and Russet Burbank originate from the potato-derived *Ubi3* promoter and terminator sequences.

A.3(d) 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) including which generations have been used.

Potato plants are cultivated by vegetative propagation and commonly maintained as disease-free plantlets in tissue culture. Shoots from the plantlets are cut and transferred to fresh medium periodically to maintain healthy stocks (Figure 11). When many plants are needed, for example for seed production, multiple shoots are cut and grown in tissue culture medium. Plantlets with roots are transferred to greenhouses to produce tubers for seed. Greenhouse tubers are planted in fields to multiply potato seed for large-scale potato production.

Potato seed is a tuber that contains buds, called “eyes”, which sprout and grow into mature potato plants. The seed tuber is planted whole or as a cut piece with eyes (Figure 11).

In vegetative (asexual) propagation, progeny arise from a single parent plant, and each progeny cell receives the same genetic material. As the parent cell divides in two, the resulting progeny cells are copies of one another and the parent plant. Vegetative propagation produces a genetically uniform crop because no new genetic material is introduced through sexual reproduction.

During seed production growers eliminate plants in the field that are “off-types” to maintain the desired characteristics of the variety. In addition, commercial seed is reinitiated from the disease-free, tissue culture source material on a continuous basis.

As a result of vegetative propagation, each potato is a genetic clone of its parent plant since the tuber (and not the true seed) is used to generate the next plants (Figure 12). An example of potato vegetative propagation is shown in (Figure 12). Tuber production begins with parent plants containing the desired traits of interest. Cuttings from the parent plants are propagated in tissue culture (plantlets). These are transferred to greenhouses to grow mini-tubers for distribution to seed farmers. Mini-tubers are planted in fields to produce 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 reverts to tissue culture parent plants that have been tested to confirm the presence of the desired traits and disease-free status.

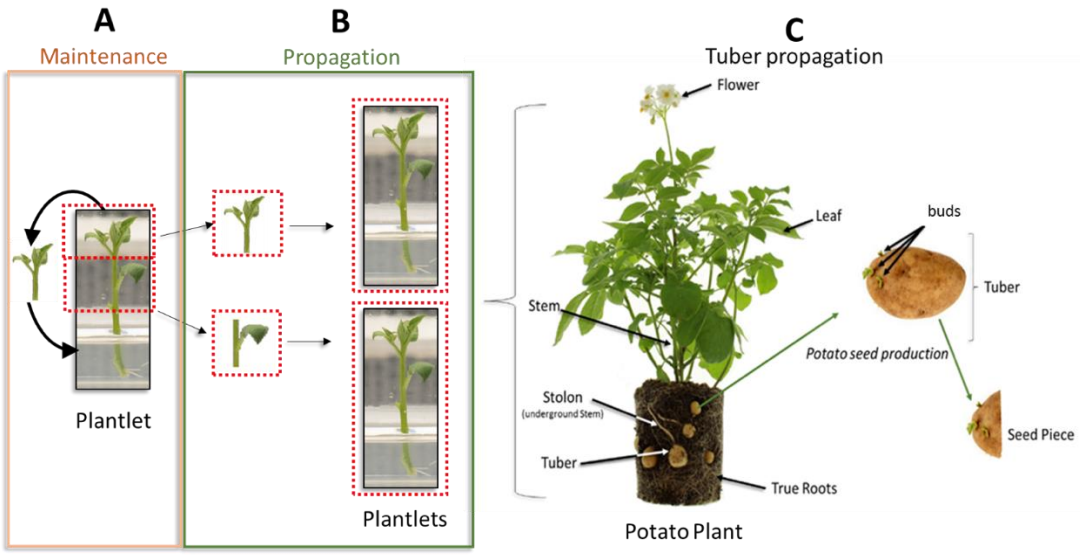


Figure 11. Potato Plant and Tuber Propagation

Potato variety stocks are maintained in tissue culture (A) and multiplied by vegetative propagation (B) to produce mature potato plants and potato seed (C).

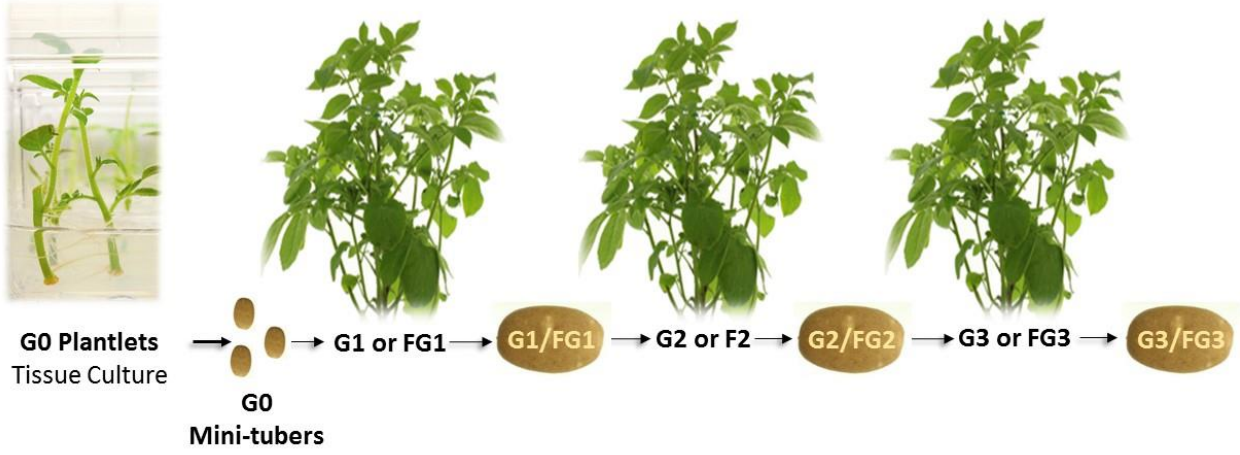


Figure 12. Commercial Production of Potatoes

Plantlets are propagated from cuttings of the stock tissue culture plantlet. Plantlets are transferred to soil or grown using Nutrient Film Technique (NFT) or hydroponics in greenhouses. Tubers from these Greenhouse-0 plants are referred to as mini-tubers. An entire mini-tuber is planted in either the greenhouse or field to produce a new potato plant. Tubers from Greenhouse-1/Field Grown-1 plants can be cut into 2-4 oz. (55-115 g) pieces, which contain lateral buds, and are used as "seed pieces" to produce Greenhouse-2/Field Grown-2 plants. The process of vegetative propagation is repeated to generate planting material.

A.3(e) Evidence of the stability of the genetic changes, including:

(i) The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored

(ii) The pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments

Because commercial potatoes are vegetatively propagated, the progeny from a parent cell are genetically identical to each other and the parent plant (Section A3(d)). Therefore, the T-DNA insert in BG25 is expected to be genetically stable during vegetative propagation. Consequently, evaluating insert stability by examining inheritance using Mendelian segregation analysis is not applicable for potatoes. Nonetheless, stability of inserted DNA in BG25 was examined across multiple vegetative propagations using digital droplet PCR and PCR (Report H).

BG25 G0, G1, and G2 plants and Russet Burbank were grown in the greenhouse from tissue culture plantlets (Figure 13).

Droplet digital PCR was used to measure the absolute copy number of amplicons across the pSIM4363 insert in BG25 plants that had undergone multiple rounds of vegetative propagation. In conjunction with ddPCR, a PCR assay was conducted to assess the left and right flanking regions of the pSIM4363 insert across three generations. Detailed methodology is provided in Report H.

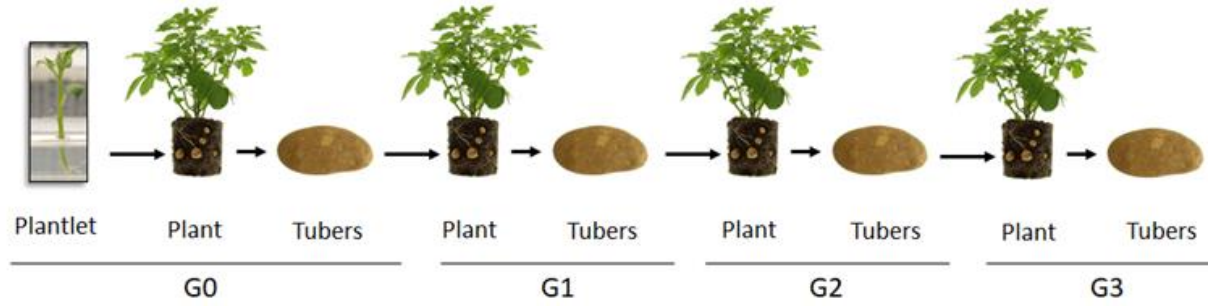


Figure 13. Vegetative Propagation of Potato Plants

Tissue culture plantlets were planted in soil to produce tubers, designated G0. G0 tubers were planted to produce G1 plants and tubers, which were used to produce G2 plants and tubers. DNA was isolated from G0 and G2 samples of vegetatively-propagated greenhouse plants for analysis.

Single copy insert amplicons in event BG25 determined by ddPCR

Droplet digital PCR was used to measure the absolute copy number of amplicons across the insert in BG25 plants. Quantitation of copy number was performed by automated counting of droplets in which the FAM fluorophore labels targets 1 to 6 (Figure 14;Table 11), and the HEX fluorophore labels the reference targets 7 or 8 (Table 11). The reference assays for vacuolar invertase, target 7, or vacuolar protein sorting, target 8, were previously validated to amplify four alleles at a single locus.

Quantification of these targets using BG25 genomic DNA from G0, G1, and G2 plants shows that each amplicon was present as a single copy in each generation of plants (Table 12).

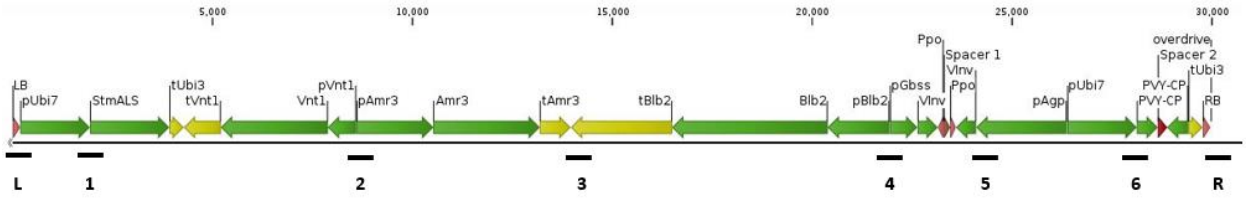


Figure 14.Regions of the insert from pSIM4363 in BG25 assayed by ddPCR and PCR

Assays for targets labelled 1 to 6 were designed to amplify junction regions in the insert for quantification relative to the reference genes (amplicons 7 and 8, not pictured). Assays for left and right flanking regions (L, R) were performed by PCR.

Table 11. Primer and probe sequences, fluorophores, restriction enzymes, and annealing temperatures utilised in ddPCR for copy number determination

Target	Primer or Probe	Primer or Probe Sequence (5' to 3')	Amplicon Size (bp)	Enzyme	Annealing Temp. (°C)	Duplexed with Reference Assay
1	Forward	<CCI>	<CCI>	<CCI>	<CCI>	9
	Reverse	<CCI>				
	Probe	<CCI>				
2	Forward	<CCI>	<CCI>	<CCI>	<CCI>	9
	Reverse	<CCI>				
	Probe	<CCI>				
3	Forward	<CCI>	<CCI>	<CCI>	<CCI>	9
	Reverse	<CCI>				
	Probe	<CCI>				
4	Forward	<CCI>	<CCI>	<CCI>	<CCI>	9
	Reverse	<CCI>				
	Probe	<CCI>				
5	Forward	<CCI>	<CCI>	<CCI>	<CCI>	9
	Reverse	<CCI>				
	Probe	<CCI>				
6	Forward	<CCI>	<CCI>	<CCI>	<CCI>	9
	Reverse	<CCI>				
	Probe	<CCI>				
7	Forward	<CCI>	<CCI>	<CCI>	<CCI>	10
	Reverse	<CCI>				
	Probe	<CCI>				
8	Forward	<CCI>	<CCI>	<CCI>	<CCI>	10
	Reverse	<CCI>				
	Probe	<CCI>				
9 VI[1]	Forward	<CCI>	<CCI>	-	-	-
	Reverse	<CCI>				
	Probe	<CCI>				
10 VPS[2]	Forward	<CCI>	<CCI>	-	-	-
	Reverse	<CCI>				
	Probe	<CCI>				

[1]VI = Vacuolar invertase reference

[2]VPS = Vacuolar protein sorting reference

Table 12. Absolute copy number per target

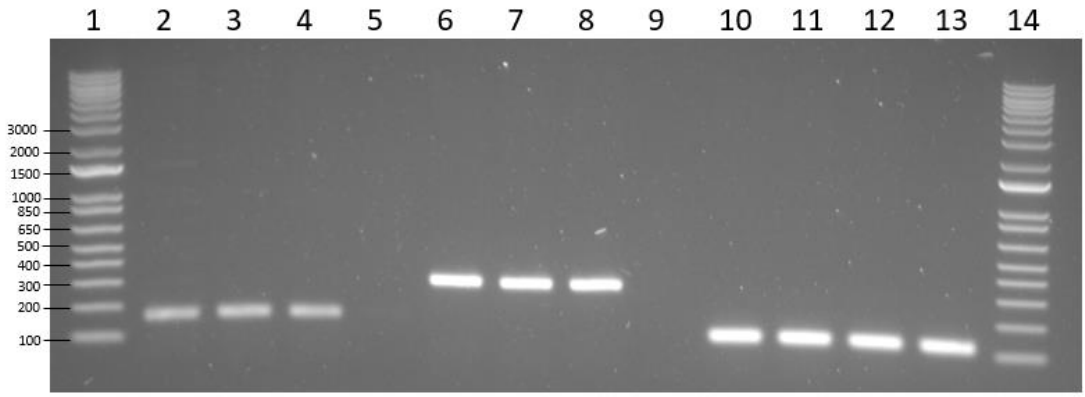
Amplicon	BG25 G0 Absolute Copy Number[1]	BG25 G1 Absolute Copy Number	BG25 G2 Absolute Copy Number	Russet Burbank Absolute Copy Number
1	0.9	1.0	0.9	0.0
2	1.0	1.0	1.1	0.0
3	1.0	1.1	1.0	0.0
4	1.1	1.1	1.1	0.0
5	1.0	1.0	1.0	0.0
6	0.8	0.8	0.9	0.0

[1]Software used reference gene values to produce the absolute quantification of the target.

PCR analysis of insert-genome junctions specific to event BG25

PCR amplification was performed across the left and right junctions of the insert from pSIM4363 in BG25 (Figure 14), across three generations of plants (G0, G1, and G2) with Russet Burbank as a negative control, and the endogenous potato gene APRT as an amplification control (Figure 15). The band sizes corresponded to the amplicon lengths for each primer pair (Table 13).

A



B

Lane	Sample	Target Sequence
1	Molecular Ladder	n/a
2	BG25-G0	Left Flank
3	BG25-G1	Left Flank
4	BG25-G2	Left Flank
5	Russet Burbank	Left Flank
6	BG25-G0	Right Flank
7	BG25-G1	Right Flank
8	BG25-G2	Right Flank
9	Russet Burbank	Right Flank
10	BG25-G0	APRT
11	BG25-G1	APRT
12	BG25-G2	APRT
13	Russet Burbank	APRT
14	Molecular Ladder	n/a

Figure 15. Event BG25 flanking regions detected using PCR analysis

(A) Left and right flank of the BG25 insertion from pSIM4363 were amplified from leaf samples from G0, G1, and G2 plants (lanes 2 to 4 and 6 to 8), and the negative control Russet Burbank (lanes 5 and 9). The reference gene APRT was amplified from all samples as a positive control (lanes 10 to 13). (B) Description of each lane in A. Molecular size markers are in bp.

The amplicons associated with each flanking region of the insert from pSIM4363 remained consistent during three vegetative propagations of BG25 (lanes 2 to 4 and 6 to 8, Figure 15).

The absence of observed differences between G0, G1, and G2 samples demonstrated that the insert from pSIM4363 is stable in vegetatively propagated BG25 plants.

Table 13. PCR primer sequences and amplicon sizes

Target	Primer Sequence		Amplicon Size (bp)
L	Primer (F)	<CCI>	<CCI>
	Primer (R)	<CCI>	
R	Primer (F)	<CCI>	<CCI>
	Primer (R)	<CCI>	
APRT	Primer (F)	<CCI>	<CCI>
	Primer (R)	<CCI>	

A.3(g) An analysis of the expressed RNA transcripts, where RNA interference has been used

The BG25 insert also contains two cassettes designed to reduce gene expression of *VInv*, *Ppo*, and the PVY-CP genes. Down regulation of these genes is not the result of any expressed proteins. Instead, expression of these sequences produces dsRNA which is recognized by the plant's RNAi pathway, cut into short pieces, and processed to capture and degrade mRNA of the targeted genes.

The activity and function of VINV and PPO in BG25 is identical to that of potato events W8, X17, Y9 and Z6, all previously reviewed by FSANZ (see A1199). Molecular data showing the down regulation of VInv and Ppo transcripts in BG25 tubers are provided below. The details of the materials and methods used in these studies are provided in Report F.

In the case of PVY-CP, the target mRNA for the PVY-CP inverted repeat transcripts are viral RNA, present only during a viral infection. As such, measuring the decrease in viral target RNA is not feasible in healthy plants. However, the effectiveness of the viral protection was shown in efficacy studies using virus inoculation and monitoring the level of infection.

The transcribed PVY-CP inverted repeat is expected to form a double-stranded RNA (dsRNA) hairpin that is processed rapidly into siRNA in the cell (Figure 16). The dsRNA is not translated into protein. The ubiquitin monomer, following translation, would be identical to ubiquitin already present in potato.

Potato virus Y is the type species of the genus potyvirus, in the family *Potyviridae*. The virus is transmitted by aphids and spread occurs rapidly when an insect probes PVY-infected potato leaves. The virus has a positive-sense, single stranded RNA genome (Figure 17). The RNA is enclosed in the viral capsid, made up of approximately 2,000 copies of a single polypeptide CP. *Potato virus Y* protection in BG25 potatoes results from RNAi that targets the CP region (ORF 10; Figure 17) and inhibits PVY replication.

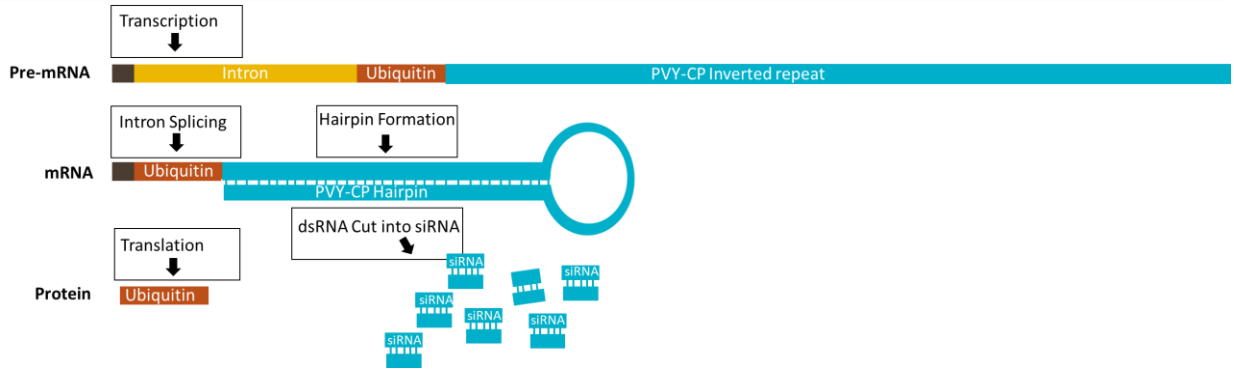


Figure 16 PVY-CP RNA Transcript

The intron would be spliced from the PVY-CP RNA transcribed from the Ubi7 promoter. The PVY-CP inverted repeat would form dsRNA and be cut quickly into siRNA. The ubiquitin monomer would result from translation of the remaining mRNA. This protein would have the same sequence as the potato ubiquitin.

Figure 17 The PVY RNA Genome

The PVY genome has ten identified open reading frames (ORFs, numbered 1-10) that encode proteins for virus replication and spread. (1) P1-Pro = a viral protease; (2) HC-Pro = a cis-acting protease and helper component (HC) involved in cell-to-cell movement; (3-4) P3-PIPO = contains two ORFs that encode a viral protease (P3) and the PIPO protein (proposed functions include replication, movement, or suppression); (5, 7) $6K_1$ and $6K_2$ = short peptides that act as membrane anchors during replication; (6) CI = an RNA helicase; (8) NIa-VPg = encodes a viral protease and the VPg protein; (9) NIb = the RNA-dependent RNA polymerase; (10) CP = coat protein. The 5´-end of the viral genome is covalently attached to the VPg protein, and there is a PolyA tail at the 3' end. (Figure modified from Revers et al., 1999).

<u>Down regulation of Ppo and VInv</u>

Reverse transcription quantitative real-time PCR (RT-qPCR) was used to measure Ppo and VInv RNA transcript levels in BG25 and Russet Burbank tuber samples. Primers and probes were specific to the target transcripts and relative expression for Ppo and VInv transcripts is shown in Figure 18 and Figure 19, respectively. Compared to Russet Burbank, BG25 showed reduced expression of Ppo transcripts in tubers (Figure 18) and reduced expression of VInv in tubers (Figure 19).

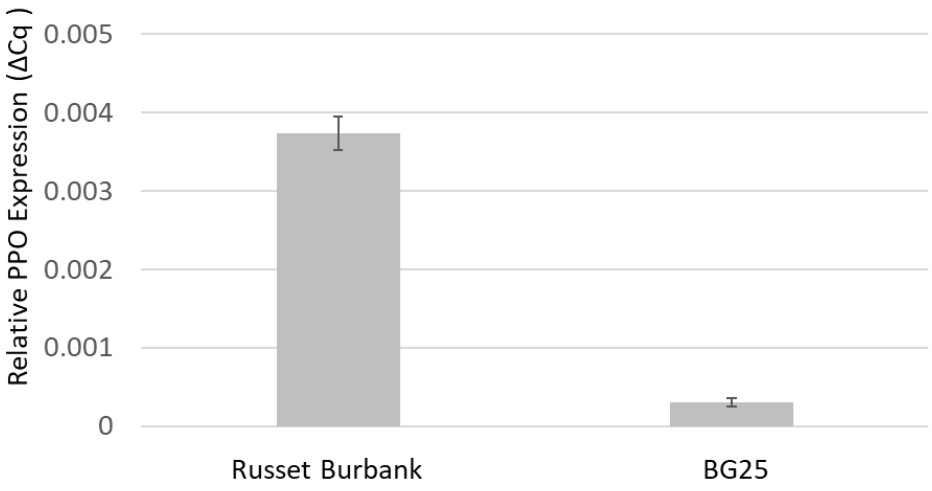


Figure 18. Ppo transcript levels in BG25 and Russet Burbank tubers measured by RT-qPCR
The Ppo RT-qPCR assay was performed on three biological replicates of event BG25 and Russet Burbank tuber samples analysed in triplicate. Expression of *Ppo* was normalised to endogenous reference genes (*Elongation Factor 1α* and *APRT)* by calculating a ΔCq value. The ΔCq for each replicate was exponentially transformed to ΔCq-Expression by $2^{-\Delta Cq}$, showing relative expression of Ppo transcripts for all samples analysed.

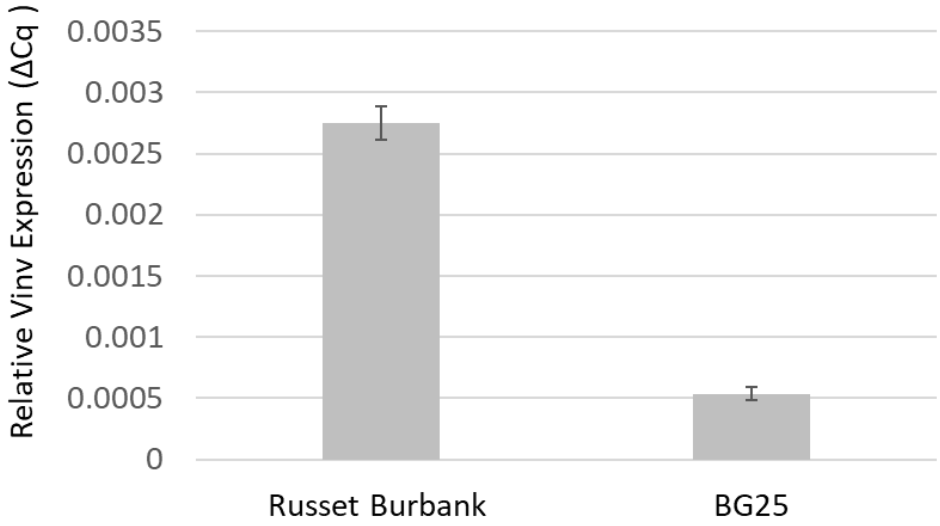


Figure 19. VInv transcript levels in event BG25 and Russet Burbank measured by RT-qPCR

The VInv RT-qPCR assay was performed on three biological replicates of event BG25 and Russet Burbank tuber samples analysed in triplicate. Expression of *VInv* was normalised to endogenous reference genes (*Elongation Factor 1α* and *APRT)* by calculating a ΔCq value. The ΔCq for each replicate was exponentially transformed to ΔCq Expression by $2^{-\Delta Cq}$, showing relative expression of VInv transcripts for all samples analysed.

<u>Summary of Ppo and VInv transcript down regulation in event BG25</u>

Inverted repeat sequences for the *Ppo* and *VInv* genes were introduced into BG25 to decrease the expression of PPO and VINV proteins in tubers using the plant's RNAi process. Compared to Russet Burbank, RT-qPCR

showed reduced expression of Ppo and VInv transcripts in BG25 tubers. This reduced expression of transcripts translates to reduced PPO and VINV in BG25 tubers.

Conclusion of the genetic characterisation of event BG25

A combination of Sanger and Illumina NGS sequencing corroborated studies using ddPCR and PCR, to demonstrate the presence of a single insert associated with transformation of BG25 using pSIM4363. The structure and sequence of the insert in BG25 is provided, with flanking DNA sequence. No backbone DNA was integrated into the Russet Burbank genome. No annotated genes were disrupted by the insertion of the T-DNA.

The insert in BG25 consisted solely of the sequences targeted for insertion and did not contain any detectable backbone sequence. The studies confirmed the stability of the DNA insert in BG25 across multiple cycles of vegetative propagation, which demonstrated that the insert will likely be maintained through vegetative propagation.

Using RT-qPCR, Russet Burbank showed reduced expression of Ppo and VInv transcripts in tubers compared to BG25 tubers. The reduced transcript expression translates to reduced PPO and VINV in BG25 tubers.

B. Characterisation and Safety Assessment of New Substances

B.1. Characterisation and Safety Assessment of New Substances

B.1(a) a full description of the biochemical function and phenotypic effects of all new substances (e.g. a protein or an untranslated RNA) that are expressed in the new GM organism, including their levels and site of accumulation, particularly in edible portions

BG25 expresses four proteins from the insert. Three of these are the R-proteins, AMR3, BLB2 and VNT1. These are summarised in Table 14 and all of them are cisgenes that use their native promoter and termination sequences. R-proteins are expressed at low levels in plants and in some cases are estimated to be as low as 18 parts per trillion (Bushey et al., 2014). In addition, R-proteins share high protein sequence similarity. Thus, it is challenging to detect or quantitate these proteins in plant extracts.

Table 14. Summary of R-genes in BG25

R-gene	R-protein	Source of gene	*Phytophthora infestans* Effector Recognized	R-protein Mode of Action
Rpi-amr3	AMR3	*S. americanum*	Avr-amr3	Recognise pathogen effectors and trigger the plant hypersensitive response
Rpi-blb2	BLB2	*S. bulbocastanum*	Avr-blb2	
Rpi-vnt1	VNT1	*S. venturii*	Avr-vnt1	

The fourth protein is StmALS, a modified version of the *S. tuberosum* acetolactate synthase protein (StALS), which was used as a selection marker during transformation. Expression of *StmAls* is under the control of the potato pUBi7 and the tUbi3. The StmALS protein is resistant to ALS-inhibiting herbicides. However, herbicide tolerance is not a commercial trait in BG25, and Simplot will not claim BG25 has resistance to ALS-inhibiting herbicides.

The expression levels of the R-proteins and StmALS in BG25 are summarised in Table 15. Data supporting these levels is provided in this section. In this submission, only the tuber data are provided as this is the only part of the potato plant that is consumed. Protein levels were measured using immunoblots and, for the R-

proteins, the levels of mRNA were measured using reverse-transcription quantitative real time polymerase chain reaction (RT-qPCR).

Table 15. Protein expression levels of R-proteins and StmALS in BG25 tubers

Protein	Units[1]	Tubers
AMR3	ng/g (FW)	<500
BLB2	ng/g (FW)	<100
VNT1	ng/g (FW)	<500
StmALS - Range	ng/g (FW)	200-570
StmALS - Mean	ng/g (FW)	420

[1] ng/g = ppb, FW – fresh weight

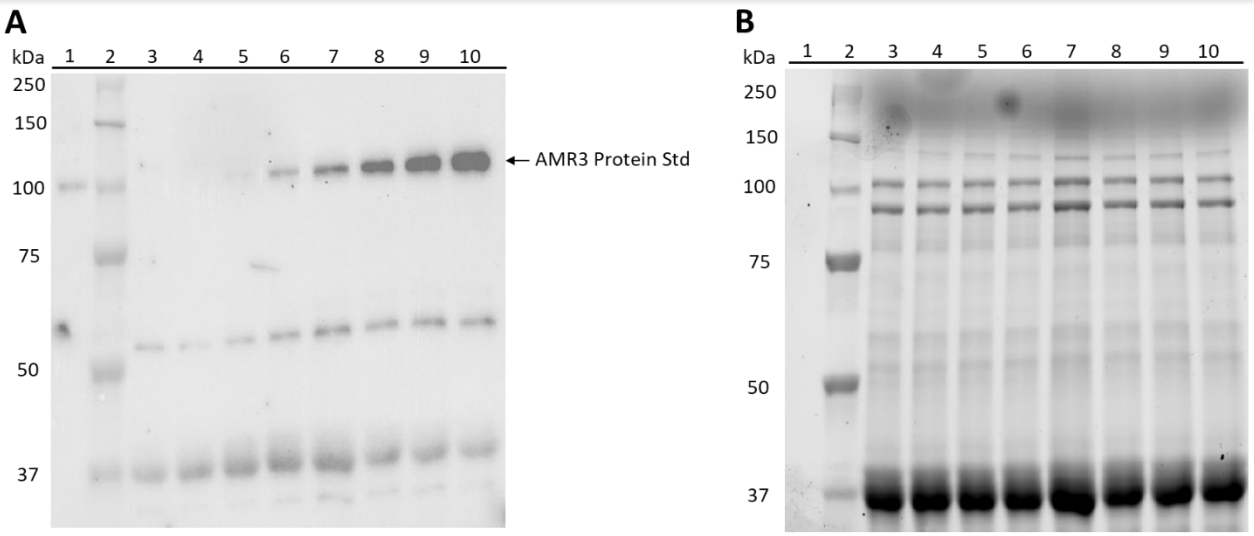
AMR3 in event BG25

The pSIM4363 T-DNA contains the *Rpi-amr3* gene (2664 bp; 887 amino acids) originating from the wild Solanum species *S. americanum.* The gene is expressed under the native *Rpi-amr3* promoter and terminator, pAmr3 and tAmr3, respectively.

A description of the safety and mode of action of AMR3 can be found in Section A.2 and Section B.1(b). The methods for the determination of AMR3 protein and transcript levels in BG25 are provided in Report B.

AMR3 immunoblot detection in BG25 tubers

An AMR3 protein standard was expressed and purified from *E. coli* and quantified. The limit of quantitation (LOQ) for AMR3 in tubers was determined by adding the AMR3 protein standard to Russet Burbank tuber extracts at different concentrations (Figure 20A). In some cases, bands corresponding to proteins other than AMR3 were observed due to cross-reactivity of these other proteins with the α-AMR3-3456 polyclonal antibody. These bands correspond to endogenous proteins present in the conventional Russet Burbank variety and in BG25 (Figure 21A). The LOQ was established as 500 ppb.



C

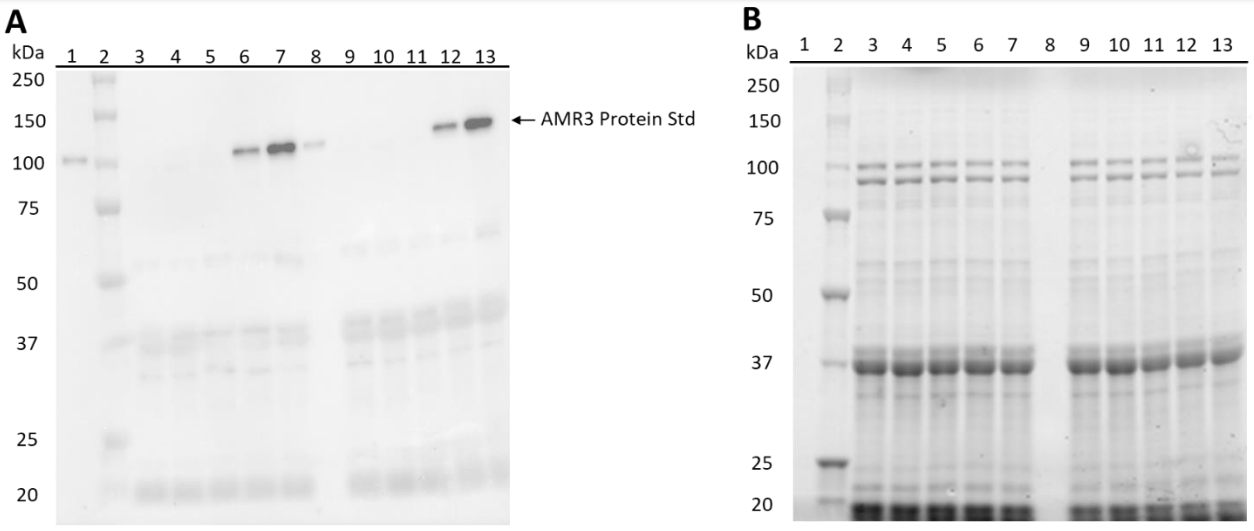
Lane	Sample	AMR3 Protein Std (ppb)
1	AMR3 Protein Std	500
2	MW Marker	N/A
3	RB	0
4	RB + AMR3	100
5	RB + AMR3	250
6	RB + AMR3	500
7	RB + AMR3	750
8	RB + AMR3	1,000
9	RB + AMR3	1,250
10	RB + AMR3	1,500

Figure 20. AMR3 LOQ in tuber samples

(A) Immunoblot analysis of an AMR3 protein standard (102 kDa) added to Russet Burbank (RB) tuber extracts showing the LOQ. The heterologous expressed AMR3 protein standard was added to RB tuber samples (10 µg total protein) at different concentrations (ppb) as indicated in C. The LOQ was established to be 500 ppb (Lane 6). (B) Stain-free gel showing total protein (10 µg) from tuber extracts. (C) Descriptions for each lane apply to A and B.

AMR3 not detected in BG25 tubers

No immunoreactive bands corresponding to the AMR3 protein were observed in BG25 tubers by immunoblot (Figure 21A). The level of the AMR3 protein in BG25 tubers was below the LOQ of 500 ppb (Figure 21A). As a positive control, the AMR3 protein standard, spiked into BG25 (Lanes 12 and 13) and Russet Burbank (Lanes 6 and 7) protein extracts at concentrations of 500 and 1,000 ppb was observed by immunoblot (Figure 21A).



C

Lane	Sample	AMR3 Protein Std (ppb)
1	AMR3 Protein Std	500
2	MW Marker	N/A
3	RB-1	0
4	RB-2	0
5	RB-3	0
6	RB-3 + AMR3	500
7	RB-3 + AMR3	1,000
8	AMR3 Protein Std	500
9	BG25-1	0
10	BG25-2	0
11	BG25-3	0
12	BG25-3 + AMR3	500
13	BG25-3 + AMR3	1,000

Figure 21. AMR3 not detected in BG25 tubers

(**A**) Immunoblot showing detection of the AMR3 protein standard spiked at concentrations of 500 and 1,000 ppb in BG25 (Lanes A12 and A13) and Russet Burbank (RB, Lanes A6 and A7) samples. The AMR3 protein is 102 kDa based on its amino acid sequence. AMR3 protein bands (corresponding to a ~100 kDa MW protein) were not observed in BG25 or RB samples. (**B**) Stain-free gel showing total protein (10 µg) from tuber extracts. (**C**) Descriptions for each lane apply to A and B.

Rpi-amr3 transcript expression in BG25 tubers

Reverse transcription quantitative real time PCR was used to measure *Rpi-amr3* transcript levels in BG25 leaf and tuber samples compared to Russet Burbank. *Solanum americanum*, which expresses *Rpi-amr3*, was used as a positive control. *Solanum americanum* does not produce tubers, so leaves were used for assessment in this plant. Primers and probes were specific to the *Rpi-amr3* region encoding the nucleotide binding site (NBS) domain of AMR3. Amplification of *Rpi-amr3* transcripts was observed in BG25 tuber and *S. americanum* leaf, but not in Russet Burbank (Figure 22).

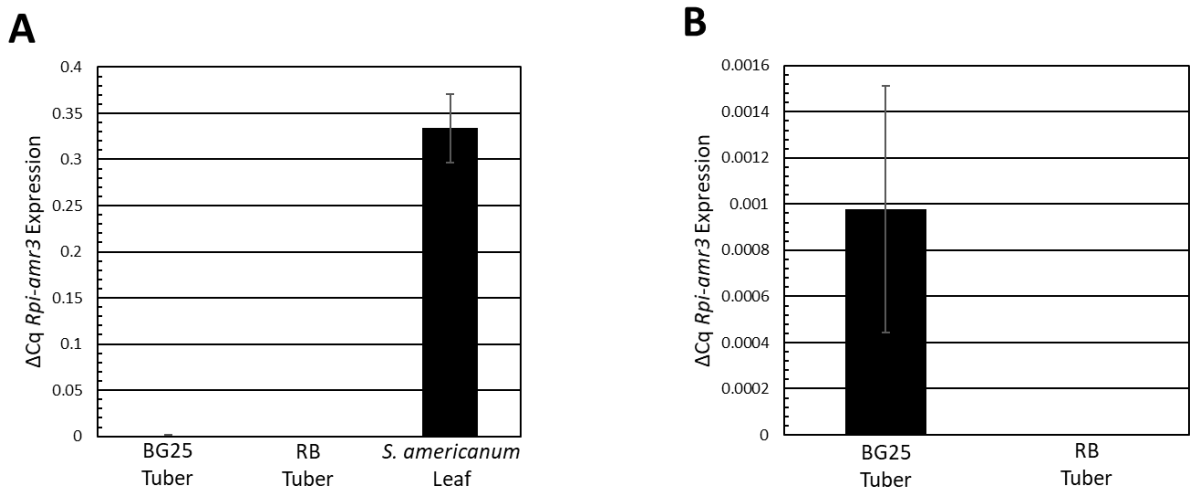


Figure 22. *Rpi-amr3* transcript levels in BG25 tubers

(**A**) Transcript expression of *Rpi-amr3* in BG25, Russet Burbank (RB) tubers, and *S. americanum* leaves. (**B**) Same as A, zoomed in to show *Rpi-amr3* transcript expression in BG25 tubers compared to RB. Reverse transcription-quantitative polymerase chain reaction assays were performed on three biological replicates of BG25 and RB tubers, analysed in triplicate. *Solanum americanum* leaf extract was used as a positive control. Expression of *Rpi-amr3* was normalised to endogenous reference genes (*EF1α* and *APRT*) by calculating a ΔCq value. The ΔCq for each replicate was exponentially transformed to ΔCq-Expression as $2^{-\Delta Cq}$, showing relative expression of *Rpi-amr3* for all samples analysed.

Conclusions on AMR3 protein characterisation

The AMR3 protein was not detected in BG25 tubers and is therefore below the LOQ. The *Rpi-amr3* transcript expression analysis demonstrated that the *Rpi-amr3* gene is present and expressed in BG25.

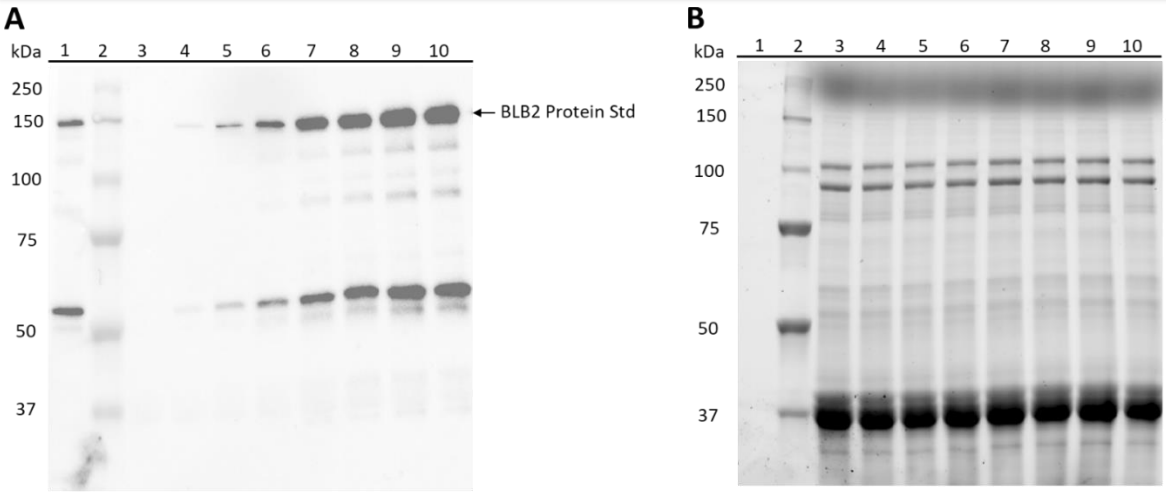
BLB2 in event BG25

The pSIM4363 T-DNA contains the *Rpi-blb2* gene (3,890 bp; 1,267 amino acids) originating from the wild Solanum species *S. bulbocastanum.* The gene is expressed under the native *Rpi-blb2* promoter and terminator, pBlb2 and tBlb2, respectively.

A description of the safety and mode of action of BLB2 can be found in Section A.2 and Section B.1(b). The methods for the determination of BLB2 protein and transcript levels in BG25 are provided in Report C.

BLB2 immunoblot detection in BG25 tubers

The LOQ for BLB2 in tuber samples was determined by adding the BLB2 protein standard to Russet Burbank tuber extracts at different concentrations (see Report C). The LOQ was established to be 100 ppb (Figure 23).



C

Lane	Sample	BLB2 Protein Std (ppb)
1	BLB2 Protein Std	500
2	MW Marker	N/A
3	RB	0
4	RB + BLB2	100
5	RB + BLB2	250
6	RB + BLB2	500
7	RB + BLB2	750
8	RB + BLB2	1,000
9	RB + BLB2	1,250
10	RB + BLB2	1,500

Figure 23. BLB2 LOQ in tubers

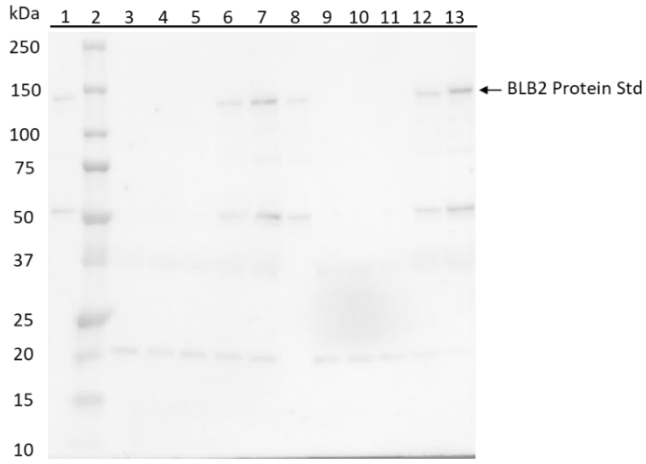
(**A**) Immunoblot analysis of a BLB2 protein standard added to Russet Burbank (RB) tuber extracts showing the LOQ. The heterologously expressed BLB2 protein standard was added to RB tuber samples (10 µg total protein) at different concentrations (ppb) as indicated in **C**. The LOQ was established to be 100 ppb (Lane A4). Note that an observed ~50 kDa band co-elutes with the BLB2 protein (~150 kDa) during purification (Lane A1). It is not present in Russet Burbank (Lane A3). The band increases in intensity proportional to the BLB2 protein and is likely a cross-reactive protein from *E. coli*. It is possible that it could be a degradation product of BLB2, however the ~50 kDa band was not detected using a His-tag specific antibody, while the ~150 kDa BLB2 band was. (**B**) Stain-free gel showing total protein (10 µg) from tuber extracts. (**C**) Description for each lane applies to A and B.

BLB2 Protein Not Detected in BG25 Tubers

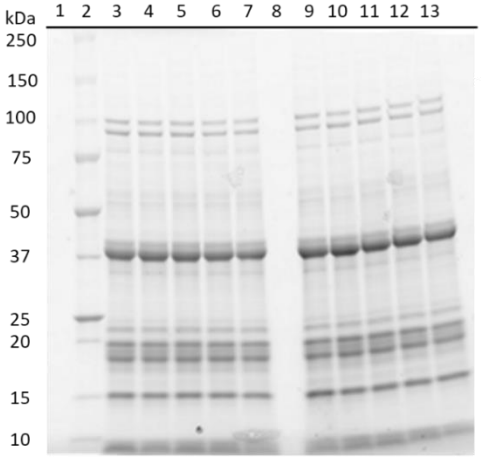
No immunoreactive bands corresponding to the BLB2 protein were observed in BG25 tuber samples by immunoblot (Figure 24). In some cases, bands corresponding to endogenous proteins other than BLB2 were observed due to cross-reactivity of these other proteins with the α-BLB2-3480 polyclonal antibody. These bands correspond to proteins present in both the BG25 event and the conventional Russet Burbank variety.

The level of the BLB2 protein in BG25 tubers was below the LOQ of 100 ppb (Figure 24A). As a positive control, the BLB2 protein standard, spiked into BG25 (Figure 24; Lanes 12 and 13) and Russet Burbank (Figure 24; Lanes 6 and 7) protein extracts at concentrations of 500 and 1,000 ppb was observed by immunoblot (Figure 24A).

A



B



C

Lane	Sample	BLB2 Protein Std (ppb)
1	BLB2 Protein Std	500
2	MW Marker	N/A
3	RB-1	0
4	RB-2	0
5	RB-3	0
6	RB-3 + BLB2	500
7	RB-3 + BLB2	1,000
8	BLB2 Protein Std	500
9	BG25-1	0
10	BG25-2	0
11	BG25-3	0
12	BG25-3 + BLB2	500
13	BG25-3 + BLB2	1,000

Figure 24. BLB2 detection in BG25 tuber samples

(**A**) Immunoblot showing detection of the BLB2 protein standard spiked at concentrations of 500 and 1,000 ppb in BG25 (Lanes A12 and A13) and Russet Burbank (RB, Lanes A6 and A7) samples. The BLB2 protein is 146 kDa based on its amino acid sequence. BLB2 protein bands (corresponding to a ~150 kDa MW protein) were not observed in BG25 or RB samples. (**B**) Stain-free gel showing total protein (10 µg) from tuber extracts. (**C**) Description for each lane applies to A and B.

Rpi-blb2 transcript expression in BG25 tubers

Reverse transcription-quantitative polymerase chain reaction was used to measure *Rpi-blb2* transcript levels in BG25 leaf and tuber samples compared to Russet Burbank. *Solanum bulbocastanum*, which expresses *Rpi-blb2*, was used as a positive control. Primers and probes were specific to the *Rpi-blb2* region encoding the C-terminal, LRR domain of BLB2. The ΔCq *Rpi-blb2* expression levels were higher in BG25 and *S. bulbocastanum* leaves compared to Russet Burbank (Figure 25), indicating that *Rpi-blb2* is present and expressed in BG25 and *S. bulbocastanum*.

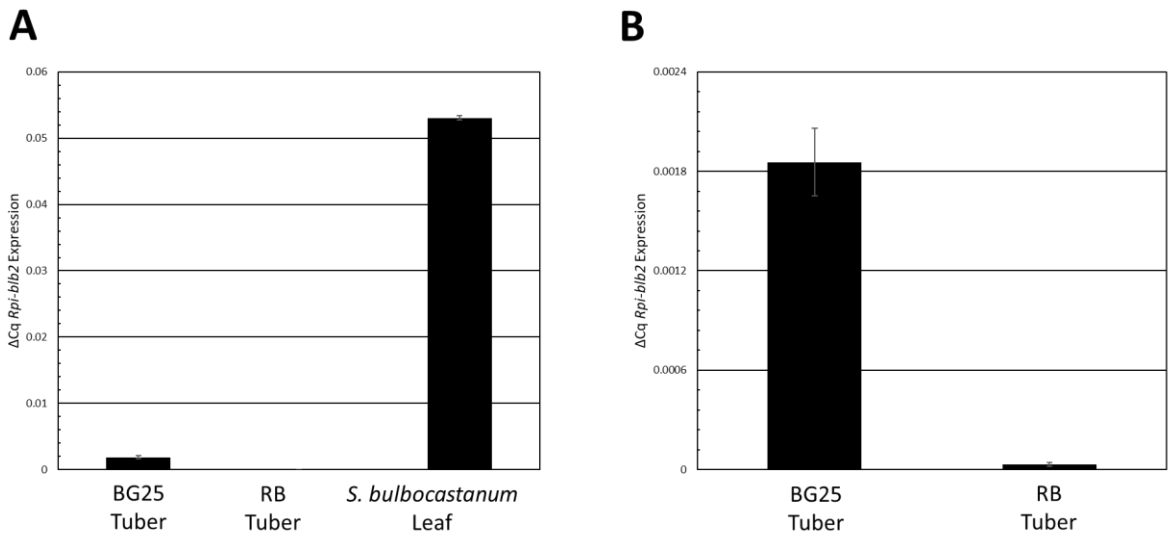


Figure 25. *Rpi-blb2* transcript levels in BG25 tubers

(**A**) Transcript expression of *Rpi-blb2* in BG25 and Russet Burbank (RB) tubers, and in *S. bulbocastanum* leaves. (**B**) Same as A, zoomed in to show *Rpi-blb2* transcript expression in BG25 tubers compared to Russet Burbank. Reverse transcription-quantitative polymerase chain reaction assays were performed on three biological replicates of BG25 and RB tubers, analysed in triplicate. Expression of *Rpi-blb2* was normalised to endogenous reference genes (*EF1α* and *APRT*) by calculating a ΔCq value. The ΔCq for each replicate was exponentially transformed to ΔCq-Expression as $2^{-\Delta Cq}$, showing relative expression of *Rpi-blb2* for all samples analysed. *Solanum bulbocastanum* leaves was used as a positive control.

Conclusions on BLB2 protein characterisation

The BLB2 protein was not detected and is therefore below the LOQ in BG25 tubers. The *Rpi-blb2* transcript expression analysis demonstrated that the *Rpi-blb2* gene is present and expressed in BG25.

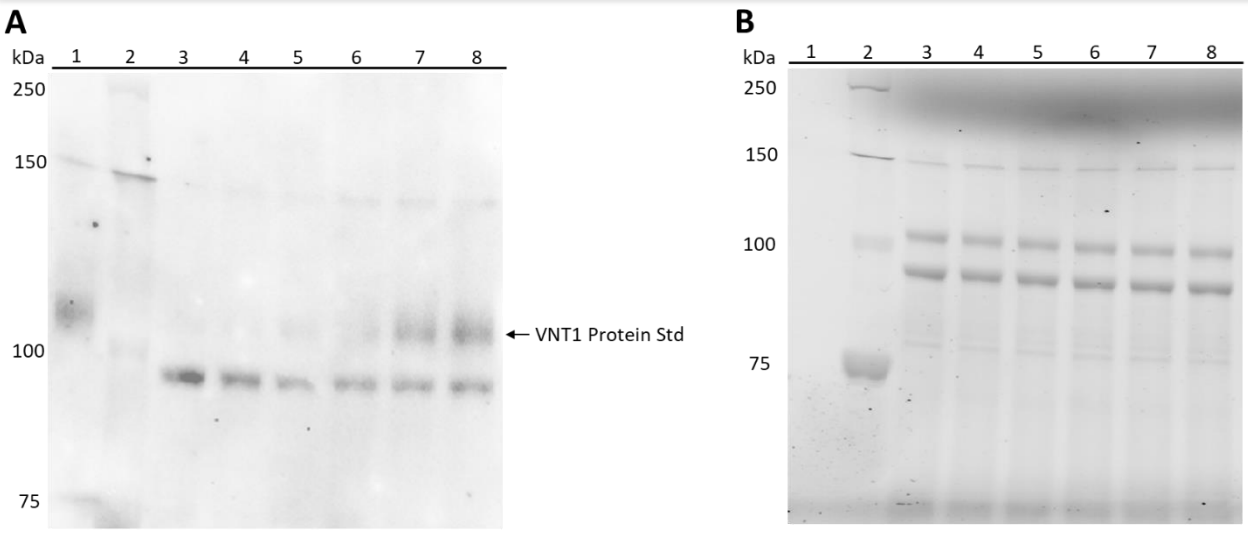
VNT1 in event BG25

The protein VNT1 is the same construct and protein expressed in potato events W8, X17, Y9 and Z6 previously assessed by FSANZ (see A1139 and A1199).

A description of the safety and mode of action of VNT1 can be found in Section A.2 and Section B.1(b). The methods for the determination of VNT1 protein and transcript levels in BG25 are provided in Report J.

VNT1 immunoblot detection in BG25 tubers

A VNT1 protein standard was expressed and purified from *E. coli* and quantified (see Report J). The LOQ for VNT1 in tubers was determined by adding the VNT1 protein standard to Russet Burbank tuber extracts at different concentrations. The LOQ was established to be 500 ppb (Figure 26).



C

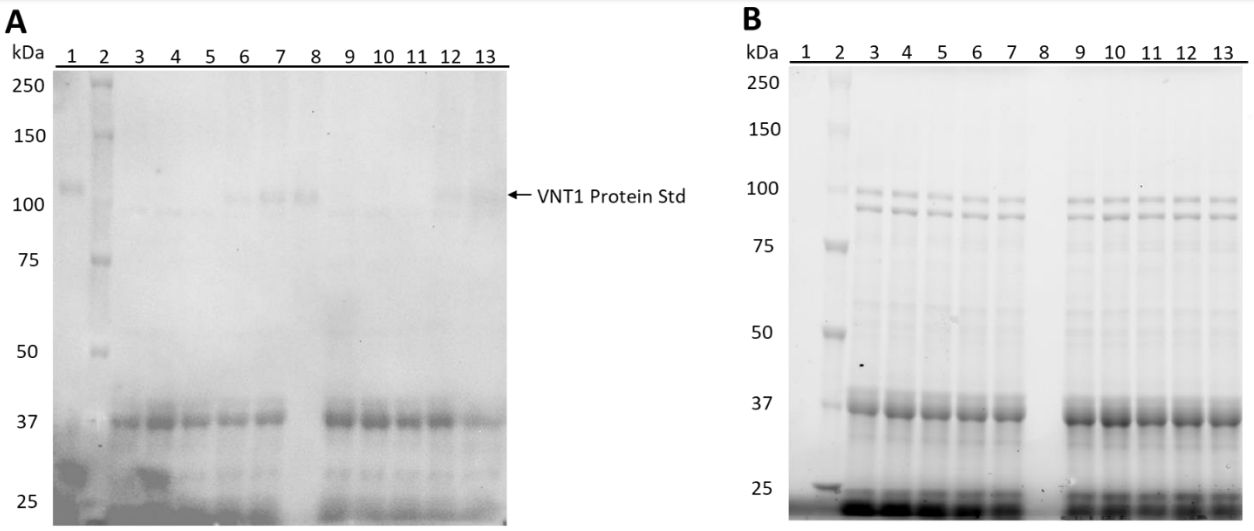
Lane	Sample	VNT1 Protein Std (ppb)
1	VNT1 Protein Std	1,000
2	MW Marker	N/A
3	RB	0
4	RB + VNT1	100
5	RB + VNT1	250
6	RB + VNT1	500
7	RB + VNT1	750
8	RB + VNT1	1,000

Figure 26. VNT1 LOQ in Tuber Samples

(**A**) Immunoblot analysis of a VNT1 protein standard (102 kDa) added to Russet Burbank (RB) tuber extracts showing the LOQ. The heterologous VNT1 protein standard was added to RB tuber samples (10 µg total protein) at different concentrations (ppb) as indicated in **C**. The LOQ was established to be 500 ppb (Lane A6). (**B**) Stain-free gel showing total protein (10 µg) from tuber extracts. (**C**) Description for each lane applies to A and B.

VNT1 not detected in BG25 tubers

No immunoreactive bands corresponding to the VNT1 protein were observed in BG25 tubers by immunoblot (Figure 27A). The level of the VNT1 protein in BG25 tubers was below the LOQ of 500 ppb (Figure 27A). As a positive control, the VNT1 protein standard, spiked into BG25 (Lanes A12 and A13) and Russet Burbank (Lanes A6 and A7) protein extracts at concentrations of 500 and 1,000 ppb were observed by immunoblot (Figure 27A).



C

Lane	Sample	VNT1 Protein Std (ppb)
1	VNT1 Protein Std	1,000
2	MW Marker	N/A
3	RB-1	0
4	RB-2	0
5	RB-3	0
6	RB-3 + VNT1	500
7	RB-3 + VNT1	1,000
8	VNT1 Protein Std	1,000
9	BG25-1	0
10	BG25-2	0
11	BG25-3	0
12	BG25-3 + VNT1	500
13	BG25-3 + VNT1	1,000

Figure 27. VNT1 detection in BG25 tubers

(**A**) Immunoblot showing detection of the VNT1 protein standard spiked at concentrations of 500 and 1,000 ppb in BG25 (Lanes A12 and A13) and Russet Burbank (RB, Lanes A6 and A7) tuber samples. The VNT1 protein is 102 kDa based on its amino acid sequence. VNT1 protein bands (corresponding to a ~100 kDa MW protein) were not observed in BG25 or RB samples. (**B**) Stain-free gel showing total protein (10 µg) from tuber extracts. (**C**) Description for each lane applies to A and B.

Rpi-vnt1 Transcript Expression in BG25 Tubers

Reverse transcription-quantitative polymerase chain reaction was used to measure *Rpi-vnt1* transcript levels in BG25 tubers compared to Russet Burbank. *Solanum venturii*, which expresses *Rpi-vnt1*, was used as a positive control. Primers and probes were specific to the Rpi-vnt1 region encoding the C-terminal, LRR domain of VNT1. The ΔCq *Rpi-vnt1* expression levels were higher in BG25 tubers and *S. venturii* leaves compared to Russet Burbank tubers (Figure 28), indicating that *Rpi-vnt1* is present and expressed in BG25 and *S. venturii*.

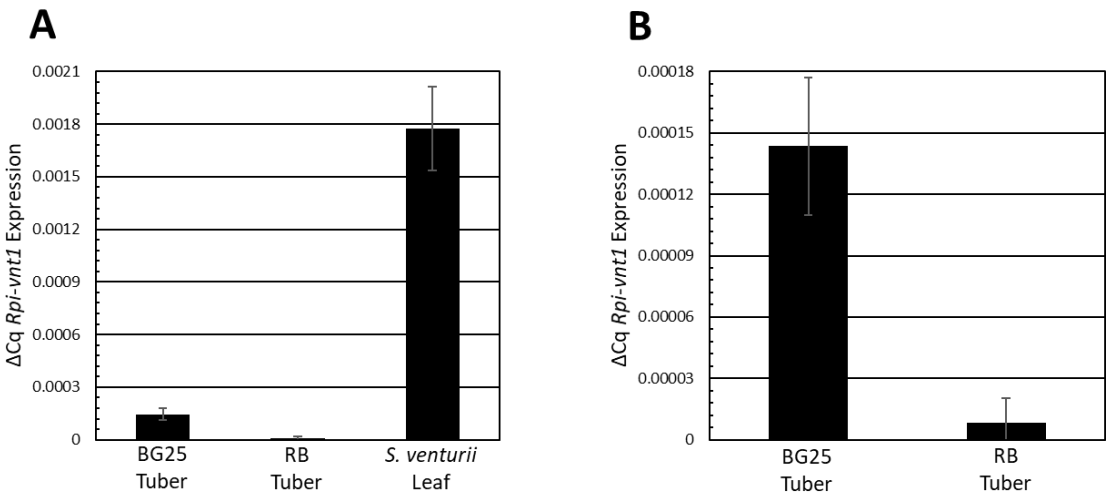


Figure 28. *Rpi-vnt1* Transcript Levels in BG25 Tubers

(**A**) Transcript expression of *Rpi-vnt1* in BG25, Russet Burbank (RB) tubers, and *S. venturii* leaves. (**B**) Same as A, zoomed in to show *Rpi-vnt1* transcript expression in BG25 tubers compared to RB. Reverse transcription-quantitative polymerase chain reaction assays were performed on three biological replicates of BG25 and RB tubers, analysed in triplicate. Expression of *Rpi-vnt1* was normalised to endogenous reference genes (*EF1α* and *APRT*) by calculating a ΔCq value. The ΔCq for each replicate was exponentially transformed to ΔCq-Expression as $2^{-\Delta Cq}$, showing relative expression of *Rpi-vnt1* for all samples analysed. *S. venturii* leaf extract was used as a positive control.

Conclusions on VNT1 protein characterisation

The VNT1 protein was not detected in BG25 tubers and is therefore below the LOQ. The *Rpi-vnt1* transcript expression analysis demonstrated that the *Rpi-vnt1* gene is present and expressed in BG25 tubers.

StmALS in event BG25

The pSIM4363 T-DNA contains the *StmALS* gene (1,977bp; 659 amino acids), a modified *S. tuberosum* acetolactate synthase gene. The gene is expressed under the potato polyubiquitin 7 promoter and potato polyubiquitin 3 terminator, pUbi7 and tUbi3, respectively.

A description of the safety and mode of action of StmALS can be found in Section A.2 and Section B.1(b). The methods for the determination of StmALS protein and transcript levels in BG25 are provided in Report I.

StmALS immunoblot detection in BG25

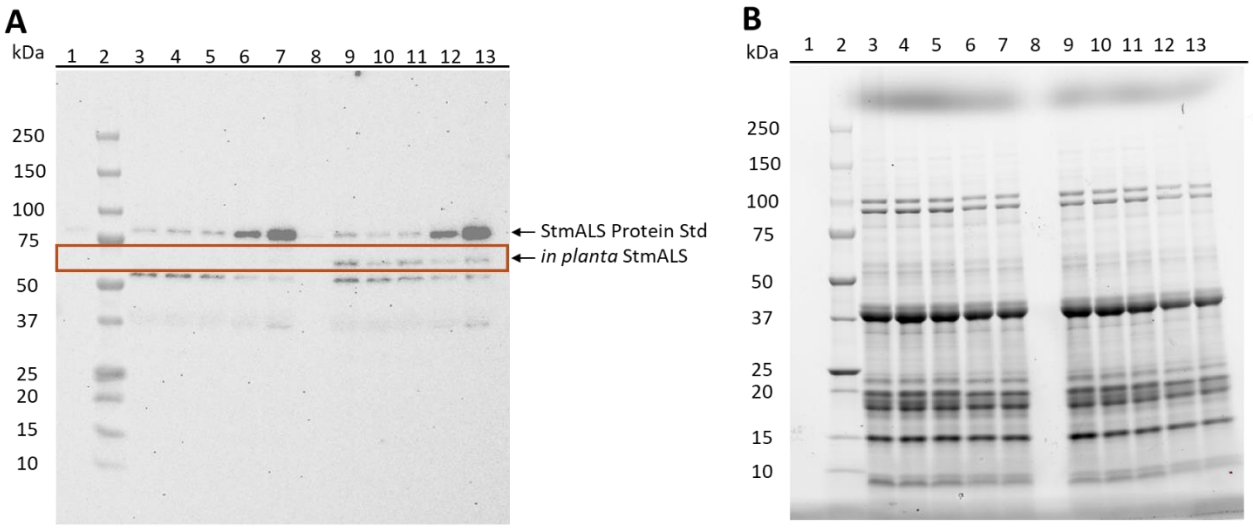
An StmALS protein standard (~80 kDa) was expressed and purified from *E. coli* and quantified (see Report I). The α-StmALS-37524 antibody detected StmALS in tuber samples.

Non-specific binding of the α-StmALS-37524 antibody also was observed in tuber samples (i.e., bands around 37 and 50 kDa). In addition, an ~80 kDa background band was observed in tuber samples, which is similar in size to the StmALS protein standard. The ~80 kDa background band is present even in samples that do not contain the StmALS protein standard (e.g., Figure 29, Lanes A3 to A5) so the antibody binds to another protein that is in conventional potato.

The StmALS protein was detected in BG25 tuber samples collected from four different field locations (Figure 29, Figure 30, Figure 31 and Figure 32). The *in planta*, mature StmALS protein is approximately 65 kDa, based

on its predicted amino acid sequence following chloroplast transit peptide cleavage (Duggleby and Pang, 2000). StmALS protein bands (corresponding to ~65 kDa MW) were observed in BG25, but not in Russet Burbank tuber samples (Figure 29, Figure 30, Figure 31 and Figure 32).

Variability in StmALS band intensity can be observed between BG25 samples, even between replicates from the same location (e.g., see Figure 29; Lanes A9 to A11, red box). The variability indicates that StmALS was expressed at different levels in the collected samples, which may be due to the maturity of the sample at the time of collection, or other biotic or abiotic factors. Expression of native *Als* genes has been shown to be higher in less mature or developing organs where protein production is active (Keeler et al., 1993).

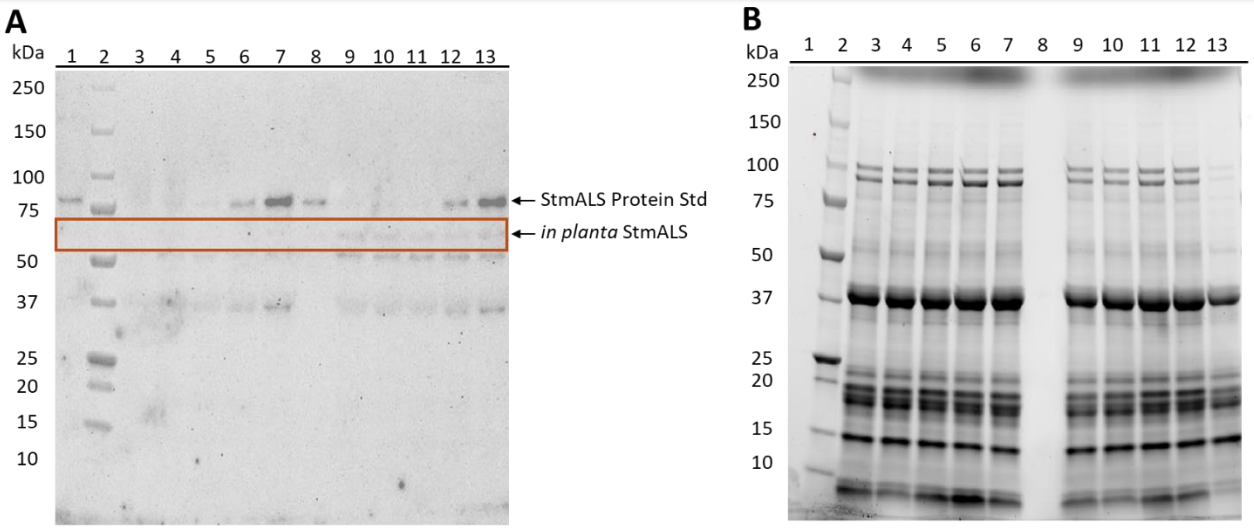


C

Lane	Sample	StmALS Protein Std (ppb)
1	StmALS Protein Std	2,500
2	MW Marker	N/A
3	RB-1	0
4	RB-2	0
5	RB-3	0
6	RB-3 + StmALS	1,000
7	RB-3 + StmALS	2,500
8	StmALS Protein Std	2,500
9	BG25-1	0
10	BG25-2	0
11	BG25-3	0
12	BG25-3 + StmALS	1,000
13	BG25-3 + StmALS	2,500

Figure 29. StmALS detection in BG25 tuber samples from Canyon County

(**A**) Immunoblot showing detection of mature StmALS (~65 kDa) in BG25 tuber samples collected at Canyon County, ID (Lanes A9 to A11, red box). Some variability in band intensity between samples was observed suggesting differences in the expression levels of StmALS (e.g., the ~65 kDa band in Lane A10 had lower intensity than the band in Lane A9). The mature StmALS (~65 kDa) was not detected in Russet Burbank tuber samples (Lanes A3 to A5, red box). The StmALS protein standard (~80 kDa) was loaded alone (Lanes A1 and 8), or at concentrations of 1,000 and 2,500 ppb in BG25-3 (Lanes A12 and A13) and RB-3 (Lanes A6 and A7) samples. A background band at ~80 kDa was observed due to non-specific binding of the α-StmALS-37524 antibody in tuber protein extracts (Lanes A3 to 5 and A9 to 11). (**B**) Stain-free gel showing total protein (10 µg) from tuber protein extracts. (**C**) Description for each lane applies to A and B.

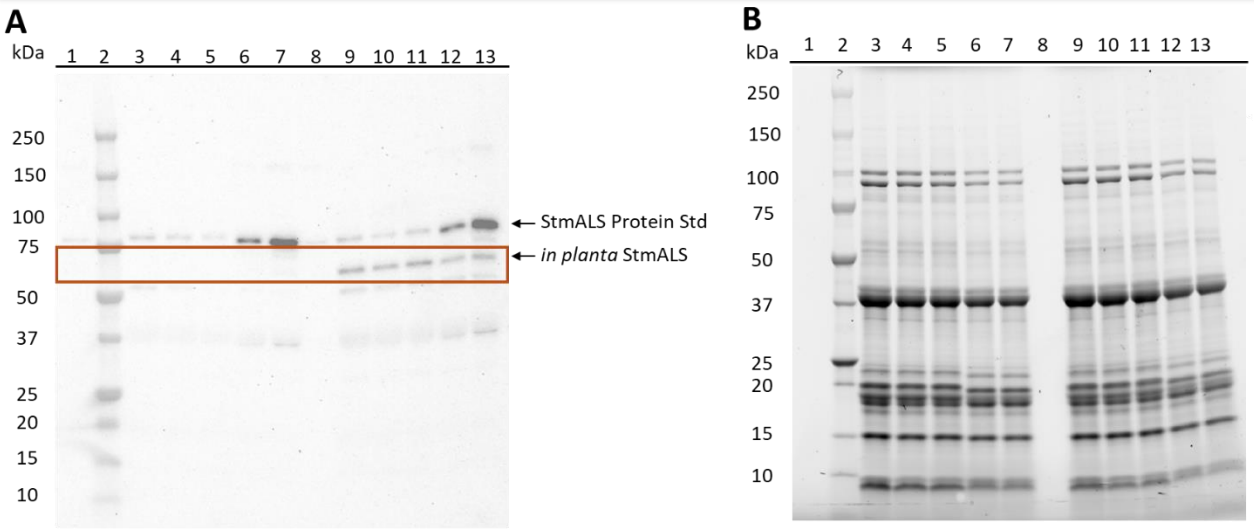


C

Lane	Sample	StmALS Protein Std (ppb)
1	StmALS Protein Std	2,500
2	MW Marker	N/A
3	RB-1	0
4	RB-2	0
5	RB-3	0
6	RB-3 + StmALS	1,000
7	RB-3 + StmALS	2,500
8	StmALS Protein Std	2,500
9	BG25-1	0
10	BG25-2	0
11	BG25-3	0
12	BG25-3 + StmALS	1,000
13	BG25-3 + StmALS	2,500

Figure 30. StmALS detection in BG25 tuber samples from Minidoka County

(**A**) Immunoblot showing detection of mature StmALS (~65 kDa) in BG25 tuber samples collected at Minidoka County, ID (Lanes A9 to A11, red box). The mature StmALS (~65 kDa) was not detected in Russet Burbank tuber samples (Lanes A3 to A5, red box). The StmALS protein standard (~80 kDa) was loaded alone (Lanes A1 and A8), or at concentrations of 1,000 and 2,500 ppb in BG25-3 (Lanes A12 and A13) and RB-3 (Lanes A6 and A7) samples. A background band at ~80 kDa is observed due to non-specific binding of the α-StmALS-37524 antibody in tuber protein extracts (Lanes A3 to A5 and A9 to A11). (**B**) Stain-free gel showing total protein (10 µg) from tuber protein extracts. (**C**) Description for each lane applies to A and B.

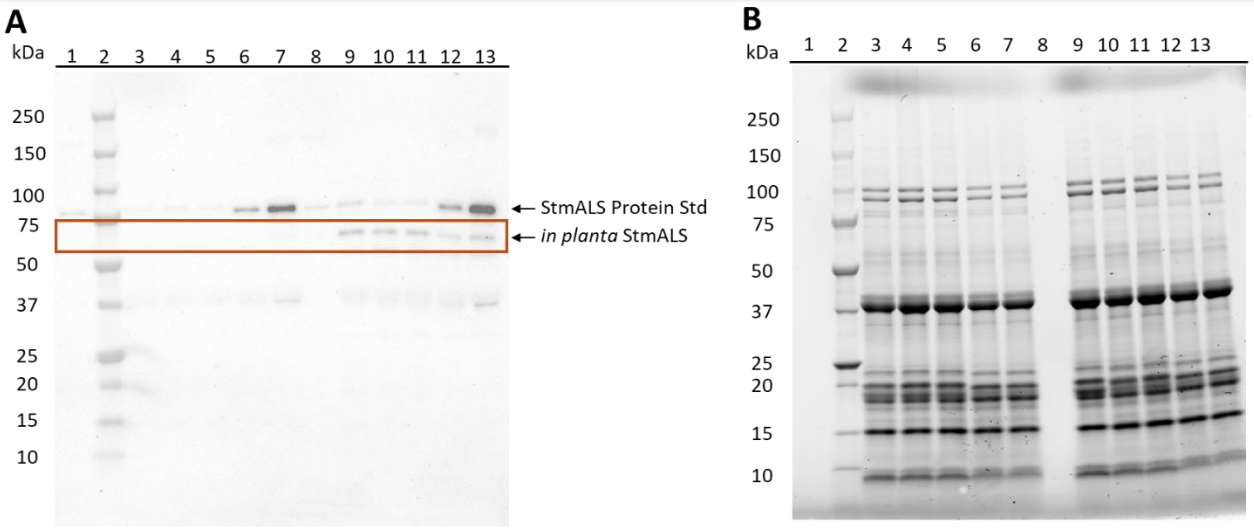


C

Lane	Sample	StmALS Protein Std (ppb)
1	StmALS Protein Std	2,500
2	MW Marker	N/A
3	RB-1	0
4	RB-2	0
5	RB-3	0
6	RB-3 + StmALS	1,000
7	RB-3 + StmALS	2,500
8	StmALS Protein Std	2,500
9	BG25-1	0
10	BG25-2	0
11	BG25-3	0
12	BG25-3 + StmALS	1,000
13	BG25-3 + StmALS	2,500

Figure 31. StmALS detection in BG25 tuber samples from Grand Forks County

(**A**) Immunoblot showing detection of mature StmALS (~65 kDa) in BG25 tuber samples collected at Grand Forks County, ND (Lanes A9 to A11, red box). The mature StmALS (~65 kDa) was not detected in Russet Burbank tuber samples (Lanes A3 to A5, red box). The StmALS protein standard (~80 kDa) was loaded alone (Lanes A1 and A8), or at concentrations of 1,000 and 2,500 ppb in BG25-3 (Lanes A12 and A13) and RB-3 (Lanes A6 and A7) samples. A background band at ~80 kDa was observed due to non-specific binding of the α-StmALS-37524 antibody in tuber protein extracts (Lanes A3 to A5 and A9 to A11). (**B**) Stain-free gel showing total protein (10 µg) from tuber protein extracts. (**C**) Description for each lane applies to A and B.



C

Lane	Sample	StmALS Protein Std (ppb)
1	StmALS Protein Std	2,500
2	MW Marker	N/A
3	RB-1	0
4	RB-2	0
5	RB-3	0
6	RB-3 + StmALS	1,000
7	RB-3 + StmALS	2,500
8	StmALS Protein Std	2,500
9	BG25-1	0
10	BG25-2	0
11	BG25-3	0
12	BG25-3 + StmALS	1,000
13	BG25-3 + StmALS	2,500

Figure 32. StmALS detection in BG25 tuber samples from Grant County

(**A**) Immunoblot showing detection of mature StmALS (~65 kDa) in BG25 tuber samples collected at Grant County, WA (Lanes A9 to A11, red box). The mature StmALS (~65 kDa) was not detected in Russet Burbank tuber samples (Lanes A3 to A5, red box). The StmALS protein standard (~80 kDa) was loaded alone (Lanes A1 and A8), or at concentrations of 1,000 and 2,500 ppb in BG25-3 (Lanes A12 to A13) and RB-3 (Lanes A6 to A7) samples. A background band at ~80 kDa is observed due to non-specific binding of the α-StmALS-37524 antibody in tuber protein extracts (Lanes A3 to A5 and A9 to A11). (**B**) Stain-free gel showing total protein (10 µg) from tuber protein extracts. (**C**) Description for each lane applies to A and B.

The amount of StmALS (~65 kDa) in BG25 tuber samples was quantified using an immunoblot method and densitometry (Figure 33 and Table 16). The concentration of StmALS in tubers was calculated from all BG25 tuber samples, from all locations, using a standard curve (Figure 33C). The conversion of these values to ppb is described in Report I.

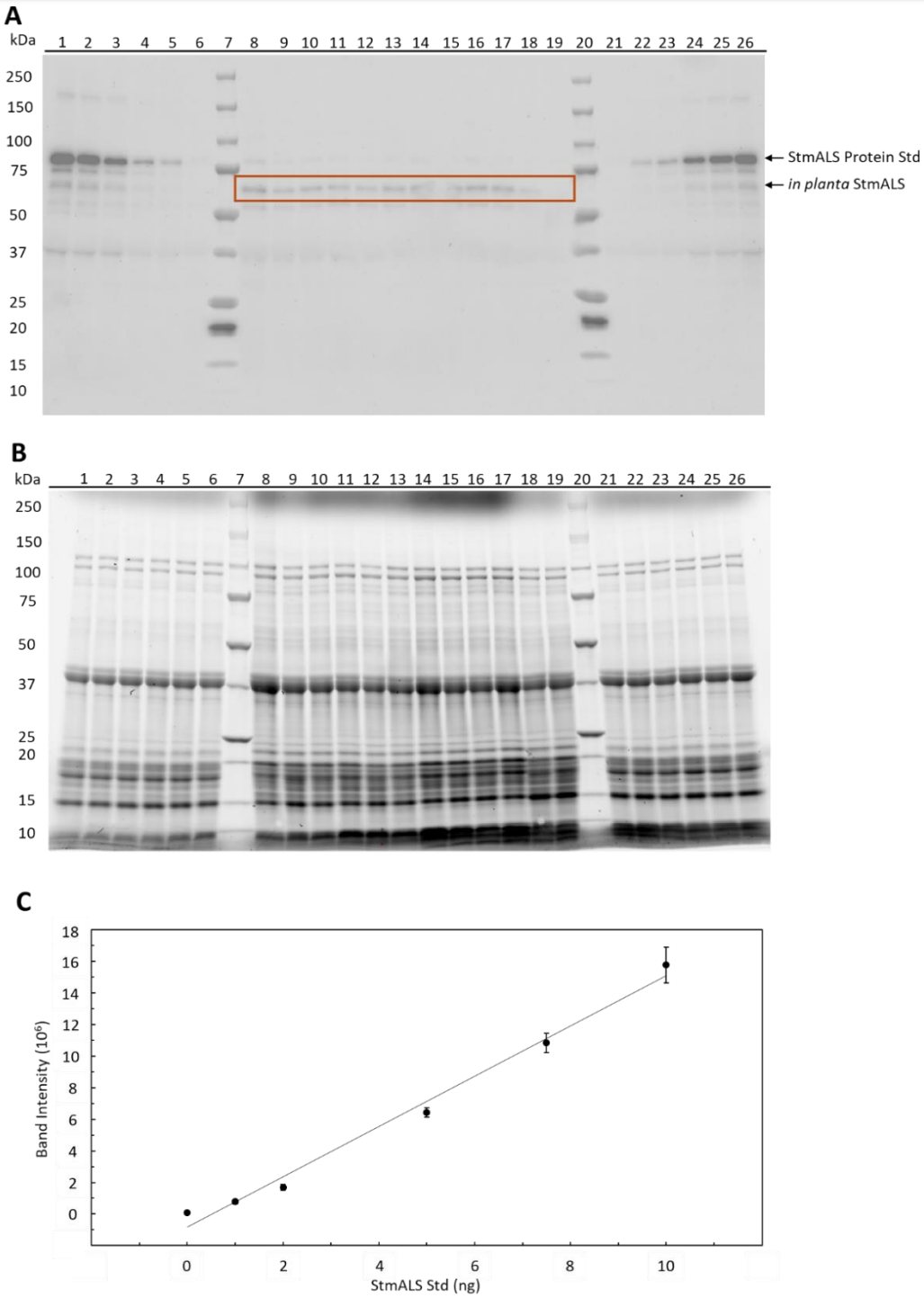


Figure 33. StmALS quantitation in BG25 tuber samples from all locations

(**A**) Immunoblot showing detection of mature StmALS (~65 kDa) in BG25 tuber samples from all locations (Lanes A8 to A19, red box). A serial dilution of StmALS protein standard (~80 kDa) was added to Russet Burbank samples and loaded in duplicate to make a standard curve (Lanes A1 to A6 and A21 to A26). (**B**) Stain-free gel showing total protein (10 µg) in each sample. (**C**) Standard curve of StmALS protein standard band intensities versus amount loaded (ng). Lane descriptions are given in Table 16.

Table 16. Descriptions of lanes in Figure 29 and densitometric analysis.

Lane	Sample	Location	Average Densitometric Volumes (10^6)	Calculated StmALS in fragment (ng)	StmALS in tuber (ppb)[1]
1	RB + 10 ng StmALS	Grant County, WA	15.8	-	
2	RB + 7.5 ng StmALS	Grant County, WA	10.8	-	
3	RB + 6.4 ng StmALS	Grant County, WA	6.4	-	
4	RB + 1.7 ng StmALS	Grant County, WA	1.7	-	
5	RB + 1 ng StmALS	Grant County, WA	0.78	-	
6	RB + 0 ng StmALS	Grant County, WA	0.082	-	
7	MW Marker	N/A	N/A	N/A	
8	BG25-1	Canyon County, ID	1.4	1.4	430
9	BG25-2	Canyon County, ID	0.62	0.91	380
10	BG25-3	Canyon County, ID	0.85	1.1	420
11	BG25-1	Minidoka County, ID	0.73	0.98	490
12	BG25-2	Minidoka County, ID	0.61	0.91	400
13	BG25-3	Minidoka County, ID	0.94	1.1	380
14	BG25-1	Grand Forks County, ND	0.96	1.1	470
15	BG25-2	Grand Forks County, ND	0.72	0.98	450
16	BG25-3	Grand Forks County, ND	1.3	1.3	570
17	BG25-1	Grant County, WA	1.2	1.3	480
18	BG25-2	Grant County, WA	0.37	0.76	340
19	BG25-3	Grant County, WA	0.071	0.57	200
20	MW Marker	N/A	N/A	N/A	
21	RB + 0 ng StmALS	Grant County, WA	0.082	-	
22	RB + 1 ng StmALS	Grant County, WA	0.78	-	
23	RB + 2 ng StmALS	Grant County, WA	1.7	-	
24	RB + 5 ng StmALS	Grant County, WA	6.4	-	
25	RB + 7.5 ng StmALS	Grant County, WA	10.8	-	
26	RB + 10 ng StmALS	Grant County, WA	15.8	-	

[1] This conversion is described in Report I.

The mean StmALS expression level in BG25 tubers was calculated to be 420 ppb, with a range of 200 to 570 ppb (Table 17).

Table 17. StmALS levels in event BG25 tubers

Sample	Mean[1] (ppb)	Range (ppb)
Tuber	420	200-570

[1] Mean rounded up.

Conclusions on StmALS expression in BG25 tubers

The StmALS protein was detected and quantified in BG25 tuber samples collected from four different field locations. The average StmALS protein expression level was 420 ppb in tubers (Table 17).

B.1(b) Information about prior history of human consumption of the new substances, if any, or their similarity to substances previously consumed in food.

Safety Assessment of R Proteins

The safety of R-proteins was addressed using standard risk assessment principles (see Report G). The hazard and the exposure of the R-proteins expressed in BG25 was assessed and this information used to assess the risk to food and environmental safety (Figure 34). This assessment is detailed below. The safety of VNT1 has previously been assessed by FSANZ (see A1139 and A1199).

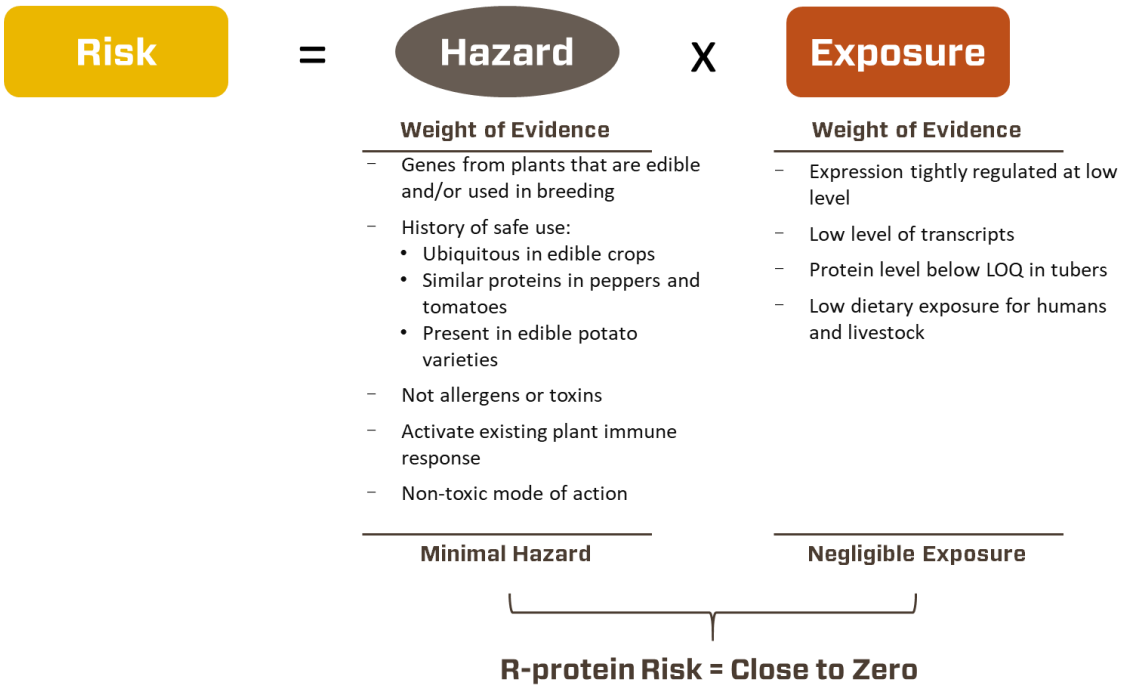


Figure 34. The risk from consuming R-proteins in BG25 potatoes is close to zero

Risk assessment principles, considering potential hazard and exposure, were applied to evaluate risk associated with consumption of R-proteins from BG25 potatoes.

R-Protein hazard assessment

R-genes and their expressed R-proteins are found throughout the plant kingdom, including in many edible crops. Standard safety studies for proteins introduced into biotech crops require purification of large amounts of protein from the host plant or a heterologous system to conduct the analyses. Resistance proteins, however, are expressed at exceptionally low levels, and purification of biologically active R-proteins from the host plant or heterologous expression systems is problematic (Habig et al., 2018). As a result, generating a standard protein safety data package for R-proteins, such as AMR3, BLB2, and VNT1, is impractical. However, R-protein safety can be comprehensively and adequately assessed without isolation of protein (Roper et al., 2021).

To assess the safety of the three R-proteins in BG25 potatoes, a weight of evidence approach was employed. This approach considered all information in a comprehensive manner to assess both hazard and exposure. The R-protein exposure assessment focused on tubers, which are the only edible part of the potato plant for humans and livestock. The safety of VNT1 was determined earlier (Habig et al., 2018; also see sheyFSANZ A1139 and A1199), and because AMR3 and BLB2 are similar R-proteins, this rationale was used to assess the

safety of all three R-proteins in BG25. Findings for the three R-proteins pointed to minimal hazard and negligible exposure, indicating that the risk from consuming AMR3, BLB2, and VNT1 in BG25 potatoes is close to zero (Figure 34).

Resistance protein hazard assessment provides a weight of evidence for safety based on the source of the proteins, a history of safe use, the lack of similarity to known allergens and toxins, activation of a conserved plant pathway, and a non-toxic mode of action.

The three R-proteins in BG25 are AMR3, BLB2, and VNT1 and are from Solanum species. Protein expression for each gene is regulated by the native promoter and terminator sequences and all three proteins share the common domain structure found in other R-proteins (Table 18).

The AMR3, BLB2, and VNT1 R-proteins come from Solanum gene donor plants that have a history of safe use in food and feed. *Solanum americanum* is eaten as a vegetable and the berries are used for jams. *Solanum bulbocastanum* has been used to breed conventional potato varieties, and the VNT1 protein from *S. venturii* is present in conventional varieties with a history of safe use. These insertions in BG25 are cisgenes derived from the Solanum gene pool.

Based on the weight-of-evidence and considering the close-to-zero risk, the AM3, BLB2 and VNT1 proteins in BG25 potato are as safe as conventional varieties for humans, livestock, and the environment.

Table 18. Source of the R-proteins in BG25

R-protein	Donor	Amino Acid Number	R-protein Domain Structure
AMR3	*S. americanum*	887	
BLB2	*S. bulbocastanum*	1267	CC-NBS-LRR[1]
VNT1	*S. venturii*	891	

[1] CC = coiled-coil, NBS = nucleotide-binding site, LRR = leucine-rich repeat

Proteins with a history of safe use, or that are structurally and functionally related to proteins with a history of safe use, are considered safe to consume (Hammond and Cockburn, 2007). As a component of the safety assessment of R-proteins, bioinformatic analyses were conducted to identify amino acid sequence similarity between AMR3, BLB2, and VNT1 in BG25 and other R-proteins in potato varieties that have a history of safe use.

While hundreds of R-proteins are found in potato (Jupe et al., 2012; Wei et al., 2016), there was similar sequence identity between AMR3 (68 to 72%), BLB2 (80 to 81%), and VNT1 (77% to 98%) and R-proteins from cultivated potatoes (Table 19).

Weight of evidence in support of R-protein safety includes:

- the source of each of the R-proteins (AMR3, BlB2 and VNT1 described in Section A.2) are from plants that are edible and/or used in potato breeding
- R-proteins have a history of safe use. They are ubiquitous in edible crops (e.g., AMR3, BLB2 and VNT1 have 71-83% sequence identity to R-proteins in tomato and pepper
- AMR3, BLB2 and VNT1 have similar sequence identity to R-proteins in cultivated potato
- The R-proteins have a non-toxic mode of action (see Section A.2 and Section B.1(b))
- They are not toxic or allergenic (see Section B.1(d)).

Table 19. Sequence identity between AMR3, BLB2, and VNT1 and other R-Proteins from cultivated potatoes

R-protein in BG25	R-protein	Accession No.	% Identical Amino Acids to R-protein in BG25	Source
AMR3	R1B-23	XP_006346790	72	*S. tuberosum* (cultivated potato)
	R1A-10	XP_015163927	68	*S. tuberosum* (cultivated potato)
BLB2	R1A-3 isoform X3	XP_015160128	81	*S. tuberosum* (cultivated potato)
	R1A-3 isoform X1	XP_015160126	80	*S. tuberosum* (cultivated potato)
	R1A-3 isoform X2	XP_015160127	80	*S. tuberosum* (cultivated potato)
VNT1	VNT1.3	ACJ66596	98	*S. tuberosum* (Alouette variety)
	St-Tm-2 ToMV	ABM05492	77	*S. tuberosum* (cultivated potato)
	RPP13-like protein 3	XP_015170548	79	*S. tuberosum* (cultivated potato)
	RPP13-like protein 3	XP_006360919	80	*S. tuberosum* (cultivated potato)

R-Protein exposure assessment

R-proteins are maintained in an inactive state at low concentrations in the cell (Spoel and Dong, 2012). They are activated by interaction with pathogen-secreted effector proteins, this initiates the plant hypersensitivity response, which triggers plant cell death (also known as apoptosis). Low levels of R-protein in plant cells help to avoid unnecessary plant cell death. Based on an assumption of one R-protein copy per plant cell, levels may be as low as 18 parts per trillion, leading to negligible human exposure (3.6 ng protein/year) (Bushey et al., 2014). The low concentration of R-proteins decreases the exposure, which reduces potential risk to human health, non-target organisms, and the environment. The exposure assessment for the three R-proteins in BG25 for North America is provided in Report G. See the relevant parts of Section B.1(a) above on history of safe use and refer to the other relevant supplemental reports. In addition, the dietary exposure of the R-proteins to humans and livestock in Australia and New Zealand was estimated.

Low dietary exposure to AMR3, BLB2, and VNT1

In event BG25, measured levels of *Rpi-amr3, Rpi-blb2, and Rpi-vnt1* mRNA were significantly lower in tubers compared to leaves (see Section B.1(a) and Reports B, C, and J; respectively). This pattern of gene expression that is higher in leaves and lower in tubers is consistent with other research on R-gene expression in potatoes (Pel, 2010). Dietary exposure of humans to AMR3, BLB2, or VNT1 in BG25 tubers is expected to be negligible because of low expression in tubers.

Animals and invertebrates (except for insects) are not known to graze on potato leaves in the field due to the high levels of glycoalkaloids in solanaceous leaves (Kozukue et al., 1987; Maga, 1994). As such, the dietary exposure analysis of humans and livestock to AMR3, BLB2, or VNT1 is focused on tubers.

Given that the AMR3, BLB2 and VNT1 were too low to detect in tubers (see Section B.1(a) and Reports Reports B, C, and J; respectively), dietary exposure is expected to be negligible. Limits of Quantitation (LOQ)

were established for each AMR3, BLB2, and VNT1 in potato tubers using western blot analyses (Reports Reports B, C, and J; respectively). The LOQ was used to calculate the potential dietary exposure for each R-protein. Since a no-observed-adverse-effect level has not been established for any R-proteins, a margin of exposure cannot be calculated.

Low Dietary Exposure to Humans of AMR3, BLB2 and VNT1 in Australia and New Zealand

Data on the dietary consumption of potatoes vary depending on the source and are difficult to estimate due to the numerous potato-based products consumed. When considering both fresh and processed potatoes, both Australia and New Zealand consumption rates are relatively high compared to other regions of the world (FAO 2023; Table 20). In New Zealand, potatoes are the most popular vegetable with data suggesting that consumption of potatoes is 11% greater than Australia (Table 20).

Data from a 2011 and 2012 health survey undertaken by the Australian Bureau of Statistics[6] estimated the median amount of potatoes consumed by Australians for various age groups (Table 21). The largest daily consumption of potatoes was from the age group 31-50 at 137 g per day (Table 21). When considering potatoes, potato products and potato mixed dishes, there appears to be predominantly more consumption by males than females (Table 22).

The potential dietary exposure of people to the R-proteins AMR3, BLB2 and VNT1 from consuming BG25 potatoes was evaluated by calculating an estimate of daily dietary intake of AMR3, BLB2 and VNT1 consumption rates and comparing this to daily protein intake. The dietary exposure estimates are based on several assumptions. Firstly, it only considers potatoes and not potato products or potato mixed dishes and assumes that 100% in the potato diet is derived from BG25 potatoes. It also assumes that there is no degradation of R-proteins during processing or cooking. Lastly, it also assumes that the proportion of protein consumed in the diet is 50% less than the recommended amount for males and females (NHMRC 2006), which results in an increased proportion of R-proteins consumed in the diet.

Table 20. Potato consumption for selected countries/regions

Region	Potato consumption (kg/capita/year)[1]	Potato consumption (g/capita/day)[2]
Africa	14.95	40.96
Asia	30.97	84.84
Europe	72.35	198.22
New Zealand	64.03	175.42
United States of America	48.65	133.29
Australia	57.62	157.86
Canada	79.29	217.23
Oceania	42.95	117.67
China Mainland	46.59	127.64
World	32.98	90.36

[1] Food and Agriculture Organization of the United Nations (FAO, 2023). Data from 2021
[2] Estimated based on annual consumption

[6] Australian Bureau of Statistics (43640DO006_20112012 Australian Health Survey: Nutrition First Results – Foods and Nutrients, 2011–12 — Australia).

Dietary exposure calculations were determined using a conservative estimate of BG25 potato consumption in New Zealand and Australia of 64 kg and 58 kg per capita per year, respectively (FAO 2023; Table 20). An estimate of dietary exposure of humans to BLB2 from BG25 was calculated assuming tubers contain 0.1 µg/g (taken as the LOQ) of BLB2. Similarly, an estimate of dietary exposure of humans to AMR3 and VNT1 was calculated assuming BG25 tubers contain 0.5 µg/g of AMR3 and VNT1.

Table 21. Median consumption of potatoes in Australia[1]

Category (grams/day	Age Group (years)									
	2-3	4-8	9-13	14-18	19-30	31-50	51-70	71+	19+	Total 2 and over
Potatoes	38.9*	65.2	115.0	122.9	128.0	137.0	123.5	126.2	130.0	122.5

1: Data from 43640DO006_20112012 Australian Health Survey: Nutrition First Results – Foods and Nutrients, 2011–12 — Australia
* estimate has a relative standard error of 25% to 50% and should be used with caution.

Table 22. Median amount of potato and potato products consumed in Australia[1]

Food Product	Males	Female	Persons
	Grams		
Potatoes	137.0	111.0	122.1
Potato products	75.9	72.0	72.0
Potato mixed dishes	181.3	147.0	181.3

1: Data from 43640DO006_20112012 Australian Health Survey: Nutrition First Results – Foods and Nutrients, 2011–12 — Australia

Based on FAO and Australian Bureau of Statistics (ABS) data, the Australian male and female population has a potato consumption rate of 1.81 and 2.19 g/kg body weight/ day respectively (g/kg bw/d) (Table 23). The highest consumers of potatoes are New Zealand females at 2.3 g/kg bw/d. Based on this, the potential exposure of humans to the three R-proteins from BG25 tubers was evaluated by calculating an estimate of daily dietary intake of each protein consumption rates and comparing this to daily protein intake (Table 23 and Table 24). For these calculations, the average weights of Australian and New Zealand males and females was estimated based on data from the ABS and Figure NZ data respectively.

Table 23. Dietary Exposure of Australian and New Zealand people to BLB2, AMR2 and VNT1 from BG25

Total Population	Gender	Potato Consumption[1] g/kg bw/d	Estimated Exposure to BLB2 in Potato[2] µg/kg bw/d	Estimated Exposure to AMR3 and VNT1 in Potato[3] µg/kg bw/d
Australia*	Male	1.81	0.201	1.005
	Female	2.19	0.230	1.150
New Zealand**	Male	2.01	0.181	0.905
	Female	2.30	0.219	1.095

[1] Consumption based on 2021 FAO data Table 20
[2]Exposure to BLB2 = (Consumption) × (0.1 µg BLB2/g fresh weight), based on a conservative estimate at the LOQ.
[3] Exposure to AMR3 and VNT1 = (Consumption) x (0.5 µg protein/g fresh weight), based on a conservative estimate at the LOQ.
* Average weight of an Australian male, 87kg, and an Australian female, 72kg (ABS 2018)
** Average weight of a New Zealand male, 87.13kg and a New Zealand female, 76.3kg; Calculated from Figure NZ (2021) Mean weight of adults in New Zealand. https://figure.nz/chart/uVNhVZ5e8itkButj Accessed 17 November 2023;

Estimated exposure to BLB2 protein in Australian males is 201 ng/kg bw/d and for females 230 ng/kg bw/d (Table 23). In addition, the percentage consumed compared to total protein intake is negligible (0.000043% for males and 0.000058% for females; Table 24). Similar negligible exposure levels were estimated for the New Zealand population (Table 23 and Table 24).

Estimated exposure to AMR3 and VNT1 protein in Australian males is 1.0 µg/kg bw/d and for females 1.15 µg/kg bw/d (Table 23). In addition, the percentage consumed compared to total protein intake is low (0.0002% for males and 0.0003% for females; Table 24). Similar low exposure levels were estimated for the New Zealand population.

Collectively, these estimates indicate that dietary exposure to R-proteins from BG25 tubers would be negligible.

Table 24. Dietary exposure of humans to BLB2, AMR2 and VNT1 from BG25 as a proportion of daily protein intake

Total Population	Gender	Percentage of daily protein consumed that is BLB2[1,2]	Percentage of daily protein consumed that is AMR3 and VNT1[3,4]
Australia	Male	0.000043%	0.0002%
	Female	0.000058%	0.0003%
New Zealand	Male	0.000048%	0.0002%
	Female	0.000061%	0.0003%

[1]Exposure to BLB2 = (Consumption) × (0.1 µg BLB2/g potato tuber), based on a conservative estimate at LOQ.
[2]Percent of daily protein consumed that is BLB2 = (exposure to BLB2/ 420 or 375 mg/kg bw/d of protein[5]).
[3]Exposure to AMR3 and VNT1 = (Consumption) x (0.5 µg protein/g potato tuber)
[4]Percent of daily protein consumed that is AMR3 and VNT1 = (exposure to AMR3 and VNT1/ 420 or 375 mg/kg bw/d of protein[5]).
[5]Proportion of protein consumed in the diet, 50% less than the recommended amount for males and females respectively (NHMRC 2006)

Dietary Exposure of Livestock to BLB2, AMR3 and VNT1

Unwanted potato material is sometimes incorporated into ruminant livestock feed. This can include potato culls (whole unpeeled potato not suited for fresh market or processing) and potato process waste (wet and dry peel, raw chip, fries, and cooked potatoes). According to (OECD, 2013), beef cattle in North America may consume up to 30% of their diet (as fed) from potatoes, and sheep may consume up to 10% of their diet (as fed) from potatoes.

Although (OECD, 2013) does not list any use of potato material in pig feed and current best practice pertaining to potato products in pig feed is not clearly documented, other sources have noted that potato steam peel can be included at a level up to 30% dry matter in feed during the growing-finishing phase (VanLunen et al., 1989). Steam peel is a by-product of potato processing where the peel is removed after steaming. Pigs also may consume cooked potatoes from restaurant and catering waste.

Data on the use of potato as an animal feed in Australia and New Zealand is lacking. However, it would be reasonable to expect that when used, the proportion of potatoes in feed would be like the proportions outlined in OECD 2013.

The potential exposure for livestock to each of the three R-proteins in BG25 potatoes was evaluated by calculating an estimate of daily dietary potato intake for cattle, sheep, and pigs, assuming incorporation rates of 30% as fed (6% dry matter), 10% as fed (2% dry matter), and 30% (dry matter), respectively (Table 25).

Table 25.Livestock Consumption of Potatoes

Livestock	Feed Intake (kg/d (dry matter))	Diet Incorporation (as fed)	Diet Incorporation (dry matter)	Animal Body Weight (kg)	Potato Consumption (g (DW)/kg bw/d)[6]
Cattle, beef	9.1[1]	30%[1]	6%[3]	500[1]	1.1
Sheep, adult	1.5[2]	10%[2]	2%[3]	85[2]	0.35
Sheep, lamb	2.0[2]	10%[2]	2%[3]	40[2]	1.0
Pigs, finishing	3.1[4]	Not applicable	30%[5]	100[4]	9.3

[1]From p. 28 of (OECD, 2013).
[2]From p. 34 of (OECD, 2013).
[3]"As fed" is converted to "dry matter" by dividing by 5, since potatoes are 20% solids and 80% water (OECD, 2002).
[4]From p. 38 of (OECD, 2013).
[5]From (VanLunen et al., 1989).
[6]Units are in g (DW)/kg bw/d and calculated as feed intake x % diet incorporation as dry matter x (1000 g/kg)/animal body weight.

The exposure estimates for each R-protein that cattle could consume from BG25 potatoes were 0.0014 mg/kg bw/d for BLB2 and 0.0028 mg/kg bw/d for AMR3 and VNT1. The exposure estimates for sheep (adults) were 0.00044 mg/kg bw/d for BLB2 and 0.00088 mg/kg bw/d for AMR3 and VNT1; and 0.0013 mg/kg bw/d for BLB2 and 0.0025 mg/kg bw/d for AMR3 and VNT1 in lambs. The exposure estimate for pigs was 0.012 mg/kg bw/d for BLB2 and 0.023 mg/kg bw/d for AMR3 and VNT1 (Table 26).

In each case, estimates were conservative (high-end) as it was assumed that BG25 potatoes made up 100% of the potato material incorporated into the animal diet. The amount of each R-protein was assumed to be at the limit of quantification, which is greater than the actual amount. In addition, these estimates assume no degradation of the R-protein during processing or cooking of the potatoes. Therefore, the estimated exposures are conservative and actual exposure is likely much lower. Like the human dietary exposure analysis, livestock have negligible exposure to R-proteins consumed from potatoes, even using conservative (high-end) assumptions.

Table 26. Livestock Exposure to BLB2, AMR3, and VNT1

Livestock	Potato Consumption[1] (g (DW)/kg bw/d)	AMR3[2] (µg/g (DW))	AMR3 Consumed[3] (mg/kg bw/d)	BLB2[2] (µg/g (DW))	BLB2 Consumed[3] (mg/kg bw/d)	VNT1[2] (µg/g (DW))	VNT1 Consumed[3] (mg/kg bw/d)
Cattle, beef	1.1	2.5	0.0028	1.25	0.0014	2.5	0.0028
Sheep, adult	0.35	2.5	0.00088	1.25	0.00044	2.5	0.00088
Sheep, lamb	1.0	2.5	0.0025	1.25	0.0013	2.5	0.0025
Pigs, finishing	9.3	2.5	0.023	1.25	0.012	2.5	0.023

[1]Calculated as feed intake x % diet incorporation as dry matter x (1000 g/kg)/animal body weight (BW) (see Table 25)
[2]LOQs of 250 ppb (BLB2) or 500 ppb (VNT1, AMR3) in fresh weight tubers = 0.25 or 0.5 µg/g (FW) = 1.25 or 2.5 µg/g (DW) as tubers are 20% solids and 80% water, (OECD, 2002).
[3]Consumption = potato consumption (g (DW)/kg bw/d) x R-protein LOQ (1.25 or 2.5 µg/g (DW)) × (0.001 mg/µg).

B.1(c) information on whether any new protein has undergone any unexpected post-translational modification in the new host

Glycosylation is an important post-translational modification, and is known to influence protein folding, localisation and trafficking, protein solubility, antigenicity, biological activity, and half-life, as well as cell-cell interactions (Mazola et al., 2011).

Glycosylation of proteins has been suggested as a distinguishing structural feature of allergenic proteins (Altmann 2007). Post-translational modifications (PTMs) to the R-proteins or StmALS cannot be directly evaluated as protein expression levels for R-proteins are below the limits of detection and isolation of StmALS from the plant has not been successful. However, the structure of R-proteins and StmALS from BG25 were searched for the signal sequence required for transport to the endoplasmic reticulum, a pre-requisite for glycosylation (Pattison and Amtmann et al., 2009) and other glycosylation sites. No such signal peptides were found in the R-proteins or StmALS from BG25 using the public algorithms SignalP-6.0 (Teufel et al., 2022) and TargetP-2.0 (Almegro Armenteros et al., 2019).

Proteins without signal peptides are unlikely to be exposed to the N-glycosylation machinery and therefore may not be glycosylated (*in vivo*) even if they contain potential glycosylation motifs.

B.1(d) where any ORFs have been identified (in subparagraph A.3(c)(v) of this Guideline (3.5.1)), bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs

BG25 was developed by transforming the Russet Burbank potato variety with pSIM4363 using *Agrobacterium*. A modified potato acetolactate synthase gene (*StmAls*) was used as a marker for selection during transformation.

An analysis was completed using bioinformatic techniques (Goodman et al., 2008; Ladics et al., 2007; Terrat and Ducancel, 2013) to determine homology between known toxins or allergens and open reading frames (ORFs) introduced into event BG25 through transformation with pSIM4363 (Report A). A summary of the methods used to identify ORF sequences and evaluate the sequences against known allergens or toxins is provided in Table 27. Most of the ORFs contained in the pSIM4363 insert already exist as part of the potato genome, as the insert is derived from potato DNA.

Table 27. Overview of Analyses Using Bioinformatics

Analysis	Purpose	Approach
Start-to-stop ORF Analysis	Identify all open reading frames associated with the pSIM4363 insert, including junction regions.	Python script: systematically identify all ORFs (≥30 amino acids) located between a start codon and a stop codon where all six reading frames are considered.
Allergenicity Analysis	Confirm that known allergenic sequences have not been introduced through transformation.	COMPARE database maintained by the Health and Environmental Sciences Institute: identify any small regions of identity or larger regions of homology between ORFs and known allergens.
Toxicity Analysis	Confirm that sequences similar to known toxins have not been introduced through transformation.	BLAST (blastp) search: identify any ORFs with homology to proteins with “toxin” in its UniProtKB annotation.

The ORFs identified from the pSIM4363 insert were assessed for homology to potential toxins and known allergens. The ORFs identified were from the pSIM4363 insert only, there were no junction ORFs identified in BG25. The sequences associated with each ORF are shown in Appendix A of Report A. The insert contained:

- **Insert sequences:** The insert contains R-genes from wild Solanum species for protection against late blight, a PVY sequence that targets the coat protein region of the PVY genome using RNAi for PVY protection, and sequences for down regulation of potato polyphenol oxidase (PPO) and potato vacuolar invertase transcripts using RNAi. The pSIM4363 insert also contains the acetolactate synthase gene (*StmAls*), which is used as a selection marker during transformation.

Identification of ORFs Associated with pSIM4363

All ORFs contained within the BG25 insert were identified, and no ORFs crossed between the insert and adjacent flanking sequences (junction ORFs). An ORF was defined as the contiguous, ≥30 amino acid sequence between a start and subsequent in-frame stop codon. A novel open reading frame was defined as not otherwise existing in the potato genome. Nucleotide sequences can be translated in three reading frames from two directions. All six reading frames within the pSIM4363 insert and flanking regions were analyzed for ORFs (shown schematically in Figure 35).

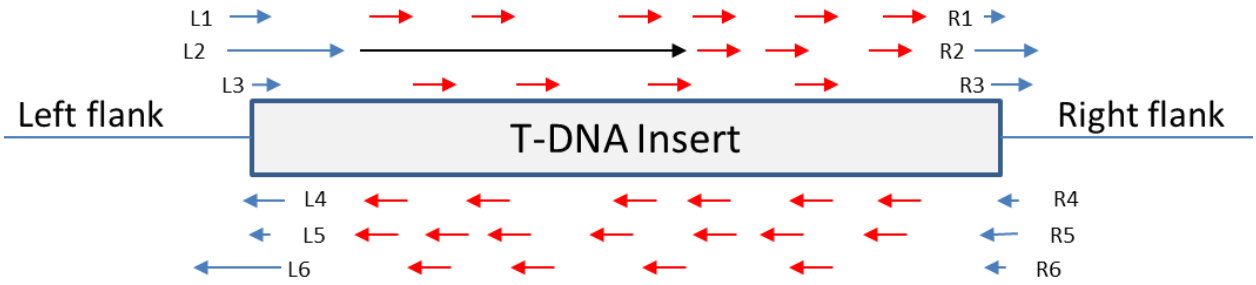


Figure 35. Complete ORF Analysis Scheme

A representative T-DNA insertion site in the plant genome. All ORFs ≥30 amino acids, contained within the DNA insert (red lines), including an introduced protein coding region (**black** line) or overlapping the junctions between the insert and plant genome (blue lines), were identified, and used in subsequent analyses. All lines are representative and do not indicate actual ORFs.

Allergenicity Searches

The allergen search was performed against the COMPARE database maintained by the Health and Environmental Sciences Institute (HESI; http://db.comparedatabase.org/). The COMPARE database (version 2023, released January 26, 2023) contained 2,631 protein sequences.

The FASTA algorithm was used to align and calculate sequence identity between input and database entries (Pearson and Lipman, 1988). The search methods used are supported by published guidance for protein allergenicity prediction in food products (Goodman et al., 2008; Ladics, 2008; Ladics and Selgrade, 2009).

Full length sequence search

A full-length search identified matches between full-length queries and the allergens contained in the COMPARE database. Similarity was determined using (1) sequence identity greater than 50% between the

query protein and database entry, and (2) an E-value of less than 10^{-4} as criteria. Lower E-values (e.g., $<10^{-4}$) indicate a high degree of sequence similarity and a lower likelihood that hits are due to chance.

80-mer Sliding Window Search

The 80-mer sliding window search identifies localised regions of similarity between the ORFs and known allergens by comparing all contiguous 80 amino acid sequences within an ORF to sequences in the COMPARE database. Matches were defined as sequences having greater than 35% identity to known allergens and an E-value of 10 was used to filter alignments containing large sequence gaps. Sequences in the databases with less than 35% identity are not considered likely candidates for cross-reactivity (Ladics, 2008; Ladics and Selgrade, 2009) and are therefore not included in the search output.

8-mer Exact Match Search

The 8-mer exact-match search identifies small, localized regions consisting of eight amino acids of identity between the queried ORF sequence and known or suspected allergens in the COMPARE database. Although the occurrence of matching 8-mer sequences can be used to assess the potential for cross-reactivity, the Food Allergy Research and Research Program warns that the 8-mer search can also lead to identification of false positive results, and suggests caution be used when interpreting findings based on this search alone (FARRP, 2023). There is presently no scientific rationale for a small region of sequence with high identity to allergens being a safety concern without substantiation by other search methods (Goodman et al., 2008).

Assessment of ORFs for allergen similarity

There were no allergen matches to the AMR3, BLB2, VNT1, and StmALS protein sequences or the PVY and PPO sequences using the full-length, 80-mer, or 8-mer searches.

Two ORFs associated with the VINV sequence matched with a minor allergen from tomato, beta-fructofuranosidase precursor-vacuolar invertase (Table 28). The ORFs are complementary because of the VInv inverted repeat and have similar sequences. This similarity results in a common match being identified from the bioinformatic analysis.

Table 28. Allergen similarity of ORFs in event BG25

ORF	Query Match	Organism	Accession
>ORF96 MLSWQRTAYHFQPQKNWMNDPNGPLYHKGWYHLF YQYNPDSAIWGNITWGHAVSKDLIHWLYLPFAMVPD QWYDINGVWTGSALEIIVTDLFLNNNFNEIKQQS >ORF101 MLSWQRTAYHFQPQKNWMNDPNGPLYHKGWYHLF YQYNPDSAIWGNITWGHAVSKDLIHWLYLPFAMVPD QWYDINGVWTGSALENNCN	Minor allergen beta-fructofuranosidase precursor – vacuolar invertase (Foetisch et al., 2003)	*Solanum lycopersicum*	AAL75449, AAL75450

Although similarity was identified between the ORFs and the tomato allergen, the match is not a safety concern. This is because the ORF sequences are like the vacuolar invertase protein sequence already expressed in potato. Therefore, BG25 potatoes are no more likely than conventional potatoes to cause an allergic reaction in individuals sensitive to the tomato vacuolar invertase. Additionally, translation of these

ORFs into proteins is unlikely because the transcripts from the inverted repeat in pSIM4363 form dsRNA that are processed by the RNAi machinery into siRNA. This sequence is not translated into protein. The siRNA generated from the VInv inverted repeat down regulate expression of potato vacuolar invertase resulting in reduced amounts of the enzyme in BG25 compared to conventional potatoes and eliminating any associated allergenicity concerns.

Toxicity Searches

Methods familiar to regulators and established in some countries include an approach modelled after the allergenicity studies where bioinformatics is used to inform on the potential of sequence similarity between protein sequences and known toxins.

Toxin similarity searches against the UniProtKB database filtered with the keyword "toxin" were performed using BLAST (blastp; https://blast.ncbi.nlm.nih.gov/Blast.cgi). An E-value cutoff of 10^{-2} was used as a criterion for similarity. Searches were performed on January 30, 2023.

Proteins that are not actual toxins may be identified as matches using the filtered UniProtKB toxin database because of how it was generated using a text-based, keyword search. The database contains the sequences of actual toxins, but also contains sequences of proteins that are not toxins, but that contain the keyword "toxin" in the accession annotation. This ensures that potential toxins are not excluded from the search. However, the database also contains proteins that may function in the synthesis of toxins, may interact with toxins, may be involved in toxin-induced defense responses or be antitoxins, or are non-toxic proteins from organisms that produce other known toxins. Only matches to actual known toxins indicate a potential safety concern. For all others, a brief explanation and rationale for safety is provided.

Assessment of ORFs for toxin similarity

R-protein Results

No toxin matches were identified for AMR3, BLB2, or VNT1. Some proteins that align with AMR3, BLB2, and VNT1 (Table 29) were identified because they contain the keyword "toxin" in their accession records and were therefore included in the database. None of the identified proteins are actual toxins. The matched proteins function like R-proteins, protecting plants against toxins or toxin-producing pathogens.

Table 29. Summary of toxin results for AMR3, BLB2 and VNT1

Query Match	Organism	Accession
LOV1[1]	*Arabidopsis thaliana*	A7XGN8, A9QGV6
RP3-like[1]	*Sorghum bicolor*	B3VTC2, B3VTC1, B3VTB7
RNA N-glycosidase[1]	*Hordeum vulgare, Triticum aestivum*	A0A3B6FZY2, A0A287K383, A0A3B6SRU1
Glutathione transferase[1]	*Brassica cretica*	A0A8S9FSH5
TIR domain-containing protein[2]	*Salix viminalis*	A0A6N2KQB0

[1]Matches were observed for AMR3, BLB2, and VNT1.
[2]Matches were observed for BLB2 and VNT1.

Proteins Matching AMR3, BLB2, and VNT1

LOV1 and RP3-like are R-protein homologs that function in host sensitivity to fungal pathogens through recognition of effector molecules (i.e., victorin, Pc toxin). LOV1 confers susceptibility to the fungus *Cochliobolus victoriae* by conditioning victorin-dependent induction of defense-associated proteins. Victorin

is a toxin synthesised by *C. victoriae*. RP3-like confers resistance to Pc toxin. A literature review confirmed that LOV1 and RP3-like proteins are not toxins or substances with toxic properties (Lorang et al., 2007; Nagy and Bennetzen, 2008; Walton, 1996).

RNA N-glycosidases function as ribosome-inactivating proteins. RNA N-glycosidases are commonly found in plants and are involved in defense responses against fungal pathogens (Peumans et al., 2001; Stirpe, 2013). Aligning sequences of these RNA N-glycosidases with R-proteins demonstrates that they both contain a nucleotide binding site (NBS) domain. R-proteins have not been associated with ribosome inactivation and are not considered toxic to humans or animals.

Glutathione transferase (GST) is not a toxin (Allocati et al., 2018). This GST enzyme functions in a cellular detoxification system that metabolises various toxic compounds, e.g., by conjugation with glutathione, which are then transported out of the cell. The accession is included in the database because it contains the keyword "toxin" due to the enzyme's role in detoxification.

Proteins Matching Only BLB2 and VNT1

A TIR domain-containing protein (A0A6N2KQB0) from *Salix viminalis* aligned with BLB2 and VNT1. The protein binds nucleotides, like R-proteins, and contains a nucleotide binding (NBS) domain, the reason it aligned with BLB2 and VNT1. The accession has a gene ontology annotation of "defense response to fungus", which is like the function of BLB2 and VNT1. The protein also contains a knottin fold, which is a common domain found in plant lectins, proteinase inhibitors, gamma-thionins, and defensins (the reason it is included in the database). The BLB2 and VNT1 proteins do not contain the knottin fold domain and are not considered toxins.

Acetolactate Synthase and PVY results

No toxin matches were identified for StmALS or the PVY-CP protein sequences. In the BG25 insert, the promoter of the *polyubiquitin 7* (*Ubi7*) gene (GenBank Accession No. U26831) includes a ubiquitin monomer. This promoter was used to drive expression of the StmALS coding sequence and the PVY-CP inverted repeat. Two ORFs from the BG25 insert, ORF 9 and ORF 117 (Figure 36), have minimal potential to form ubiquitin-StmALS fusions, because the ubiquitin monomer is cleaved post-translation (Garbarinao et al., 1992), or ubiquitin-PVY-CP fusions, because the inverted repeat sequence mRNA rapidly folds into dsRNA and is cut up by the RNA interference system.

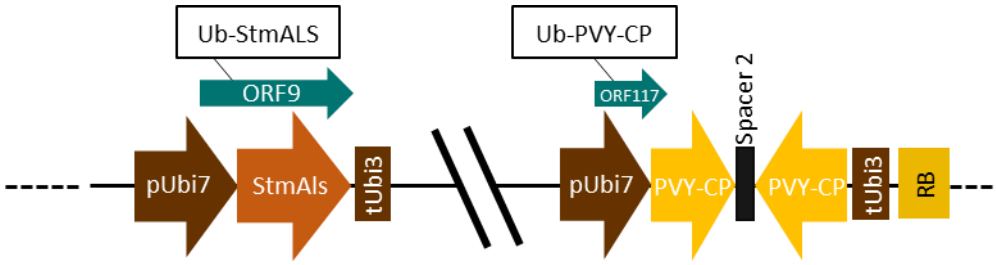


Figure 36. Location of pUbi7-StmALS and pUbi7-PVY-CP ORFs in the BG25 Insert

The structures are shown for the StmALS cassette (on the left border end of the T-DNA) and PVY-CP cassette (adjacent to the T-DNA right border) in the BG25 insert. The promoter of the *polyubiquitin 7* (pUbi7) gene was used to drive expression of the StmALS coding sequence and the PVY-CP inverted repeat. The locations of ORF9 (pUbi7-StmALS) and ORF117 (pUbi7-PVY-CP) are shown (green arrows).

None of the matches to ORF9 or ORF117 (Table 30) are actual toxins and are all false positive hits due to these sequences having similarity to StmALS and/or ubiquitin. They are included in the database because they contain the keyword "toxin" in their accession records (Table 30), and due to how the database was generated using a text-based, keyword search. The matched proteins are acetolactate synthase or similar homologs from microbial species, or related to ubiquitin, which are both commonly expressed in microorganisms, including those that may produce toxins but these homologs of acetolactate synthase and ubiquitin are not themselves toxins.

VINV Results

Some proteins that are homologs of sucrose-degrading enzymes and ubiquitously expressed in bacteria, both pathogenic and non-pathogenic strains, align with potato vacuolar invertase (Table 31). The accession records for these proteins contained the keyword "toxin" due to the pathogenicity of the host organism and not the sucrose degrading activity of the proteins. The pSIM4363 insert contains an inverted repeat with sequence from the potato vacuolar invertase gene.

The alignment of ORFs containing sequence from the vacuolar invertase gene identified non-toxic proteins whose annotation includes the keyword "toxin". These proteins are broadly expressed in pathogenic and non-pathogenic organisms but are not related to toxicity. None of these matches pose a safety concern. Accession records for proteins are often annotated with the keyword "toxin" because of the source organism's toxicity, and the search criteria used to generate the toxin database (see Materials and Methods of Report A) can result in the identification of false-positives. No toxin matches were identified for any other ORF associated with the pSIM4363 insert in BG25.

Conclusions from allergenicity and toxicity potential

Bioinformatic methods were used to assess the allergen and toxin potential of ORFs associated with the pSIM4363 insert in BG25. There were no safety concerns identified in terms of allergenicity or toxicity potential.

Table 30. Summary of toxin results for pUbi7-StmALS and pUbi7-PVY

ORF[1]	Query Match	Source Organism	Accession
>ORF9 **MNLQMQIFVKTLTGKTITLEVESSDTIDNVKAKIQDKEGIPPDQQRLIF AGKQLEDGRTLADYNIQKESTLHLVLRLRGGGSM**AAAASPSPCFSKTL PPSSSKSSTILPRSTFPFHNHPQKASPLHLTHTHHHRRGFAVSNVVISTTT HNDVSEPETFVSRFAPDEPRKGCDVLVEALEREGVTDVFAYPGGASMEI HQALTRSNIIRNVLPRHEQGGVFAAEGYARATGFPGVCIATSGPGATNL VSGLADALLDSIPIVAITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVM DVEDIPRVVREAFFLAKSGRPGPVLIDVPKDIQQQLVIPNWDQPMRLP GYMSRLPKLPNEMLLEQIIRLISESKKPVLYVGGGCLQSSEELRRFVELTGI PVASTLMGLGAFPTGDELSLQMLGMHGTVYANYAVDGSDLLLAFGVR FDDRVTGKLEAFASRAKIVHIDIDSAEIGKNKQPHVSICADIKLALQGLNSI LEGKEGKLKLDFSAWRQELTEQKVKYPLSFKTFGEAIPPQYAIQVLDELT NGNAIISTGVGQHQMWAAQYYKYKKPHQWLTSGGLGAMGFGLPAAI GAAVGRPGEIVVDIDGDGSFIMNVQELATIKVENLPVKIMLLNNQHLG MVVQLEDRFYKANRAHTYLGPANEEEIFPNMLKFAEACGVPAARVSHR DDLRAAIQKMLDTPGPYLLDVIVPHQEHVLPMIPIGGAFKDVITEGDGR RSY >ORF117 **MNLQMQIFVKTLTGKTITLEVESSDTIDNVKAKIQDKEGIPPDQQRLIF AGKQLEDGRTLADYNIQKESTLHLVLRLRGGGSM**KRYNLHTA	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase	*Bacillus* sp.	A0A0F5RIA4, A0A7T3JEH9
		Cronobacter sakazakii	A0A7V7RAZ8, A0A6L3RV70
		Escherichia coli	A0A8H0IT73, A0A0A1A7K6, A0A6D1AZQ7
		Vibrio cholerae	A0A0H5UKL7
	3D-(3,5/4)-trihydroxycyclohexane-1,2-dione hydrolase	*Bacillus thuringiensis*	A0A7T3JQE4
	(3, 5/4)-trihydroxycyclohexane-1,2-dione acylhydrolase (Decyclizing)	*Citrobacter freundii*	A0A7X8S2W7
		Cronobacter sakazakii	A0A2S9UIT0
		Escherichia coli	A0A6D0J210
		Paraburkholderia edwinii	A0A8F9G1V3
	Acetolactate synthase, acetolactate synthase 2/3 catalytic subunit, acetolactate large subunit	*Bacillus* sp.	A0A0F5RZ67, A0A0F5RU87, A0A0F5RL81, A0A0F5RII6
		Bacillus thuringiensis	A0A7T3JK51, A0A7T3JL35, A0A7T3JLZ9
		Brevibacillus laterosporus	A0A518V7G5, A0A502HXQ1
		Citrobacter freundii	A0A7X8P299, A0A7X8NXS0, A0A256E8T2
		Cronobacter sakazakii	A0A6L3P7Z9, A0A2S9UE64, A0A6L3PG31, A0A2S9UAL8, A0A7V7REC8
		Escherichia coli	A0A1Q4P8G2, C3SKW8, A0A2A6DR48, A0A0L7ALV4, K4Y7S6, A0A0F3WGS1, A0A0H0L9E8, A0A0E0Y7L4, A0A093DRN0, A0A0C2EG85, J7R7Z3, A0A5F1T0D6, A0A0A0GPP2, A0A0L7AIM2, A0A0G9FZ05, C3SLY2, A0A0A6VBN1, A0A037YIK2, A0A4Y9WVM1, A0A0E0XTN6, J7QIM0, K4VZK4, A0A0F3WKG6, A0A478G5I1, A0A0B0VNN7, A0A0J2EBM4, A0A1V3W797, A0A085P6E0, A0A0C2EF31, K4Y0H9, A0A0E0Y7I9, A0A066RE04, A0A0K3JAS5, A0A1Q4PT50, A0A0L7ALG4, A0A4Z0TKY8, A0A862ZP53, A0A8E2M5S9, A0A2A6Q4I3,

ORF[1]	Query Match	Source Organism	Accession
			A0A1L4J346, A0A3W4GNY2, A0A8H1DLV7, A0A862ZNX3, A0A2A3VQH4, A0A8H0PZY7, A0A2J5Z2U3, A0A8F9G1X6, A0A862ZCD0, A0A8H0F9Z3, A0A8H0EVP2, A0A787Q9I0, A0A8H0NPL8, A0A8H0EXB5
		Lactococcus lactis	A0A4V0YXX6, A0A089ZDN5
		Shigella boydii	A0A376V2A7, A0A4T7HCB2
		Shigella dysenteriae	A0A2S8DCF1, A0A2S8DG25, A0A2S8DEI1
		Paraburkholderia edwinii	A0A8F9CB57, A0A8F9G0A0
		Pectobacterium carotovorum	A0A0N9N073
		Spiroplasma poulsonii	A0A3S0SED8
		Staphylococcus aureus	A0A6F9YRN6
		Vibrio cholerae	A0A0F4FBK0, A0A0H6USU4, A0A085S1P0
	Alpha-keto acid decarboxylase	*Bacillus thuringiensis*	A0A7T3JIQ8
		Lactococcus lactis	A0A8I2H601
	Benzoylformate decarboxylase	*Pectobacterium carotovorum*	A0A0N9NDM5
	E3 ubiquitin-protein ligase parkin	*Crotalus adamanteus*	A0A0F7Z269[2]
	Gcl protein	*Escherichia coli*	Q1HMD0, Q1HMC3, Q1HMC6, Q1HMB3, Q1HMB7, Q1HMB6, Q1HMB4, Q1HMC0, Q1HMC5
	Glyoxylate carboligase	*Escherichia coli*	A0A0L7AGC9, A0A4D3BMR1, A0A0E0Y4A4, A0A6D0XKC9, K4XGX9, A0A0F3WKS5, A0A0B1JEY6, A0A5F1T1Q8, A0A8H1NCZ2, A0A4Q9CSJ7, A0A8H0SUK4, A0A093DK16, A0A1L4SYS2, C3TKV2, A0A862ZCS1
		Paraburkholderia edwinii	A0A8F9C5Z6
		Shigella dysenteriae	A0A2S8DFY0
	Indolepyruvte decarboxylase, Indole-3-pyruvate decarboxylase	*Bacillus* sp.	A0A0F5RUM2
		Citrobacter freundii	A0A7X8S4X2
		Cronobacter sakazakii	A0A6L3PDM0, A0A7V7RBV4

ORF[1]	Query Match	Source Organism	Accession
		Pectobacterium carotovorum	A0A0N9N097
	ORF11 Protein	*Staphylococcus aureus*	Q8GAX2
	Oxalyl-CoA decarboxylase, oxyalyl-CoA decarboxylase, ThDp-dependent	*Escherichia coli*	A0A0L7AN87, A0A0P7MSF1, A0A0B1KXE7, C3T1I2, K4VRZ9, J7Q8P7, A0A0E0XW73, A0A1J0ZCU7, A0A7R6ZJU0, A0A862ZII9, A0A8H0IP98
		Paraburkholderia edwinii	A0A8F9G2Q6
		Shigella dysenteriae	A0A2S8D879
	NEDD8	*Anguilla anguilla*	A0A0E9WQ96[2], A0A0E9XI78[2]
	Polyubiquitin-B	*Centruroides hentzi*	A0A2I9LPH5[2]
	Pyruvate oxidase	*Bacillus* sp.	A0A0F5RW09, A0A7T3JJ76
		Escherichia coli	A0A8E2MC68
		Lactococcus lactis	A0A4V0YXJ7
	Pyruvate dehydrogenase	*Escherichia coli*	C3TGT7, A0A0F3TZP7, A0A2U9KN90, A0A0H0RV87, A0A478G2Y9, J7R2L8, K4XA94, A0A0E0Y517, A0A093D8T3, A0A4Y9X5R5, A0A8B5KKX2, A0A862ZED6
		Citrobacter freundii	A0A427LFI7
		Cronobacter sakazakii	A0A2S9UEJ2, A0A7V7PVF1
	RING-type E3 ubiquitin transferase	*Crotalus adamanteus*	)A0F7Z391[2]
	ribosomal protein S27a, 40S ribosomal protein S30	*Anguilla anguilla*	)A0E9WPB0[2], A0A0E9WPF1[2], A0A0E9WIH1[2]
		Crotalus adamanteus	S0S4[2], J3S4E0[2]
		Centruroides hentzi	A0A2I9LPN2[2], A0A2I9LPP4[3]
		Hadrurus spadix	A0A1W7R9P2[2]
		Micrurus fulvius	U3F6K5[2], U3FWY9[2]
	60S ribosomal protein	*Centruroides hentzi*	A0A2I9LPM5[2]
	ribosomal protein L40	*Anguilla anguilla*	A0A0E9WI12[2]
		Crotalus adamanteus	J3S9G5[2]
		Hadrurus spadix	A0A1W7R9R2[2], A0A1W7R9R4[2]
		Micrurus fulvius	U3FBE5[2]

ORF[1]	Query Match	Source Organism	Accession
	Sulfoacetaldehyde acetyltransferase	*Paraburkholderia edwinii*	A0A8F9C039
	Thiamine pyrophosphate-binding protein	*Brevibacillus laterosporus*	A0A502I6B0, A0A518V6B7
		Cronobacter sakazakii	A0A6L3S0E0, A0A7V7URN5
		Paraburkholderia edwinii	A0A8F9C5T9, A0A8F9BYS9, A0A8F9BV93, A0A8F9C722, A0A8F9C7J9
	quinone-dependent pyruvate dehydrogenase	*Escherichia coli*	A0A8H1ISS8, A0A8H0NGV9, A0A8H0LAQ9, A0A8H0EPY5
		Paraburkholderia edwinii	A0A8F9C0K0
		Shigella dysenteriae	A0A2S8D5Q1
	Ubiquitin C	*Crotalus adamanteus*	A0A0F7Z3D0[2]
	Ubiquitin-like domain containing protein	*Anguilla anguilla*	A0A0E9W2E1[2], A0A0E9XAF2[2], A0A0E9WUH5[2], A0A0E9WWB5[2], A0A0E9WS89[2], A0A0E9Y2L5[2]
		Crotalus adamanteus	A0A0F7Z3D6[2], A0A0F7Z8H0[2]
	Uncharacterised protein	*Paraburkholderia edwinii*	A0A8F9G4K8
	excision repair protein RAD23	*Crotalus adamanteus*	J3S9D2[2], A0A0F7Z214[2]
		Hadrurus spadix	A0A1W7R9V3[2]
		Micrurus fulvius	A0A0F7YZU5[2]

[1]Bold sequence indicates region of identity between ORFs derived from the pUbi7 sequence.
[2]Toxin hits matching to ORF9 and ORF117.
[3]Toxin hit matching to ORF117 only.

Table 31.Summary of Toxin Results for VINV

ORF	Query Match	Organism	Accession
>ORF96 MLSWQRTAYHFQPQKNWMNDPNGPLYHKGWYHLFYQYNPDSAIWGNITWGHAVSKDLIHWLYLPFAMVPDQWYDINGVWTGSALEIIVTDLFLNNNFNEIKQQS >ORF101 MLSWQRTAYHFQPQKNWMNDPNGPLYHKGWYHLFYQYNPDSAIWGNITWGHAVSKDLIHWLYLPFAMVPDQWYDINGVWTGSALENNCN	Glycosyl Hydrolase Family 32, Glycoside hydrolase Family 32, Sucrose-6-Phosphate Hydrolase	*Escherichia coli*	A0A0L7AN64, A0A0K4J7Y8, A0A0J2BDG4, K4XMF7, A0A0E0XW81, O86076, A0A0V7X3J6, A0A854K415, A0A8E2MC43, A0A8H0P7Y7, A0A8H0M6U4
	Glycosyl hydrolase family 32	*Sinorhizobium americanum*	A0A4R2B3Q6
	Sucrose-6-phsophate hydrolase	*Cronobacter sakazakii*	A0A6L3RQB6, A0A7V7RCM5
	Sucrose-6-phsophate hydrolase	*Lactococcus lactis*	A0A8I2H7T9
	Sucrose-6-phosphate hydrolase	*Citrobacter freundii*	A0A7X8NXI9, A0A0D7KZS1
	Sucrose-6-phosphate hydrolase	*Vibrio cholerae*	A0A0H7HPC8

B.2. New Proteins

B.2 (a) Information on potential toxicity

The pSIM4363 T-DNA contains cassettes for the expression of four genes. A detailed description of the history and mode of action of the genes can be found in Section B.1(b).

B.2(a)(i) a bioinformatic comparison of the amino acid sequence of each of the new proteins to known protein toxins and anti-nutrients (e.g. protease inhibitors, lectins)

Results of bioinformatic analyses comparing the amino acid sequence of VNT1, AMR3, BLB2 and StmALS to proteins identified as "toxins" from the NCBI protein databases are presented in:

- Section A.2(a)(i) and
- Section B.1(d)

No toxin matches were identified for AMR3, BLB2, or VNT1. Some proteins that align with AMR3, BLB2, and VNT1 (Table 29) were identified because they contain the keyword "toxin" in their accession records and were therefore included in the database. None of the identified proteins are actual toxins. The matched proteins function like R-proteins, protecting plants against toxins or toxin-producing pathogens.

As resistance proteins exist in most plants including food crops (McHale et al. 2006) and to date, have not been shown to have adverse effects after consumption of food or feed, it can be concluded that the four proteins are not homologous to any biologically relevant toxins. Bio-informatic analysis demonstrated no relevant similarity between the putative peptides and known toxins.

B.2(a)(ii) information on the stability of the protein to proteolysis in appropriate gastrointestinal model systems

The R-proteins are expressed in extremely low concentrations. Therefore, isolation of sufficient quantities of protein from BG25 potatoes was not feasible. Furthermore, protein stability analysis using *E. coli* was also not possible as *E. coli* expressed R-proteins are insoluble and could only be recovered from bacterial inclusion bodies. As such, to confirm the digestibility of R-proteins, potential cleavage sites were investigated using the amino acid sequences of each of the R-proteins and the PeptideCutter tool in the ExPASy Proteomics Site (Gasteiger et al., 2005).

VNT1 digestibility

VNT1 digestibility has previously been assessed by FSANZ (see A1139). VNT1 has multiple cleavage sites for pepsin (214 sites at pH 1.3 and 251 sites at pH >2), trypsin (111 sites), chymotrypsin (69 high-specificity sites, 217 low-specificity sites) and endopeptidases (166 sites). On this basis, VNT1 is considered likely to be as susceptible to digestion as most dietary proteins.

AMR3 digestibility

AMR3 has multiple cleavage sites for pepsin (191 sites at pH 1.3 and 236 sites at pH >2), trypsin (98 sites), chymotrypsin (71 high-specificity sites, 200 low-specificity sites) and endopeptidases (186 sites). On this basis, AMR3 is considered likely to be as susceptible to digestion as most dietary proteins.

BLB2 digestibility

BLB2 has multiple cleavage sites for pepsin (350 sites at pH 1.3 and 409 sites at pH >2), trypsin (142 sites), chymotrypsin (95 high-specificity sites, 341 low-specificity sites) and endopeptidases (331 sites). On this basis, BLB2 is considered likely to be as susceptible to digestion as most dietary proteins.

StmALS digestibility

StmALS has multiple cleavage sites for pepsin (121 sites at pH 1.3 and 144 sites at pH >2), trypsin (59 sites), chymotrypsin (40 high-specificity sites, 130 low-specificity sites) and endopeptidases (104 sites). This is as compared to the native StALS protein which also has multiple cleavage sites for pepsin (119 sites at pH 1.3 and 144 sites at pH >2), trypsin (59 sites), chymotrypsin (41 high-specificity sites, 130 low-specificity sites) and endopeptidases (104 sites).

On this basis, StmALS is considered likely to be as susceptible to digestion as the StALS protein and most other dietary proteins.

B.2(a)(iii) an animal toxicity study if the bioinformatic comparison and biochemical studies indicate either a relationship with known protein toxins/anti-nutrients or resistance to proteolysis.

The bioinformatic analyses did not indicate any relationships with known protein toxins/anti-nutrients and does not indicate any resistance to proteolysis. Therefore, no animal toxicity studies were undertaken.

B.2 (b) Information on potential allergenicity

Details of the potential allergenicity of the VNT1, AMR3, BLB2 and StmALS proteins as well as other putative ORFs are presented in the following Sections:

- Section A.2(a)(i) and
- Section B.1(d)

The bio-informatic analysis demonstrated no relevant similarity between the putative peptides and known allergens. Additional information is provided below.

B.2(b)(i) Source of the new protein

VNT1 protein identity

The *Rpi-vnt1* gene (accession: FJ423044) is one of three isoforms identified in the wild species *S. venturii*. The *Rpi-vnt1* gene sequence is identical to the *Rpi-phu1* gene from the related species, *S. phureja,* and a homolog of the Tm-2^2 tomato mosaic virus (ToMV) disease R-genes in tomato (Foster et al., 2009; Śliwka et al., 2013). The gene encodes the 891 amino acid R-protein designated VNT1 (Figure 37).

The recognition of pathogen-secreted effectors (e.g. Avr-vnt1) by R-proteins is one of the most studied mechanisms in plant defense response (Panstruga et al., 2009). R-proteins such as VNT1 are signal transduction ATPases with homologs found in all domains of life (Leipe et al., 2004). Most known disease resistance R-proteins contain a nucleotide-binding site (NBS or NB) and leucine-rich repeat (LRR) domain (Lozano et al., 2012). Two classes of R-proteins with distinct motifs have been identified that contain the NBS/NB-LRR domains:

- N-terminal toll/interleukin 1 receptor (TIR)(TIR-NB-LRR)

- N-terminal coiled-coil (CC-NB-LRR).

The *Rpi-Vnt1* gene and other R-genes that provide protection against *P. infestans* (known as *Rpi* genes) typically encode immune receptor proteins of the coiled coil, nucleotide binding, leucine rich repeat (CC-NB-LRR) class of intracellular plant proteins (Vleeshouwers et al., 2011).

```
001 MNYCVYKTWA VDSYFPFLIL TFRKKKFNEK LKEMAEILLT AVINKSIEIA
051 GNVLFQEGTR LYWLKEDIDW LQREMRHIRS YVDNAKAKEV GGDSRVKNLL
101 KDIQQLAGDV EDLLDEFLPK IQQSNKFICC LKTVSFADEF AMEIEKIKRR
151 VADIDRVRTT YSITDTSNNN DDCIPLDRRR LFLHADETEV IGLEDDFNTL
201 QAKLLDHDLP YGVVSIVGMP GLGKTTLAKK LYRHVCHQFE CSGLVYVSQQ
251 PRAGEILHDI AKQVGLTEEE RKENLENNLR SLLKIKRYVI LLDDIWDVEI
301 WDDLKLVLPE CDSKIGSRII ITSRNSNVGR YIGGDFSIHV LQPLDSEKSF
351 ELFTKKIFNF VNDNWANASP DLVNIGRCIV ERCGGIPLAI VVTAGMLRAR
401 GRTEHAWNRV LESMAHKIQD GCGKVLALSY NDLPIALRPC FLYFGLYPED
451 HEIRAFDLTN MWIAEKLIVV NTGNGREAES LADDVLNDLV SRNLIQVAKR
501 TYDGRISSCR IHDLLHSLCV DLAKESNFFH TEHNAFGDPS NVARVRRITF
551 YSDDNAMNEF FHLNPKPMKL RSLFCFTKDR CIFSQMAHLN FKLLQVLVVV
601 MSQKGYQHVT FPKKIGNMSC LRYVRLEGAI RVKLPNSIVK LKCLETLDIF
651 HSSSKLPFGV WESKILRHLC YTEECYCVSF ASPFCRIMPP NNLQTLMWVD
701 DKFCEPRLLH RLINLRTLCI MDVSGSTIKI LSALSPVPRA LEVLKLRFFK
751 NTSEQINLSS HPNIVELGLV GFSAMLLNIE AFPPNLVKLN LVGLMVDGHL
801 LAVLKKLPKL RILILLWCRH DAEKMDLSGD SFPQLEVLYI EDAQGLSEVT
851 CMDDMSMPKL KKLFLVQGPN ISPISLRVSE RLAKLRISQV L
```

Figure 37. Amino Acid Sequence of VNT1

The *Rpi-vnt1* gene encodes an 891 amino acid R-protein (102 Kda). Individual domains are highlighted, coiled-coil (CC) (green), nucleotide binding (NBS) (blue), and leucine rich repeat (LRR) (brown).

AMR3 protein identity

The AMR3 protein (Accession No.: AMY98955), encoded by the *Rpi-amr3* gene (Accession No.: KT373889), has a typical coiled coil-nucleotide binding -leucine-rich repeat (CC-NBS-LRR) domain structure and 887 amino acids (Figure 38). The *S. americanum Rpi-amr3* gene was cloned using R-gene enrichment sequencing (RenSeq) combined with single-molecule real-time (SMRT) sequencing using Illumina technology (Witek et al., 2016).

Solanum americanum is a wild Solanum species from the Morelloid clade, which is commonly referred to as the black nightshades. The native range for this plant includes North, Central, and South America, Melanesia (Tonga, Fiji, Vanuatu, the Solomon Islands, Papua New Guinea), New Guinea, and Australia (NBN Atlas, 2021).

Black nightshades, including *S. americanum*, are found naturally growing on disturbed ground, but are also cultivated by humans for medicinal and food use (Särkinen et al., 2018). *Solanum americanum* does not produce tubers, but both leaves and berries of *S. americanum* are consumed by humans.

Solanum americanum is a source of R-genes for late blight protection and has been shown to be naturally resistant to *P. infestans* (Witek et al., 2016).

```
001 MAAYSAVISL LQTLIDQQNI SELFHGHTAQ TLDSLHTTAE YFQHVLENIT
051 RFDSEKIKSL EEKIRVVVSY AEDVVAMKIS QIIIGSSWTF GILQHQDLLP
101 LVEKMDTTKK QVMDILSHDD DQILELTAGD SLIGTSSTTY PMLEDDIVQG
151 IDDDLEIIVK RLTGPPRDLD VVTITGMGGI GKTTLARKAY DHLTIRYHFD
201 ILVWITISQE FRCRNVLLEA LHCISKSTDI VNTKDYDKKD DNELADIVQK
251 KLKGPRYLVV VDDIWSRDVW DSIRGIFPNY NNGSRILLTT RENEVAMYAN
301 TCSPHEMSLL SLENGWRLLC DKVFGPKHDH PPELEEIGKE IVEKCQGLPL
351 TISVIAGHVS KMPRTLECWK DVARTLSEII SSHPDNCLGV LGLSYHHLPN
401 HLKPCFLSMS SFPEDFQVET RRLIYLWIAE GFIRTCENGK SLEEVAVDYL
451 EDLISRNLIQ ARKRRFNGEI KACGIHDLLR EFCLIEAEIT KHMHVERTYP
501 TLPTQKNNVR RFSFQTKFYS VDDCNKLLPP VARSIYFFSQ LDLPVVPYKR
551 YLRCCLPIHR DDRIIHDFYS RFNLLRVLVI SKTNEYFESF PLVITKLFHL
601 RYLQVRFLGD IPESISNLQN LQTLICSGGT LPGKIWMMKN LRYISIIGNK
651 VTYLPSPRTE SLVNLEEFSV LCYRSCTKEV ISGIPNLKRL TIDVLSSINN
701 YFPNGLIDMS SLTKLEAFKC NRCLYSNFNS SVIPTSLKDF VFPTSLKRLS
751 LNYYASHFFW EEISSTIIML PNLEELKLKD CRSDEYDEWS LSDKDKFKSL
801 KLLVLTDIFF DRWEATSDNF PNLKRLVLNK CDLEIPSDFG EICTLESIEL
851 HDCSTSAEDS AREIEQEQEE MGNNILKVYI HGSRSKF
```

Figure 38. Amino acid sequence of the AMR3 protein

The amino acid sequence of the AMR3 protein from pSIM4363. Individual domains are highlighted, coiled-coil (CC) (green), nucleotide binding (NBS) (blue), and leucine rich repeat (LRR) (red).

BLB2 protein identity

The pSIM4363 T-DNA contains the *Rpi-blb2* gene (3,890 bp; 1,267 amino acids) originating from the wild Solanum species *S. bulbocastanum.* The gene is expressed under the native *Rpi-blb2* promoter and terminator, pBlb2 and tBlb2, respectively.

The BLB2 protein (Accession No.: AAZ95005), encoded by the *Rpi-blb2* gene (Accession No.: DQ122125), has a typical CC-NBS-LRR domain structure and 1,267 amino acids (Figure 39). The *Rpi-blb2* gene was cloned by mapping late blight protection in a tetraploid hybrid designated ABPT (van der Vossen et al., 2005). ABPT stands for the four wild species (*S. acaule*, *S. bulbocastanum*, *S. phureja*, and *S. tuberosum*) involved in the hybrid crosses. The source of the *Rpi-blb2* gene was confirmed as *S. bulbocastanum* using markers developed from the ABPT hybrid to amplify the sequence from an *S. bulbocastanum* accession. The protein sequence is 82% identical to the tomato gene *Mi-1* (van der Vossen et al., 2005).

The source of the BLB2 protein is the wild Solanum species *S. bulbocastanum*. The first formal description of *S. bulbocastanum* was published in 1814 by Dunal, and the species is distributed from northern Mexico to Honduras (Spooner et al., 2004).

Solanum bulbocastanum is not typically cultivated or grown for commercial use, but it does produce tubers (Cultivariable, 2023).

Solanum bulbocastanum is highly resistant to late blight infection and has been a primary source of late blight protection in breeding efforts to transfer this trait to cultivated *S. tuberosum* potato varieties. However, because *S. bulbocastanum* is a diploid species and has an endosperm balance number of 1 (OECD, 1997a), interspecific crosses with *S. tuberosum* are difficult. Using a series of bridge crosses (Hermsen, 1966; Hermsen and Ramanna, 1973), as well as somatic hybridizations (Helgeson et al., 1998), several R-genes from *S. bulbocastanum* were successfully bred into *S. tuberosum* varieties. Due to challenges with crossing *S. bulbocastanum* and *S. tuberosum*, it took almost 50 years to produce potato varieties with *S. bulbocastanum*-sourced late blight protection. Two recently released, conventionally bred varieties, Toluca and Bionica, contain the BLB2 R-protein and are protected against late blight. These varieties are cultivated on a small scale, primarily in Europe (Haverkort et al., 2009)

```
0001 MEKRKDNEEA NNSLESFSAL RKDAANVLDF LERLKNEEDQ KAVDVDLIES
0051 LKLKLTFICT YVQLSYSDLE KFEDIMTRKR QEVENLLQPI LDDDGKDVGC
0101 KYVLTSLAGN MDDCISLYHR SKSDATMMDE QLGFLLLNLS HLSKHRAEKM
0151 FPGVTQYEVL QNVCGNIRDF HGLIVNCCIK HEMVENVLSL FQLMAERVGR
0201 FLWEDQADED SQLSELDEDD QNDKDPQLFK LAHLLLKIVP TELEVMHICY
0251 KTLKASTSTE IGRFIKKLLE TSPDILREYL IHLQEHMITV ITPNTSGARN
0301 IHVMMEFLLI ILSDMPPKDF IHHDKLFDLL ARVVALTREV STLVRDLEEK
0351 LRIKESTDET NCATLKFLEN IELLKEDLKH VYLKVPDSSQ YCFPMSDGPL
0401 FMHLLQRHLD DLLDSNAYSI ALIKEQIGLV KEDLEFIRSF FANIEQGLYK
0451 DLWERVLDVA YEAKDVIDSI IVRDNGLLHL IFSLPITRKK MMLIKEEVSD
0501 LHENISKNRG LIVVNSPKKP VESKSLTTDK IIVGFGEETN LILRKLTSGP
0551 ADLDVISIIG MPGLGKTTLA YKVYNDKSVS SHFDLRAWCT VDQVYDEKKL
0601 LDKIFNQVSD SNSKLSENID VADKLRKQLF GKRYLIVLDD VWDTNTWDEL
0651 TRPFPDGMKG SRIILTTREK KVALHGKLYT DPLNLRLLRS EESWELLEKR
0701 AFGNESCPDE LLDVGKEIAE NCKGLPLVVD LIAGIIAGRE KKKSVWLEVV
0751 NNLHSFILKN EVEVMKVIEI SYDHLPDHLK PCLLYFASAP KDWVTTIHEL
0801 KLIWGFEGFV EKTDMKSLEE VVKIYLDDLI SSSLVICFNE IGDYPTCQLH
0851 DLVHDFCLIK ARKEKLCDRI SSSAPSDLLP RQISIDYDDD EEHFGLNFVL
0901 FGSNKKRHSG KHLYSLTING DELDDHLSDT FHLRHLRLLR TLHLESSFIM
0951 VKDSLLNEIC MLNHLRYLSI GTEVKSLPLS FSNLWNLEIL FVDNKESTLI
1001 LLPRIWDLVK LQVLFTTACS FFDMDADESI LIAEDTKLEN LTALGELVLS
1051 YWKDTEDIFK RLPNLQVLHF KLKESWDYST EQYWFPKLDF LTELEKLTVD
1101 FERSNTNDSG SSAAINRPWD FHFPSSLKRL QLHEFPLTSD SLSTIARLLN
1151 LEELYLYRTI IHGEEWNMGE EDTFENLKCL MLSQVILSKW EVGEESFPTL
1201 EKLELSDCHN LEEIPSSFGD IYSLKIIELV RSPQLENSAL KIKEYAEDMR
1251 GGDELQILGQ KDIPLFK
```

Figure 39. Amino acid sequence of the BLB2 protein

The amino acid sequence of the BLB2 protein from pSIM4363. Individual domains are highlighted, coiled-coil (CC) (green), nucleotide binding (NBS) (blue), and leucine rich repeat (LRR) (red).

StmALS protein identity

Acetolactate synthase (ALS), which is also known as acetohydroxyacid synthase (AHAS), is an enzyme expressed in bacteria, fungi, algae, and all plant species, but not in animals. Acetolactate synthase catalyzes the first common step in the biosynthesis pathway of the branched-chain amino acids isoleucine, leucine, and valine in plants. Humans do not have this pathway and must obtain these amino acids from their diet.

The native potato *StAls* gene, which includes a chloroplast transit sequence, encodes a 659 amino acid polypeptide that interacts with a second StALS polypeptide to form a homodimer. This homodimer is the active form of the enzyme. StALS converts two pyruvate molecules to 2-acetolactate (a precursor of leucine and valine), or pyruvate and 2-ketobutyrate to 2-aceto-2-hydroxy-butyrate (a precursor of isoleucine; Figure 40).

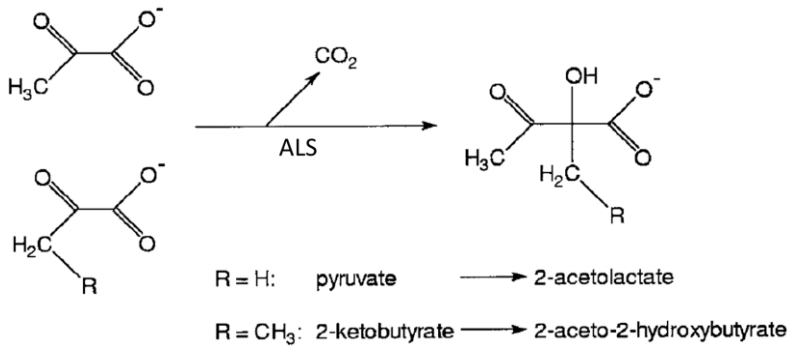


Figure 40. Acetolactate synthase enzymatic reaction

Acetolactate synthase converts two molecules of pyruvate to 2-acetolactate or converts pyruvate and 2-ketobutyrate to 2-aceto-2-hydroxy-butyrate (Duggleby and Pang, 2000).

Acetolactate synthase-inhibiting herbicides prevent branched-chain amino acid synthesis by binding to ALS and blocking access of the substrates, pyruvate or 2-ketobutyrate, to the active site (McCourt et al., 2006). By blocking the first step in branched-chain amino acid synthesis, these herbicides cause an accumulation of 2-ketobutyrate and a deficiency in amino acids necessary for growth and survival (Duggleby and Pang, 2000), which results in the death of the plant.

The modified version of potato StALS in BG25 is called StmALS and is resistant to ALS herbicides. This resistance was used as a selection marker during transformation. The full length StmALS is 659 amino acids, and StmALS differs from StALS by two amino acid substitutions: the tryptophan residue at 563 is changed to leucine (W563L), and the serine residue at 642 is changed to isoleucine (S642I). An alignment of the modified and unmodified potato ALS sequences is provided in Figure 41. The full-length StmALS has a predicted molecular weight of approximately 72 kDa.

```
StALS          MAAAASPSPCFSKTLPPSSSKSSTILPRSTFPFHNHPQKASPLHLTHTHHHRRGFAVSNV 60
StmALS         MAAAASPSPCFSKTLPPSSSKSSTILPRSTFPFHNHPQKASPLHLTHTHHHRRGFAVSNV 60
               ************************************************************

StALS          VISTTTHNDVSEPETFVSRFAPDEPRKGCDVLVEALEREGVTDVFAYPGGASMEIHQALT 120
StmALS         VISTTTHNDVSEPETFVSRFAPDEPRKGCDVLVEALEREGVTDVFAYPGGASMEIHQALT 120
               ************************************************************

StALS          RSNIIRNVLPRHEQGGVFAAEGYARATGFPGVCIATSGPGATNLVSGLADALLDSIPIVA 180
StmALS         RSNIIRNVLPRHEQGGVFAAEGYARATGFPGVCIATSGPGATNLVSGLADALLDSIPIVA 180
               ************************************************************

StALS          ITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVMDVEDIPRVVREAFFLAKSGRPGPVL 240
StmALS         ITGQVPRRMIGTDAFQETPIVEVTRSITKHNYLVMDVEDIPRVVREAFFLAKSGRPGPVL 240
               ************************************************************

StALS          IDVPKDIQQQLVIPNWDQPMRLPGYMSRLPKLPNEMLLEQIIRLISESKKPVLYVGGGCL 300
StmALS         IDVPKDIQQQLVIPNWDQPMRLPGYMSRLPKLPNEMLLEQIIRLISESKKPVLYVGGGCL 300
               ************************************************************

StALS          QSSEELRRFVELTGIPVASTLMGLGAFPTGDELSLQMLGMHGTVYANYAVDGSDLLLAFG 360
StmALS         QSSEELRRFVELTGIPVASTLMGLGAFPTGDELSLQMLGMHGTVYANYAVDGSDLLLAFG 360
               ************************************************************

StALS          VRFDDRVTGKLEAFASRAKIVHIDIDSAEIGKNKQPHVSICADIKLALQGLNSILEGKEG 420
StmALS         VRFDDRVTGKLEAFASRAKIVHIDIDSAEIGKNKQPHVSICADIKLALQGLNSILEGKEG 420
               ************************************************************

StALS          KLKLDFSAWRQELTEQKVKYPLSFKTFGEAIPPQYAIQVLDELTNGNAIISTGVGQHQMW 480
StmALS         KLKLDFSAWRQELTEQKVKYPLSFKTFGEAIPPQYAIQVLDELTNGNAIISTGVGQHQMW 480
               ************************************************************

StALS          AAQYYKYKKPHQWLTSGGLGAMGFGLPAAIGAAVGRPGEIVVDIDGDGSFIMNVQELATI 540
StmALS         AAQYYKYKKPHQWLTSGGLGAMGFGLPAAIGAAVGRPGEIVVDIDGDGSFIMNVQELATI 540
               ************************************************************

StALS          KVENLPVKIMLLNNQHLGMVVQWEDRFYKANRAHTYLGDPANEEEIFPNMLKFAEACGVP 600
StmALS         KVENLPVKIMLLNNQHLGMVVQLEDRFYKANRAHTYLGDPANEEEIFPNMLKFAEACGVP 600
               ********************* ************************************

StALS          AARVSHRDDLRAAIQKMLDTPGPYLLDVIVPHQEHVLPMIPSGGAFKDVITEGDGRRSY  659
StmALS         AARVSHRDDLRAAIQKMLDTPGPYLLDVIVPHQEHVLPMIPIGGAFKDVITEGDGRRSY  659
               ************************************** ****************
```

Figure 41. Sequence alignment between StALS and StmALS

The native full-length potato acetolactate synthase (StALS) protein sequence (Accession: ADI56521) was aligned to the modified StmALS sequence in BG25 using Clustal Omega (Sievers et al., 2011). The modified potato ALS (StmALS) has two amino acid substitutions, W563L and S642I (highlighted in red). StALS and StmALS are 99.7% identical.

B.2(b)(ii) a bioinformatics comparison of the amino acid sequence of the novel protein to known allergens

See Section B1(d) and Report A.

B.2(b)(iii) the new protein's structural properties, including, but not limited to, its susceptibility to enzymatic degradation (e.g. proteolysis), heat and/or acid stability

Bioinformatic analysis confirmed the expressed proteins are unlikely to be allergenic or toxic and would be as susceptible to digestion as most dietary proteins. Although protein characterisation studies were unable to be performed due to the intractability of the proteins, expression of gene transcripts was confirmed for BG25. Even though the protein expression levels are very low, they are sufficient to provide resistance to *P. infestans* strains in BG25.

B.2(b)(iv) specific serum screening where a new protein is derived from a source known to be allergenic or has sequence homology with a known allergen

Not applicable. The new proteins are not from sources known to be allergenic nor do they display sequence homology with known allergens.

B.2(b)(v) information on whether the new protein(s) have a role in the elicitation of gluten-sensitive enteropathy, in cases where the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains.

Not applicable. The new proteins are not from wheat, rye, barley, oats or related cereal grains.

B.3. Other (non-protein) new substances

If other (non-protein) substances are produced as a result of the introduced DNA, information must be provided on the following:

B.3(a) the identity and biological function of the substance

B.3(b) whether the substance has previously been safely consumed in food

B.3(c) potential dietary exposure to the substance

The T-DNA contains two down-regulation cassettes, that result in the production of siRNA in BG25 using the plant's RNAi pathway. As described in Section A.3(b), each down-regulation cassette is comprised of DNA sequence arranged as an inverted repeat.

Transcription of the inverted repeats leads to down regulation of polyphenol oxidase and vacuolar invertase. The PPO-VInv inverted repeat is derived from the DNA sequences from two potato genes (*Ppo5*, and *VInv*; Table 2). Similarly, PVY-CP inverted repeat is expected to form a double-stranded RNA (dsRNA) hairpin that is processed rapidly into siRNA in the cell that target the CP region and inhibit PVT replication.

Due to the nature of the inverted repeat sequences, their transcripts form dsRNA through complementary binding. The dsRNA act as a precursor for the plant's own RNAi post-transcriptional regulatory pathway. A cellular RNase III enzyme, Dicer, recognises and processes the precursor dsRNA into 21-24 bp duplexes termed siRNA. The siRNA bind with cellular proteins forming RNA Induced Silencing Complexes (RISC). The RISC selectively degrades one of the siRNA strands, referred to as the passenger strand. The remaining strand, referred to as the guide strand is used to target the complementary sequence in mRNA molecules. Once the guide strand pairs with an mRNA in a RISC complex, the mRNA molecule is cleaved and degraded, preventing protein translation (Chau and Lee, 2007; Fire et al., 1998).

FSANZ have previously assessed potato event E12 (A1128), transformed with pSIM1278 and events W8, X17 and Y9, transformed with both pSIM1278 and pSIM1678 (A1139 and A1199). FSANZ did not identify any potential public health and safety concerns with the PPO and VInv down-regulation cassettes in these events.

Details of a safety assessment of small RNA generated in Innate® potatoes was presented in A1139. According to scientific literature, RNA and siRNA are labile during processing and digestion and biological barriers further reduce potential exposure by limiting uptake of siRNA into the cells of mammals (Fabre et al., 2014; Hickerson et al., 2008; McAllan, 1982). There is no mechanism for harm in consuming siRNA in Innate® potatoes due to RNA lability during processing and digestion, extensive biological barriers that limit uptake and activity in cells, and the lack of complementarity between the potential siRNA in Innate® and the transcriptome and genome of humans. In addition, conservation of the potato *VInv* gene is limited to plants, providing additional evidence that potatoes containing small RNA directed against the potato *VInv* gene are as safe for consumption as conventional potatoes.

Based on the estimated exposure analysis, humans have very high margins of exposure for the siRNA consumed from BG25 tubers. Given that small RNA are ubiquitous in nature, present in all food, and unlikely to accumulate in the environment, consumption of BG25 potatoes and their associated siRNA is as safe as the consumption of conventional potatoes.

B.3(d)(i) where RNA interference has been used: the role of any endogenous target gene and any changes to the food as a result of silencing that gene

Glucose and fructose can accumulate in tubers during cold storage in a process known as cold-induced sweetening (Bhaskar et al., 2010). If potatoes contain high levels of reducing sugars, they can become undesirably dark and develop bitter flavours after frying (Halterman et al., 2016).

Two potato transcripts targeted for reduced expression are polyphenol oxidase and potato vacuolar invertase. The down regulation of these genes has been assessed previously by FSANZ (see Applications A1128 and A1139). The aim of the suppression of polyphenol oxidase results in tubers with reduced blackspot bruising. The potato vacuolar invertase (VINV) is also targeted for reduced expression through RNAi, resulting in lower reducing sugars. Following starch breakdown in the amyloplast, glucose, glucose-6-phophsate, and maltose are transferred to the cytoplasm. From there, the sugars are further metabolised and shuttled into the glycolysis pathway for mitochondrial respiration or converted into sucrose (Malone et al., 2006; Sowokinos, 2001). Invertase enzymes including VINV hydrolyse the sucrose into glucose and fructose. Down regulation of VINV reduces the conversion of sucrose to fructose and glucose during cold storage, which inhibits formation of sugar-related defects in fries and chips (Halterman et al., 2016; Ye et al., 2010).

Lower reducing sugars are within the range for conventional potatoes and are considered substantially equivalent to edible potatoes (Section B5).

B.3(d)(ii) where RNA interference has been used: the expression levels of the RNA transcript

The expression levels of transcripts from the potato enzymes are presented in Section A.3(g).

The reduced expression of polyphenol oxidase and vacuolar invertase were consistent with the compositional and trait efficacy data in Sections B.1 and B.5. The inserted cassette was successful in reducing expression of polyphenol oxidase and vacuolar invertase transcripts in BG25 tubers. Down regulation of the targeted transcripts was not observed in BG25 leaves.

B.3(d)(iii) where RNA interference has been used: the specificity of the RNA interference

The reduced expression of the potato genes is facilitated by the *Agp* promotor of the ADP glucose pyrophosphorylase gene (*Agp*) and the *Gbss* promoter of the granule-bound starch synthase gene (*Gbss*). Both promotors are primarily active in tubers. The specificity of reduced expression is demonstrated in Section A.3(g). The inserted cassette was successful in reducing expression of polyphenol oxidase and vacuolar invertase transcripts in BG25 tubers. Down regulation of the targeted transcripts was not observed in BG25 leaves.

B.5 Compositional analyses of the food produced using gene technology

This must include all of the following:

B.5(a) the levels of relevant key nutrients, toxicants and anti-nutrients in the food produced using gene technology compared with the levels in an appropriate comparator (usually the non-GM counterpart). A statistical analysis of the data must be provided.

B.5(b) information on the range of natural variation for each constituent measured to allow for assessment of biological significance should any statistically significant differences be identified

B.5(c) the levels of any other constituents that may potentially be influenced by the genetic modification, as a result, for example, of downstream metabolic effects, compared with the levels in an appropriate comparator as well as the range of natural variation.

Compositional Assessment of BG25

Compositional analysis of BG25 (Report D) was conducted to evaluate the levels of key nutrients (proximates, vitamins, amino acids, and minerals) and glycoalkaloids compared to the non-transformed control variety Russet Burbank.

Analytes were measured from tubers harvested from six field trials conducted in 2021 for phenotypic and agronomic assessments (Table 32). Additional details for the composition and statistical methods can be found in:

- Report D

The analytes selected for the compositional assessment were based on the recommendations in the OECD consensus document on compositional considerations for new varieties of potatoes: key nutrients, anti-nutrients, and toxicants (OECD, 2021).

Table 32. Field Trial Locations and Study Design for BG25 and Russet Burbank

Year	Site Code	USDA Notification #	State	County	Trial Design	Rows x Seed Pieces per Row[1]
2021	ID-BONN	20-364-101n	Idaho	Bonneville	RCB, 4 Reps	4x20
2021	ID-MINI	20-364-101n	Idaho	Minidoka	RCB, 4 Reps	4x20
2021	MI-MONT	21-047-102n	Michigan	Montcalm	RCB, 4 Reps	4x20
2021	ND-GRAN	20-364-101n	North Dakota	Grand Forks	RCB, 4 Reps	4x20
2021	WA-FRAN	20-364-101n	Washington	Franklin	RCB, 4 Reps	4x20
2021	WA-GRAN	20-364-101n	Washington	Grant	RCB, 4 Reps	4x20

[1]All planting material was G2 field-grown tuber pieces.

Field-grown tuber seed-pieces (G2) were used as planting material at all sites. The cultivation practices, including soil preparation, fertilizer application, irrigation, and pesticide-based control methods, were location-specific, recommended by regional potato extension specialists and agronomists, and typical for potato.

Field trials were planted using a randomised complete block (RCB) design. Four blocks (replicates) at each site included plots of the test and control variety as treatments. Each plot contained four rows. Rows were

approximately 20 ft (6.1 m) long and seed spacing was one tuber every 10 to 12 in (25.4 to 30.5 cm). The seed tubers were placed by hand, or machine-planted, to a depth of approximately 6 in (15.2 cm).

Each sample was composed of six randomly selected tubers from each replicate at each site (four replicates per site). Because there were six sites used for the field trial, a total of 24 samples were collected for analysis at harvest and an additional set of 24 samples was stored at 7 °C for six months for a second analysis for sugars as part of the trait efficacy determinations.

Analysis was conducted to confirm that composition of BG25 remained within the normal levels for potato when compared to Russet Burbank and conventional potatoes. All results are reported in FW. Compositional assessments were performed for the following:

- Proximates, vitamins, minerals, and starch (Table 33)
- Feed related fibre (Table 34)
- Glycoalkaloids (Table 35)
- Reducing sugars (Table 36).

When an analyte value was below the LOQ, half of the LOQ was used for that value when calculating the mean. The rationale behind this is the expectation that the analyte is present, and its real value, though not quantifiable, is somewhere between 0 and the LOQ. In cases where the values were below the LOQ, the number of observations made below the LOQ was indicated in parentheses beside the total number of observations (N) in each table.

Total glycoalkaloids were reported as the sum of α-solanine and α-chaconine. Since the LOQs for α-solanine and α-chaconine are each 2.50 mg/100 g, total glycoalkaloid values below 5.00 mg/100 g were considered below the LOQ.

When all observations were below the LOQ, every observation got assigned a value of half the LOQ. In cases like this, calculating a standard deviation and analysis via a linear mixed model were not possible. The p-value and standard deviation in these cases were reported as “NA” (not applicable).

Proximates, vitamins, minerals, and starch

No statistical differences between BG25 and Russet Burbank were observed for proximates, vitamins, minerals, and starch (Table 33). These results indicated that BG25 was equivalent to Russet Burbank for these analytes.

Feed related fibre measures

Acid detergent fibre (ADF) and neutral detergent fibre (NDF) were analysed in BG25 compared to Russet Burbank (Table 34). ADF and NDF were both below the limit of quantitation in BG25 and Russet Burbank on a fresh weight basis (LOQ <1%; see Report D). Levels of NDF were not significantly different in BG25 and Russet Burbank.

Limited literature ranges were found for ADF and NDF in potatoes. Guidance from Canada indicates that typical levels of ADF in potato culls for animal feed is as low as 0.06% on an as fed basis (3% of DM) (Snowdon, 2017). Average values for raw tubers of 3.6% of DM for ADF and 8.3% of DM for NDF have been reported (Heuzé et al., 2018).

Table 33. Proximates, vitamins, minerals and starch in BG25 and Russet Burbank tubers

Variable	Variety	Mean	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Protein (%)	BG25	2.26	0.2710	0.162	24	1.93	2.69	1.22	4.02
	Russet Burbank	2.21		0.174	24	1.86	2.50		
Total Fat (%)	BG25	0.194	0.3169	0.058	24	0.113	0.319	0.080	0.740
	Russet Burbank	0.215		0.062	24	0.120	0.335		
Ash (%)	BG25	0.759	0.3713	0.106	24	0.536	0.913	0.150	2.00
	Russet Burbank	0.743		0.106	24	0.594	0.982		
Total Dietary Fiber (%)	BG25	1.55	0.9109	0.186	24	1.17	2.03	1.40	3.60
	Russet Burbank	1.54		0.279	24	1.25	2.48		
Carbohydrates (%)	BG25	16.2	0.1794	0.970	24	14.2	17.9	3.68	25.1
	Russet Burbank	15.7		1.27	24	13.5	18.0		
Starch (%)	BG25	13.5	0.0946	1.70	24	10.7	18.6	13.4	18.1
	Russet Burbank	13.0		1.62	24	9.81	15.1		
Moisture (%)	BG25	80.6	0.1733	0.988	24	79.1	82.7	71.8	85.9
	Russet Burbank	81.1		1.37	24	79.2	83.5		
Vitamin B6 (mg/100 g)	BG25	0.177	0.8110	0.023	24	0.144	0.226	0.065	0.204
	Russet Burbank	0.175		0.023	24	0.145	0.242		
Vitamin C (mg/100 g)	BG25	16.5	0.5033	2.07	24	12.3	19.2	6.97	51.4
	Russet Burbank	15.9		2.96	24	8.70	23.6		
Magnesium (mg/100 g)	BG25	20.4	0.1088	4.15	24	16.7	31.9	14.6	40.6
	Russet Burbank	19.4		4.25	24	15.4	29.6		
Potassium (mg/100 g)	BG25	411	0.5625	53.2	24	341	508	291	765
	Russet Burbank	402		49.5	24	350	511		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges are mostly from (AFSI, 2023). Total dietary fiber range is from (OECD, 2021) and the starch range is from (USDA, 2019).

Table 34. Feed related fibre in tubers of BG25 and Russet Burbank

Variable	Variety	Mean (mg/100 g)	P-Value[1]	Standard Deviation	N[2]	Range (mg/100 g)	
						Min	Max
Acid Detergent Fibre (%)	BG25	0.500	NA	NA	24 (24)	0.500	0.500
	Russet Burbank	0.500		NA	24 (24)	0.500	0.500
Neutral Detergent Fibre (%)	BG25	0.523	0.7918	0.112	24 (23)	0.500	1.05
	Russet Burbank	0.534		0.165	24 (23)	0.500	1.31

[1]P-values indicating significant differences are underlined and in bold.
[2] The number of samples with values below the LOQ are recorded in brackets.

Glycoalkaloids

Glycoalkaloids are toxins commonly found in solanaceous crops, including potato, and 95% of the total glycoalkaloids in potato tubers consists of α-solanine and α-chaconine (OECD, 2021). The safety limit for total glycoalkaloids in tubers is 20 mg/100 g fresh weight (Smith et al., 1996).

The mean total glycoalkaloid levels were statistically higher in BG25 across the six sites compared to Russet Burbank (Table 35). However, there was no significant difference in glycoalkaloid levels at each of the sites individually. Both BG25 and Russet Burbank mean total glycoalkaloid concentrations were lower than the safety limit and fell within the combined literature range (Table 35).

Table 35. Glycoalkaloids in tubers of BG25 and Russet Burbank across six sites

Site	Variety	Mean[1] (mg/100 g)	P-Value[2]	Standard Deviation	N[3]	Range (mg/100 g)		Combined Literature Range (mg/100 g)[4]	
						Min	Max	Min	Max
Bonneville County, Idaho	BG25	11.0	0.1308	3.59	4	7.90	15.7	3.20	210
	Russet Burbank	7.60		1.44	4	5.80	9.30		
Minidoka County, Idaho	BG25	2.50	NA	NA	4	2.50	2.50		
	Russet Burbank	2.50		NA	4	2.50	2.50		
Montcalm County, Michigan	BG25	14.9	0.1373	1.77	4	12.6	16.5		
	Russet Burbank	11.7		3.19	4	7.80	15.6		
Grand Forks County, North Dakota	BG25	29.9	0.1258	4.83	4	24.0	35.8		
	Russet Burbank	23.2		5.75	4	17.5	13.6		
Franklin County, Washington	BG25	12.2	0.2157	0.294	4	11.8	12.5		
	Russet Burbank	10.2		2.84	4	6.90	13.8		
Grant County, Washington	BG25	5.90	0.1131	2.12	4	4.10	8.20		
	Russet Burbank	3.78		0.877	4 (1)	2.50	4.50		
All Sites Combined	BG25	12.7	**0.0242**	9.20	24 (4)	2.50	35.8		
	Russet Burbank	9.84		7.45	24 (5)	2.50	30.6		

[1]Total of α-solanine and α-chaconine.
[2]P-values indicating significant differences are underlined and in bold.
[3] The number of calculated totals with values below the LOQ are recorded in brackets.
[4]Combined literature range from Kozukue et al. (2008).

Reducing sugars

Modifications in BG25 included a mechanism to lower reducing sugars in fresh and stored potato tubers. BG25 showed significantly lower levels of reducing sugars, fructose, and glucose, at harvest and after six months storage (Table 36). Mean levels of sucrose were significantly higher in BG25 after six months storage. The results can be attributed to down regulation of vacuolar invertase in BG25. Down regulation of vacuolar invertase slows the breakdown of sucrose into glucose and fructose in the vacuole, resulting in decreased levels of glucose and fructose and increased sucrose levels in tubers.

Mean levels of fructose, glucose, and sucrose in fresh and stored BG25 tubers were within the combined literature range for conventional potatoes.

Table 36. Sugars in tubers of BG25 and Russet Burbank at harvest and after storage at 7 °C

Variable	Variety	Mean	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Fructose and Glucose (mg/100 g)									
Fresh	BG25	90.7	**0.0021**	39.1	24	25.2	171	4.37	905
	Russet Burbank	229		102	24	93.7	492		
6 Months Storage	BG25	172	**0.0005**	79.9	24	72.0	438	4.37	905
	Russet Burbank	328		77.4	24	84.2	435		
Sucrose (mg/100 g)									
Fresh	BG25	186	0.0628	56.3	24	133	346	16.0	1,597
	Russet Burbank	150		27.2	24	117	212		
6 Months Storage	BG25	170	**0.0069**	27.3	24	116	217	16.0	1,597
	Russet Burbank	135		17.0	24	106	169		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges from Amrein et al., 2003, Vivanti et al., (2006), and AFSI (2023). For fructose and glucose, combined literature range theoretical minimum values were obtained by adding fructose and glucose minimum values from AFSI (2023).

Patatin in BG25

Potatoes are not among the "Big Eight" group of foods that account for ~90% of all food allergies in the U.S. (FARRP, 2023). There are a few reports of allergies to cooked potato in children (DeSwert et al., 2002, 2007). However, most children with potato allergy develop tolerance at mean age of four years (De Swert, et al., 2007). Patatin (Sol t 1) has been identified as the primary 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 tubers (Mignery et al., 1988). There is no mechanistic reason to suggest that the level of patatin would be changed in BG25. Because potato protein naturally contains a relatively large proportion of patatin, any unexpected change in patatin levels would be unlikely to affect allergenicity enough to alter consumption patterns for people allergic to potatoes.

Conclusion of the Compositional Assessment of BG25

A composition assessment was conducted on BG25 and Russet Burbank samples from field trials in 2021. The analytes assessed were important for potato nutrition and related to trait efficacy.

The nutrition assessment evaluated proximates, vitamins, and minerals, and feed-related fibre and showed no statistical differences between values for BG25 and Russet Burbank. The glycoalkaloid levels in BG25 were higher than in Russet Burbank, but the mean concentrations were within the range for conventional potatoes and were below the safety limit.

The trait efficacy assessment evaluated reducing sugars in fresh and stored tubers. These results demonstrated that BG25 has significantly lower levels of reducing sugars than Russet Burbank at harvest and after storage.

The results of this study demonstrated that BG25 is compositionally equivalent to conventional potatoes and is as safe and nutritious for food and feed as potatoes that have a long history of safe consumption. The results also demonstrated that the down regulation of reducing sugars was successful in fresh and stored BG25 tubers.

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

Potato has a long history of safe use. Global production in 2021[7] was more than 376 million tonnes. Two thirds were consumed directly by humans and the remaining fed to animals or used to produce starch.

The BG25 event in this submission has been developed via transformation with a T-DNA designed to down-regulate endogenous potato genes, protect against PVY, and to produce R-proteins for potato blight resistance. The introduction of the RNAi sequences and the R-proteins have no nutritional impact on the potato event. This is supported by the fact that:

- Molecular characterisation demonstrated stability of the inserts during vegetative propagation cycles
- The R-proteins and STmALS have homologues with a history of safe consumption and no significant homology to known allergens and toxins; and
- Compositional analysis did not indicate biologically significant changes to the levels of nutrients in events compared to their conventional counterparts. Event composition is within the normal variation of potato cultivars and varieties and is substantially equivalent to conventional potato varieties.

D. Other Information

Where a biotech food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies will add little to the safety assessment and generally are not warranted (see e.g. Bartholomaeus et al., 2013; Herman and Ekmay, 2014; OECD, 2003).

The only new polypeptides produced by the insert in potato event BG25 are AMR3, BLB2, VNT1 and StmALS. These proteins have a non-toxic mode of action and occur at very low levels in the plants. Their safety is supported by a weight-of-evidence that indicates safety for human consumption. Considering the compositional equivalence between the BG25 potato event and its conventional variety, and the lack of any observed phenotypic characteristics indicative of unintended effects arising from the genetic modification process, there was no plausible risk hypothesis that would indicate the need for animal feeding studies.

[7] Food and Agriculture Organization of the United Nations, http://faostat3.fao.org; data retrieved 23rd November 2023.

Reports

Report A: BG25_Allergen_Toxin_xx-x-SPS-MOL_CCI_Deleted

Report B: BG25_AMR3-Expression_ xx-xx-SPS-MOL_CCI-Deleted

Report C: BG25_BLB2-Expression_ xx-xx-SPS-MOL_CCI_deleted

Report D: BG25_Composition_xx-xx-SPS-COMP-01_CCI-Deleted

Report E: BG25_Insert_Characterization_xx-xx-SPS-MOL-03_CCI-Deleted

Report F: BG25_PPO_VINV_transcript_Expression_xx-x-SPS-MOL_CCI_deleted

Report G: BG25_R-protein_Safety_Assessment_xx-xx-SPS-MOL-CCI-Deleted

Report H: BG25_Stability_ddPCR-PCR_xx-xx-SPS-MOL_CCI_deleted

Report I: BG25StmALSExpression_ xx-xx-SPS-MOL_CCI_deleted

Report J: BG25_VNT1-Expression_ xx-xx-SPS-MOL_CCI_deleted

Report K: StmALS_Protein_Safety_Assessment_xx-xx-SPS-REG_CCI-deleted

Appendix A. The pSIM4363 insert sequence

pSIM4363 T-DNA Insert in event BG25 including 1498 nucleotide of left flanking sequence (lowercase), T-DNA insert (uppercase), and 1498 nucleotide right flanking sequence (lowercase). Primer regions are in yellow.

<Sequence redacted CCI>

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