FOOD STANDARDS
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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Safety assessment – Application A1289

Food derived from disease-resistant, low-reducing sugars and reduced browning potato line BG25

Executive summary

Application A1289 seeks approval for the sale and use of food derived from potato line BG25 which has been genetically modified to have resistance to late blight and *Potato Virus Y* (PVY), low-reducing sugars and reduced browning.

Resistance to late blight is conferred by the expression of three R-proteins: VNT1, AMR3 and BLB2, encoded by *Rpi-vnt1*, *Rpi-amr3* and *Rpi-blb2* genes respectively. They allow the plant to detect the presence of the infecting organism *Phytophthora infestans*, leading to the induction of the plant's immune response and elimination of the fungus.

BG25 contains novel dsRNA molecules that are expressed to trigger the RNA interference (RNAi) silencing pathway. Protection against PVY infection is achieved by silencing the PVY-CP gene encoding viral coat protein. Two endogenous genes are also silenced via RNAi: *Vlnv* encoding vacuolar invertase *and Ppo* encoding polyphenol oxidase. Silencing of *Vlnv* and *Ppo* genes results in lower reducing sugars and reduced browning, respectively.

BG25 also contains the modified acetolactate synthase (*StmAls*) gene from *S. tuberosum* that confers tolerance to imidazolinone herbicides. Imidazolinone tolerance was only used for selection of putative transformants during the transformation stage. The applicant has advised that herbicide tolerance is not a commercial trait in BG25.

This safety assessment addresses food safety and nutritional issues associated with the GM food. It therefore does not address:

- risks related to the environmental release of GM plants used in food production
- the safety of animal feed, or animals fed with feed, derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

History of use

On a global scale, potato is the fourth most produced food crop following maize, rice and wheat and is cultivated in over 100 countries. It has been cultivated for human consumption for thousands of years and has a long history of safe use in the food supply. Potatoes are typically cooked before consumption and are processed into different food commodities.

Molecular characterisation

Comprehensive molecular analyses indicate that a single insertion is present at a single site in the BG25 genome with the expected organisation. There are no extraneous plasmid sequences or antibiotic resistance genes present in this line.

Molecular analyses also confirmed the silencing of *Vlnv* and *Ppo* genes and the expected changes to phenotype: low-reducing sugars and reduced browning.

Characterisation and safety assessment of novel substances

The VNT1, AMR3 and BLB2 proteins are present in potato line BG25 at very low levels. The safety of the VNT1 protein has been assessed by FSANZ in previous applications (A1139 and A1199). FSANZ has not previously assessed the AMR3 and BLB2 proteins, however, they are R-proteins from the Solanaceae family with a history of safe human exposure, and are structurally and functionally similar to VNT1 and other R-proteins that are ubiquitous in edible crops. Bioinformatic analyses confirmed the expressed proteins are unlikely to be allergenic or toxic to humans.

The StmALS protein is present in BG25 tuber and leaf. FSANZ has not previously assessed the StmALS protein, however this protein is derived from *S. tuberosum* which has a history of safe use as food. StmALS is 99.7% identical to the native ALS protein present in *S. tuberosum*. Food derived from several plants resistant to ALS inhibiting herbicides through modifications to the ALS protein are commercially available and safely consumed. Bioinformatic analyses confirm that StmALS does not have any significant similarity to known allergens or toxins.

There are no safety concerns regarding the presence of the dsRNA molecules in BG25. The available data do not indicate the dsRNAs expressed in BG25 possess different characteristics, or are likely to pose a greater risk, than other RNAi mediators naturally present in potato.

Compositional analyses

Compositional analyses were undertaken on BG25 tubers and compared to the non-GM control. These analyses confirmed the expected changes to the levels of sucrose and reducing sugars as a result of RNAi mediated silencing in BG25. These changes and all other identified differences were within the range of natural variation. Overall, the compositional data support the conclusion there are no biologically significant differences in the levels of key constituents in tubers from BG25 compared to non-GM potatoes cultivars available on the market.

Conclusion

No potential public health and safety concerns have been identified in the food safety assessment of potato line BG25. On the basis of the data provided in the present application, and other available information, food derived from potato line BG25 is considered to be as safe for human consumption as food derived from conventional potato varieties.

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List of Abbreviations

Abbreviation	Definition
ADF	Acid Detergent Fibre
A. tumefaciens	*Agrobacterium tumefaciens*
ALS	Acetolactate synthase
APHIS	Animal and Plant Health Inspection Service
BLAST	Basic Local Alignment Search Tool
CC-NBS-LRR	Coiled-Coil-Nucleotide-Binding-Site-Leucine-Rich Repeat
CFIA	Canadian Food Inspection Agency
COMPARE	COMprehensive Protein Allergen REsource
CP	Coat protein
ddPCR	Droplet digital PCR
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
FASTA	Fast Alignment Search Tool - All
FDA	Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
FW	Fresh Weight
ipt	Isopentenyl transferase gene
LOQ	Limit of Quantitation
NDF	Neutral Detergent Fibre
NGS	Next Generation Sequencing
OECD	Organisation for Economic Cooperation and Development
ORF	Open Reading Frame
PCR	Polymerase chain reaction
P. infestans	*Phytophthora infestans*
PGSC	Potato Genome Sequence Consortium
PPO	Polyphenol oxidase enzyme
PVY	*Potato virus Y*
RNAi	RNA interference
RT-qPCR	Reverse transcription - quantitative polymerase chain reaction
R-Protein	Resistance protein
siRNA	Small interfering RNA
T-DNA	Transfer DNA
UniProtKB	Universal Protein Resource Knowledgebase
US	United States
USDA	United States Department of Agriculture
VINV	Vacuolar invertase protein

1 Introduction

FSANZ received an application from SPS International, Inc. (SPSII) to vary Schedule 26 in the Australia New Zealand Food Standards Code. The variation is to include food from a new genetically modified (GM) potato line BG25, with the OECD Unique Identifier SPS-ØBG25-7. This potato line is resistant to a fungal pathogen that causes late blight and *Potato Virus Y* (PVY). Potato line BG25 also has low-reducing sugars and reduced browning.

Resistance to late blight is conferred by the expression of three R-proteins: VNT1, AMR3 and BLB2, encoded by *Rpi-vnt1*, *Rpi-amr3* and *Rpi-blb2* genes respectively. The introduced genes are derived from wild potato species *Solanum venturi*, *Solanum americanum* and *Solanum bulbocastanum.* The introduced R-proteins do not directly inhibit the fungal pathogen. They allow the plant to detect the presence of the infecting organism *Phytophthora infestans*, leading to the induction of the plant's immune response and elimination of the fungus.

Protection against PVY is achieved by introducing inverted repeat DNA sequences which, through the RNA interference (RNAi) pathway, silences the PVY-CP gene encoding the viral coat protein (CP) (Hannon 2002). The introduced DNA sequences were derived from PVY.

The low-reducing sugar trait is achieved by introducing inverted repeat DNA sequences that silence the expression on the endogenous vacuolar invertase (*Vlnv*) gene through RNAi. The introduced DNA sequences were derived from *Solanum tuberosum* var. Ranger Russet. The vacuolar invertase enzyme catalyses the conversion of sucrose into glucose and fructose, and its activity increases with decreasing temperature, leading to an increase in reducing sugar formation at the cold temperatures normally used for storage of potatoes (Sowokinos 2001; Bhaskar et al. 2010). Silencing of the *Vlnv* gene leads to reduced conversion of stored sucrose into glucose and fructose, particularly on the outer edge of the potatoes, which has been associated with darkening on the ends of potato chips (crisps) and French fries (Zhu et al. 2014).

The lowering of reducing sugars in BG25 decreases the potential for acrylamide formation during cooking at high temperatures. Acrylamide[1] is a known carcinogen that has been shown to form in high carbohydrate-rich foods such as potatoes when cooked at high temperatures (120-200 °C) (Başaran et al. 2023). The acrylamide forms when the amino acid asparagine reacts with reducing sugars such as glucose and fructose (Raffan and Halford 2019). Therefore, silencing of the *Vlnv* gene in BG25 may reduce the potential for acrylamide formation upon cooking.

The reduced browning trait is achieved by introducing inverted repeat DNA sequences that silence the expression of the endogenous polyphenol oxidase (*Ppo*) gene through RNAi. The introduced DNA sequences were derived from the wild edible potato, *Solanum verrucosum*. Silencing of the *Ppo* gene leads to reduced discolouration that sometimes forms in damaged or cut potatoes and is associated with PPO activity. PPO converts colourless polyphenols in the plant tissue to coloured quinones, which further react to produce dark melanin pigments. This discoloration results in reduced organoleptic properties, leading to increased food wastage by growers, processers and consumers.

If approved, food derived from potato line BG25 may enter the Australian and New Zealand food supply as imported food products such as French fries, potato crisps, potato flour or potato starch.

[1] For more information: https://www.foodstandards.gov.au/consumer/chemicals/acrylamide

2 History of use

2.1 Host organism

The potato (*Solanum tuberosum*) originated from South America, where it has been cultivated for human consumption for thousands of years (Ugent and Patterson 1988). Potato is propagated vegetatively using small tubers or pieces of tuber typically referred to as seed or seed potatoes. Potato can also be propagated via sexually produced seed, contained within tomato-like berries. However, seed production and breeding are challenging.

Internationally, potato is the fourth most produced food crop following wheat, maize and rice and is cultivated in over 160 countries (FAOSTAT 2024). Global potato production in 2022 was ~ 375 million tonnes (MT), with China the top producer at ~ 96 MT. Potato is not a major crop in Australia and New Zealand, with production around 1.1 MT and 0.43 MT in 2022, respectively (FAOSTAT 2024).

Whole potatoes are typically cooked before consumption or are processed into food commodities such as potato chips (crisps), pre-cooked French fries and dehydrated potato products (diced, flaked or granules). Potato is also used for the production of industrial starch and alcohol, with the by-products and residues having the potential to be used to supplement animal feedstock.

The Russet Burbank potato variety chosen by the applicant for genetic modification is highly susceptible to late blight infection, a current problem facing potato growers in North America and around the world (Ivanov et al. 2021). A genetic modification process was chosen to address these issues because the Russet Burbank variety is infertile (Cultivariable 2021) and therefore standard crossbreeding cannot be performed in this variety.

Potato has a long history of safe use as food. Potato and other members of the Solanaceae family, such as tomatoes and eggplants, naturally produce a pesticidal group of compounds called glycoalkaloids (GAs). GAs can be toxic to humans if consumed in high quantities (greater than 1 mg GA per kg bodyweight). However, humans are rarely exposed to such high levels of GA. A maximum limit of 200 mg/kg fresh potato is the widely accepted safe limit for total GA in registered potato varieties. Proper storage conditions and peeling the potato before use help reduce GA levels.

2.2 Donor organisms

2.2.1 Solanum tuberosum

The *VInv* and *StmAIs* DNA sequences are derived from *Solanum tuberosum*. *S. tuberosum* has a long history of safe use as food. Majority of the non-coding DNA sequences are from this source including the ADP glucose pyrophosphorylase gene promoter (p*Agp*), the granule-bound starch synthase promoter (p*Gbss*), the polyubiquitin promoter, ubiquitin terminator, some of the left and right border region and spacer DNA (see Appendix 1).

2.2.2 Solanum verrucosum

The *Ppo* DNA sequences were derived from *S. verrucosum*. This is a wild, edible species of potato from Mexico (CFIA 2015) that has been used as a bridging species for the conventional breeding of desirable traits into the domesticated *S. tuberosum* potato (Jansky and Hamernik 2009).

2.2.3 Solanum venturii

The *Rpi-vnt1* DNA sequence was derived from *S. venturi.* This is a wild species of potato with high resistance to late blight (Park 2013). This species 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 late blight resistance gene *Rpi-vnt1* belongs to a family of resistance genes found in many plant species, including food crops like the potato (Xu et al. 2011; Marone et al. 2013). This diploid species would not produce viable offspring if bred with tetraploid *S. tuberosum* varieties.

2.2.4 Solanum americanum

The *Rpi-amr3* DNA sequence was derived from *S. americanum,* a species commonly known as American black nightshade. This species does not yield tubers, but its leaves and ripe berries are consumed as food and used for medicinal purposes in parts of Africa and Asia (Särkinen et al. 2018). *S. americanum* is not known to cause allergies or toxicity in humans (Edmonds et al. 1997; Särkinen et al. 2018). The glycoalkaloid contents in *S. americanum* and other nightshade species are low and do not pose a safety risk when consumed as green vegetables or ripe fruits (Yuan et al. 2018). The leaves and berries also lack tropane alkaloids, a potent toxicant associated with nightshade berries (Pigatto et al. 2015). *S. americanum* is a source of R-proteins for late blight protection and has been shown to be naturally resistant to *P. infestans* (Witek et al. 2016).

2.2.5 Solanum bulbocastanum

The *Rpi-blb2* DNA sequence was derived from *S. bulbocastanum*. This wild potato species has been used as the primary source of the late blight protection trait in breeding efforts to transfer protection to cultivated *S. tuberosum* potato varieties (Hermsen 1966; Hermsen and Ramanna 1973; Helgeson et al. 1998). Two conventionally bred varieties of potatoes, Toluca and Bionica, contain the BLB2 R-protein from *S. bulbocastanum* and are protected against late blight. These varieties are cultivated for food use on a small scale, primarily in Europe (Haverkort et al. 2009).

2.2.6 Potato virus Y

The PVY-CP DNA sequences were derived from potato plants infected with PVY strain N. PVY is an RNA virus belonging to the potyvirus group of plant viruses and is found in most regions in the world where potato is grown (Onditi et al. 2022). The virus is aphid transmissible and commonly infects potatoes, causing serious disease (Quenouille et al. 2013). There is a long history of safe human exposure to PVY through the consumption of PVY-infected potatoes (Torrance and Talianksy 2020). PVY does not have any known allergenicity or toxicity to humans (APHIS 2024).

3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA, including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

3.1 Transformation method

An *Agrobacterium*-mediated transformation method was used to generate potato line BG25. The methodology is outlined in a flowchart in Appendix 2 and summarised below.

Internode segments from 4 week-old *in vitro* grown potato plantlet from the Russet Burbank variety were inoculated with *A. tumefaciens* AGL1 harbouring the pSIM4363 plasmid (Figure 1). The internode segments were then transferred to regeneration medium to promote callus growth. The regeneration medium contained the antibiotic timentin to suppress the growth of excess *Agrobacterium*. Callus tissue was transferred every four weeks to fresh regeneration medium until visible plantlets developed.

Regenerated plantlets were transferred to rooting medium containing imazamox, an ALS-inhibiting herbicide. Plantlets that did not produce roots were discarded as this indicates the absence of T-DNA integration, i.e. no *StmAls* expression. To identify transformants that had inadvertently taken up the plasmid backbone, explants that produced abnormal or stunted shoots indicative of *ipt* gene expression (found in the backbone region; see Figure 1) were discarded. The IPT protein, encoded by the *ipt* gene, catalyses the formation of cytokinin, a plant growth hormone that causes abnormal plant growth phenotypes.

Polymerase chain reaction (PCR) analysis was used to identify transformants for further propagation that had incorporated the T-DNA insert. The absence of backbone sequences was later confirmed by Illumina Next Generation Sequencing (NGS) and the results were further confirmed using digital droplet PCR (ddPCR).

Successful transformants were tested for polyphenol oxidase (PPO) activity, reducing sugar levels, as well as PVY and late blight protection. Transformants expressing low levels of PPO and reducing sugars, as well as the highest level of protection against PVY and late blight were advanced further.

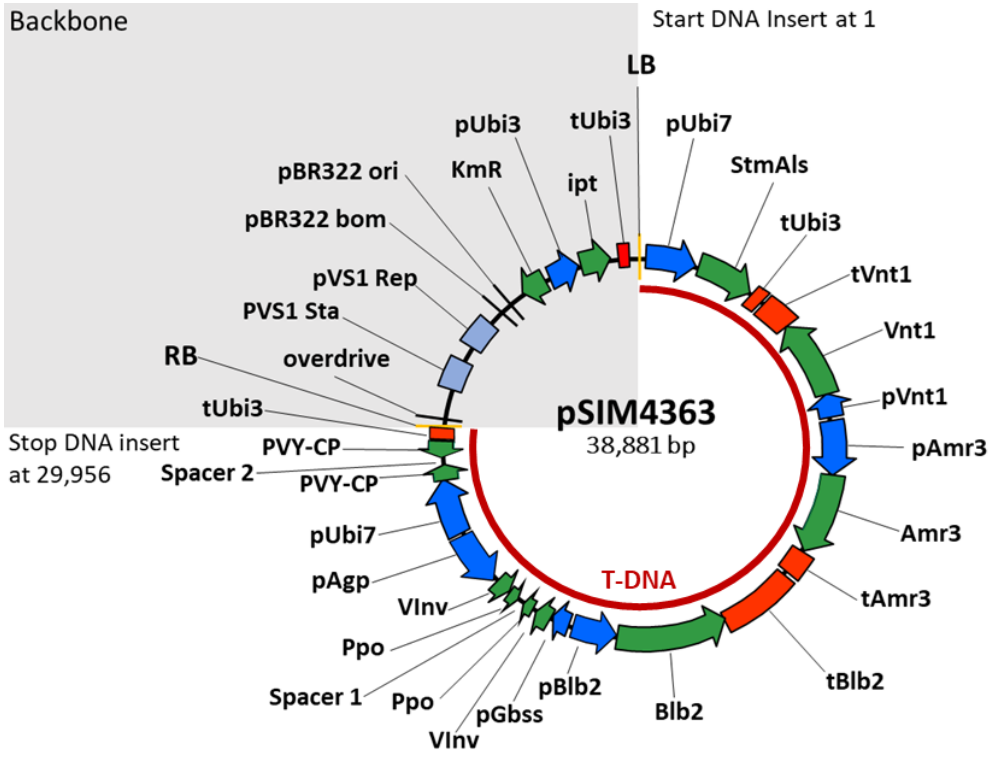

Figure 1. Map of *plasmid pSIM4363. The region between LB and RB, indicated by a maroon line, was inserted into the potato genome. The region contains the Rpi-vnt1, Rpi-amr3, Rpi-blb2 and StmAls expression cassettes as well as the VInv / Ppo and PVY-CP inverted repeat sequences.*

3.2 Detailed description of inserted DNA

Potato line BG25 contains the T-DNA from pSIM4363 plasmid (Figure 1). The *Rpi-vnt1*, *Rpi-amr3*, *Rpi-blb2* and *StmAls* expression cassettes as well as the *VInv / Ppo* and PVY-CP inverted repeat sequences are all derived from pSIM4363 and are summarised in Table 1. Additional details, including plasmid backbone and intervening sequences used to assist with cloning, mapping and sequence analysis, can be found in Appendix 1.

Table 1. Expression cassettes contained in T-DNA of pSIM4363

Expression cassette	Promoter	Enhancer/Regulatory sequences	Coding sequences	Terminator	Notes
StmAls	Polyubiquitin promoter (p*Ubi7*) from *S. tuberosum* (potato)	Intron from Ubiquitin gene (*Ubi7*) and ubiquitin gene monomer from *S. tuberosum*	Modified acetolactate synthase (*StmAls*) from *S. tuberosum*	Ubiquitin-3 gene terminator (t*Ubi3*) from *S. tuberosum*	Full length modified protein
Rpi-vnt1	*Rpi-vnt1* native promoter (p*Vnt1*) from *S. venturi* (potato)	-	*Rpi-vnt1* gene from *S. venturii*	*Rpi-vnt1 native* terminator (t*vnt1*) from *S. venturii*	Full length native protein

Expression cassette	Promoter	Enhancer/Regulatory sequences	Coding sequences	Terminator	Notes
Rpi-amr3	*Rpi-amr3* native promoter from *S. americanum* (potato)	-	*Rpi-amr3* gene from *S. americanum*	*Rpi-amr3* native terminator from *S. americanum*	Full length native protein
Rpi-blb2	*Rpi-blb2* native promoter from *S. bulbocastanum* (potato)	-	*Rpi-blb2* gene from *S. bulbocastanum*	*Rpi-blb2* native terminator from *S. bulbocastanum*	Full length protein native protein
Vlnv/Ppo inverted repeat	Granule-bound starch synthase gene promoter (p*Gbss*) and ADP glucose pyrophosphorylase gene promoter (p*Agp*) from *S. tuberosum*	-	Partial sequence of vacuolar invertase (*Vlnv*) gene from *S. tuberosum* in sense and antisense orientation. 3'UTR region of polyphenol oxidase (*Ppo*) gene from *S. verrucosum* in sense and antisense orientation	-	*Vlnv* and *Ppo* Inverted repeat partial sequences
PVY-CP inverted repeat	Polyubiquitin promoter (p*Ubi7*) from *S. tuberosum*	Intron from Ubiquitin gene (*Ubi7*) and ubiquitin gene monomer from *S. tuberosum*	Partial sequence of potato virus coat protein (PVY-CP) in sense and antisense orientation	Ubiquitin-3 gene terminator (t*Ubi3*) from S. tuberosum	PVY-CP inverted repeat partial sequence

3.3 Development of the potato line from the original transformant

After the transformation and selection process, plantlets were maintained in tissue culture (Figure 2A). Shoots from the plantlets were cut and cultured in fresh media to allow root formation (Figure 2B). Plantlets with roots were transferred to soil in greenhouses to produce tubers. Tubers from greenhouse-grown potatoes were then planted in fields, to multiply potato propagules for large scale potato production (Figure 2C). The use of the tuber as a seed is a characteristic of potato, allowing cultivation by vegetative propagation rather than by sexual reproduction[2]. Potato seed contains buds that can sprout and grow into mature potato plants.

[2] The progeny arising from this form of asexual reproduction will be genetically the same as the parent plant.

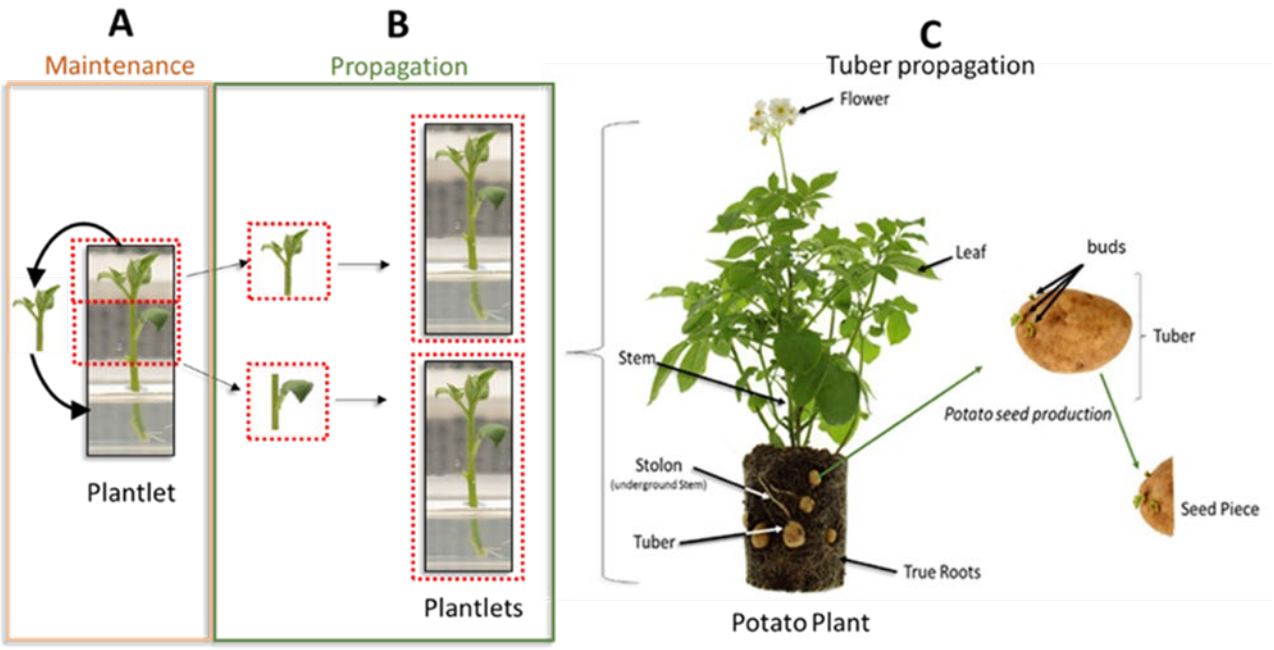

Figure 2. Potato plantlet stocks are maintained in tissue culture (**A**) and multiplied by vegetative propagation (**B**) to produce mature potato plants and potato seed (**C**).

When characterising the BG25 line, different generations of plants were analysed. Plants and tubers arising from the initial planting of plantlets into soil are referred to as G0 and the plants and tubers arising from the planting of G0 tubers are referred to as G1 and so forth (Figure 3). The type of characterisations performed and at what generation they were analysed are summarised in Table 2.

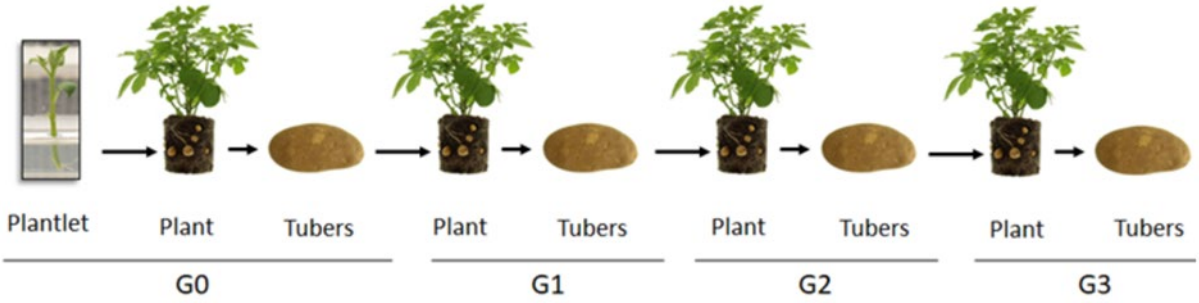

Figure 3. Generations of potato derived from vegetative propagation.

Table 2. BG25 generations used for various analyses

Analysis	Generation analysed	Control(s) used
Insertion copy number and organisation, absence of backbone (NGS analysis)	G0 leaf	Russet Burbank
Inheritance and genetic stability	G0, G1, G2 leaf	Russet Burbank
Insert integrity and site of integration	G0 leaf	Russet Burbank
Compositional analysis	G3 tuber	Russet Burbank
RNAi silencing of targeted genes		
Suppression of RNA transcripts	G2 tuber and leaf	Russet Burbank
Reducing sugar levels	G3 tuber	Russet Burbank

3.4 Characterisation of the inserted DNA and site(s) of insertion

A range of analyses were undertaken to characterise the genetic modification in BG25. These analyses focused on the nature and stability of the insertion and whether any unintended re-arrangements or expression products may have occurred as a consequence of the transformation procedure.

To characterise the number of integration sites, insert integrity and absence of extraneous sequences, the applicant made use of Next Generation Sequencing (NGS) with a targeted sequence capture technique. Sanger sequencing was used to determine the sequence of the inserted DNA and flanking sequences from the potato genome.

3.4.1 Number of integration site(s)

Leaf-derived genomic DNA (gDNA) from G0 generation of BG25 was analysed by NGS, along with DNA from a non-GM Russet Burbank plant as a control. Plasmid DNA from pSIM4363 was used as the positive control.

NGS with a targeted sequence capture technique utilising the transformation plasmid pSIM4363 was used to determine the T-DNA insert organisation and to confirm the absence of any unintended plasmid sequences in BG25.

A set of probes of 80 nucleotides in length covering the entire pSIM4363 plasmid, including the backbone, were hybridised to the gDNA extracted from the leaves of the G0 plants, and the captured samples were sequenced using NGS. The targeted sequence capture technique yielded paired-end reads (2x 150 bp) with an average of 99X depth of coverage across the entire pSIM4363 T-DNA insert, including the junctions and flanking genomic DNA.

The sequence reads obtained by NGS were compared to the potato reference genome[3] (Sharma et al. 2013) to identify unique junctions attributable to inserted DNA. Two unique insert-flank DNA junction sites were identified, indicating a single T-DNA insertion site in BG25. The right and left insert-flank DNA sequences were further analysed by PCR and Sanger sequencing, corroborating the NGS result. A reference sequence was assembled from the known T-DNA plasmid sequence and the Sanger sequenced right and left insert-flank DNA junctions.

The sequencing coverage in the control was limited to regions derived from potato endogenous sequences present in the inserted T-DNA. No insert-flank DNA junctions were identified in the control confirming that the reads were only identifying endogenous sequences.

Further alignment of the NGS reads to the assembled reference sequence, pSIM4363 sequence and the Russet Burbank draft genome v1.0[4] (control) confirmed that a single copy of the pSIM4363 T-DNA, was integrated into the genome of BG25 with the intended organisation.

The T-DNA insert copy number in BG25 was further confirmed using ddPCR, which quantified the expression of six targets spanning the T-DNA insertion relative to a reference gene (either *Vlnv* or the vacuolar protein sorting gene). All of the T-DNA targets had a copy

[3] Potato Genome Sequencing Consortium (PGSC) *S. tuberosum* group Phureja clone DM1-3 pseudomolecules (v4.03), http://spuddb.uga.edu/pgsc_download.shtml

[4] The Russet Burbank draft genome (v1.0) was developed in collaboration with Corteva (Iowa), utilising PacBio CLR sequencing reads with an average length of 34 kb to develop a genome assembly from ~37x coverage of the tetraploid genome.

number of one, indicating that BG25 contained a single T-DNA insertion.

3.4.2 Absence of backbone and other sequences

NGS sequencing analysis was performed on leaf-derived genomic DNA to detect possible plasmid backbone. NGS reads from BG25 or the control were aligned to pSIM4363 backbone sequences. A small number of reads mapped to backbone sequences, however this is due to the presence of two endogenous potato sequences in the plasmid backbone. No other backbone sequences including any antibiotic resistance genes were identified in BG25 genome from this alignment. PCR analyses with six primer pairs targeting the backbone sequence of pSIM4363 further confirmed the absence of plasmid backbone sequence in BG25.

3.4.3 Insert integrity and site of integration

The NGS, Sanger sequencing and ddPCR analyses indicated that BG25 contains a single copy of the intended T-DNA insert, with the expected organisation and no unintended sequences or rearrangements.

Alignment of NGS reads with the reference sequence and the control confirmed that BG25 contains the intended T-DNA insert, with deletions in the right and left border regions. Compared to the intended insertion, 34 bp from the right border and 330 bp from the left border are not present in BG25. Such changes during T-DNA insertion are common during *Agrobacterium*-mediated plant transformation due to double-stranded break repair mechanisms (Anderson et al. 2016). Given these sequences are not part of the gene cassettes, it is unlikely they would affect the function of the inserted genetic elements.

To determine the location of the T-DNA insertion in the potato genome, the native locus in the control (non-GM Russet Burbank) was amplified using primers that hybridise to the genomic regions flanking the T-DNA insert. The identified sequences were then compared to the BG25 sequence and the reference genome of the control. These searches located the single T-DNA insert on chromosome 12. A 55 bp deletion of the potato genome at the T-DNA integration site was identified which correspond to an intergenic region. The insertion did not disrupt any endogenous genes or any other known annotated feature in the potato genome.

3.4.4 Inheritance and genetic stability of the inserted DNA

As commercial potatoes are vegetatively propagated, standard Mendelian segregation analysis could not be used to determine inheritance. In order to confirm that the progeny were genetically the same as the parent and to ensure the stability of the inserted DNA over time, ddPCR and PCR analysis was performed on leaf-derived genomic DNA obtained from G0, G1 and G2 plants. The ddPCR measured the absolute copy number of six target amplicons across the T-DNA insert in BG25 and the PCR analysis assessed the left and right flanking regions of the T-DNA insert. The results confirmed that the inserted T-DNA was stably incorporated over 3 successive clonal generations and remained stable over this time period in BG25.

3.4.5 Open reading frame (ORF) analysis

A proprietary Python script was used to identity all start-to-stop open reading frames (ORFs) in both the inserted DNA and junctions between the insert and gDNA. All six reading frames were analysed. ORFs of 30 or more amino acids were captured for further analysis, as proteins shorter than 29 amino acids would not meet the minimum requirements of a 35% match over an 80 amino acid sequence (Codex 2009).

In total, 125 putative ORF were identified in BG25. These were all associated with the pSIM4363 insert only, i.e. there were no junction putative ORFs identified in BG25. Amino acid sequences from the 125 identified ORFs (putative peptides) were used as query sequences in homology searches for known allergens and toxins in established databases. These analyses are theoretical only as there is no reason to expect that any of the identified ORFs would, in fact, be expressed.

3.4.5.1 Bioinformatics analysis for potential allergenicity

The 125 putative peptides were compared to known allergenic proteins listed in the COMprehensive Protein Allergen REsource (COMPARE[5]) database, from the Health and Environmental Science Institute. At the date of the search (January 2023), there were 2361 sequences in the allergen database (AD_2023).

Three types of analyses were performed for this comparison:

a) Full length sequence search using a FASTA (Fast Alignment Search Tool – All) alignment was performed comparing the whole sequence to the database entries. Significant homology was determined when there was (i) greater than 50% homology between query protein and database entry and (ii) E-value < 10^{-4}. The lower the E-value, the less likely the similarity is due to chance.

b) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids to the database entries. Only matches of greater than 35% similarity over ≥ 80 amino acids (E-value of 10) were considered.

c) 8-mer exact match FASTA search was performed comparing contiguous 8 amino acids to the database entries. Only matches of 100% similarity over 8 amino acids were considered.

Two putative peptides coinciding with the inverted *Vlnv* DNA sequences in the pSIM4363 insert matched a minor allergenic vacuolar invertase protein from tomato (*S. lycopersicum*; AAL75449[6], AAL75450[7]). This match is not a safety concern, rather, the putative peptides sequences are similar to the vacuolar invertase protein sequence that is already expressed in potatoes. Therefore, BG25 potatoes are no more likely than conventional potato to cause an allergic reaction in individuals sensitive to the tomato vacuolar invertase. This match therefore does not raise any allergenicity concerns.

3.4.5.2 Bioinformatic analysis for potential toxicity

The putative peptides were compared to known toxin proteins identified in the UniProtKB[8] database that were selected using a keyword search for toxins. A BLASTP search[9] (with an E-value < 10^{-2}) was used as a criterion for similarity.[10]

The BLASTP search identified 9 potential toxins in the UniProtKB database with alignment to putative peptides associated with VNT1, AMR3, BLB2, StmALS proteins and with PVY-CP and *Vlnv* inverted repeats. While the identified potential toxins in the database contained the key word "toxin" in their accession records, none of them are actual toxins. This was confirmed by a literature review. There were no other putative peptides identified with

[5] https://comparedatabase.org
[6] www.ncbi.nlm.nih.gov/protein/18542113
[7] www.ncbi.nlm.nih.gov/protein/AAL75450
[8] https://www.uniprot.org/
[9] https://blast.ncbi.nlm.nih.gov/Blast.cgi
[10] Searches were performed on 30 January 2023.

similarity to any known human toxins. The putative peptides / ORFs in BG25 therefore do not present a toxicity concern.

3.4.6 Silencing of targeted genes

To confirm that targeted genes had been silenced as intended, the applicant provided data examining RNA transcript levels in BG25 compared to the non-GM Russet Burbank line (control), as well data showing the effects of target gene silencing.

3.4.6.1 RNA transcripts

Reverse transcription quantitative real-time PCR (RT-qPCR) analysis was used to examine RNA transcript levels of *Vlnv* and *Ppo* genes in BG25 compared to the control. In comparison to the control, BG25 showed a reduction of *Vlnv* and *Ppo* transcripts in tubers.

The viral CP RNA transcript is the target of PVY-CP double-stranded RNA (dsRNA). It is not present in healthy potato plants, but introduced during viral infection. Therefore, quantifying viral CP RNA transcript levels in healthy plants would be challenging. However, the application describes a PVY resistance assay (Appendix 2) performed during the development of the BG25 potato line. In this assay, G0 plants and susceptible non-transformed control plants were inoculated with PVY and symptoms were examined visually. Plants protected against PVY were advanced to molecular screening and field trials. Therefore, viral CP RNA transcripts are expected to be silenced if BG25 were to be infected by PVY.

3.4.6.2 Gene silencing effects on reducing sugar levels

Reducing sugars are simple sugars like glucose and fructose that contain an aldehyde group, which can act as a reducing agent in a redox reaction. Reducing sugar levels are increased by the hydrolysis of starch and sucrose, two major carbohydrate forms found in plants. Vacuolar invertase, encoded by the *Vlnv* gene, converts stored sucrose into glucose and fructose. The activity of this enzyme increases with decreasing temperature, leading to an increase in reducing sugar formation at the cold temperatures normally used for storage of potatoes (Sowokinos 2001; Bhaskar et al. 2010).

The applicant showed there are reduced levels of RNA transcripts for the *Vlnv* target gene. Further analysis was done to confirm that the reduced RNA transcript levels correspond to reduced levels of reducing sugars.

As shown in Table 3, the level of reducing sugars (fructose and glucose) in BG25 tubers were consistently lower at harvest and after storage compared to the control. The mean levels of sucrose were significantly higher in BG25 after storage compared to the control. These data indicate the breakdown of sucrose at lower temperatures has been impaired in BG25 and this is correlated to the suppression of the *Vlnv* gene.

Table 3. The change in sucrose and reducing sugar levels between BG25 and the control in freshly harvested or cold-stored potatoes at 7 °C.

Temperature	Variable	Sucrose Fold change	Reducing sugars Fold change
	Fresh	↑ 1.24	↓ **2.52**
7 °C	6 months	↑ **1.26**	↓ **1.91**

1. Bolded and underlined results indicate statistical significance between BG25 and the control.

3.4.7 Conclusion

Data provided by the applicant showed that an integration event has occurred at a single locus in the potato genome. Sequencing data confirmed that the VNT1, AMR3, BLB2, StmALS expression cassettes and the PVY-CP and *Vlnv* / *Ppo* inverted repeat cassettes are present with the expected sequence and organisation in the genome of BG25. No plasmid backbone sequences, including antibiotic resistance genes, from the transforming pSIM4363 plasmid are present. The introduced genetic elements are stably maintained through clonally propagated generations. None of the new ORFs created by the insertion raise any allergenicity or toxicity concerns.

Molecular analysis also confirmed the suppression of the *Vlnv* and *Ppo* genes in BG25 with corresponding changes to phenotype resulting in lower levels of reducing sugars in BG25.

4 Characterisation and safety assessment of novel substances

Four novel proteins are expressed in BG25: the three R-proteins, VNT1, AMR3 and BLB2, which provide protection against the late blight disease; and the StmALS protein which acts as a selectable marker during the transformation process. BG25 also contains novel dsRNA molecules which mediate silencing (by RNAi) of endogenous *Vlnv* / *Ppo* genes and exogenous PVY-CP during an infection.

4.1 Newly expressed proteins

In considering the safety of newly expressed substances it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, because of anti-nutrient properties or triggering of allergies in some consumers (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutrient or allergenic effects.

To effectively identify any potential hazards, knowledge of the characteristics, concentration and localisation of all newly expressed proteins in the organism as well as a detailed understanding of their biochemical function and phenotypic effects is required. It is also important to determine if the newly expressed protein is expressed in the plant as expected, including whether any post-translational modifications have occurred.

4.1.1 R-proteins

VNT1, AMR3 and BLB2 belong to a group of common plant resistance proteins (R-proteins) that contain a typical Coiled-Coil-Nucleotide-Binding-Site-Leucine-Rich Repeat (CC-NBS-LRR) domain structure. These CC-NB-LRR proteins are used by the plant to detect the presence of pathogenic molecules known as effectors that are indicative of an infection (Jones and Dangl 2006; McHale et al. 2006). Once the presence of an effector is detected the plant's defence responses are activated resulting in the eventual development of immunity to the pathogen.

The R-proteins introduced into BG25 recognise and mediate a response to the oomycete *P. infestans* (Foster et al. 2009; Jones et al. 2014). This pathogen causes late blight, a devastating fungal disease responsible for the Great Irish Potato Famine and which still poses a major threat to global potato cultivation.

VNT1

The VNT1 protein is encoded by the native *Rpi-vnt1* gene, promoter and terminator from *S. venturii*, a wild relative to *S. tuberosum*. The protein consists of 891 amino acids and is one of three variants expressed in *S. venturii* (Foster et al. 2009). The predicted size of the mature protein is ~102 kDa.

VNT1 has a typical CC-NBS-LRR domain structure and shares high homology to several R-proteins, actual and predicted, from edible food crops across the Solanaceae family, including potatoes, tomatoes and capsicums (sweet and chilli peppers) (Foster et al. 2009). It is therefore similar to proteins that have a history of safe human exposure.

VNT1 has been assessed previously by FSANZ in applications A1139[11] and A1199[12]. The expression product in BG25 is identical to that expressed in the previous applications.

AMR3 and BLB2

The AMR3 protein in encoded by the native *Rpi-amr3* gene from *S. americanum*, a wild Solanum species. The protein consist of 887 amino acids and the predicted size of the mature protein is ~102 kDa.

The BLB2 protein is encoded by the native Rpi-blb2 gene from *S. bulbocastanum*, a wild Solanum species. The protein consist of 1267 amino acids and the predicted size of the mature protein is ~146 kDa

FSANZ has not previously assessed the AMR3 and BLB2 proteins, however, both AMR3 and BLB2 have the typical CC-NBS-LRR domain structure found in VNT1 and other R-proteins that are ubiquitous in edible crops (Oh et al. 2014; Zhao et al. 2015). Similar to VNT1, AMR3 and BLB2 recognises and mediates a response to oomycete *P. infestans*, which activates the plant cell death pathway to inhibit the pathogen's growth and spread (Lin et al. 2022).

4.1.1.1 Expression of the R-proteins in potato tubers

RT-qPCR analyses were used to investigate the presence of *Rpi-vnt1*, *Rpi-amr3* and *Rpi-blb2* mRNAs in tuber tissue of BG25 and the control (non-GM Russet Burbank). Leaf tissues from *S. venturii*, *S. americanum* and *S. bulbocastanum* were used as positive controls, respectively. *Rpi-vnt1*, *Rpi-amr3* and *Rpi-blb2* mRNA were not detected in the leaf or tuber tissue of the control, which is expected, as this variety does not have these genes. *Rpi-vnt1*, *Rpi-amr3* and *Rpi-blb2* mRNA were detected in BG25 in the leaf and tuber tissue. These results confirm that the *Rpi-vnt1*, *Rpi-amr3* and *Rpi-blb2* genes are expressed in BG25.

Immunoblotting was used to examine the expression of VNT1, AMR3 and BLB2 proteins in tuber tissues of BG25 and the control. Polyclonal antibodies were generated for detecting VNT1, AMR3, BLB2. The recombinant forms of AMR3, BLB2 and VNT1 proteins expressed in *Escherichia coli* (*E. coli*) were used as protein standards to determine the limit of quantification (LOQ). The VNT1 protein has been considered by FSANZ in previous safety assessments (A1139 and A1199). Similar to those assessments, VNT1 was below the LOQ in BG25, as were AMR3 and BLB2. These results are in line with the published literature as R-proteins are known to be expressed at low levels and are considered to be intractable proteins[13] (McHale et al. 2006; Bushey et al. 2014).

4.1.1.2 Safety of the introduced R-proteins

FSANZ has previously assessed the VNT1 protein in A1139 and A1199's safety assessments and concluded that VNT1 is unlikely to be allergenic or toxic. The bioinformatics analyses for VNT1 have been updated for this application.

AMR3 and BLB2 are derived from the Solanaceae family and are structurally and functionally similar to VNT1 and other R-proteins found ubiquitously in other food crops. Given this structural and functional similarity to proteins with a history of safe human consumption, bioinformatic analysis is considered sufficient to confirm the safety of these proteins.

[11] https://www.foodstandards.gov.au/food-standards-code/applications/A1139
[12] https://www.foodstandards.gov.au/food-standards-code/applications/A1199
[13] Intractable proteins are those that are extremely difficult to isolate and purify. Without the ability to obtain a high amount of purified product, protein characterisation studies cannot be performed.

4.1.1.3 Bioinformatic analyses of R-proteins

The applicant provided the results of bioinformatic analyses comparing the VNT1, AMR3, and BLB2 amino acid sequence to known allergenic proteins in the COMPARE database, using the same search criteria as outlined in Section 3.4.5. The FASTA search did not identify any known allergens with homology to VNT1, AMR3 or BLB2.

The applicant has provided the results of bioinformatic analyses comparing the amino acid sequence of VNT1, AMR3, and BLB2 to proteins identified as "toxins" from the UniProtKB database. A BLASTP search identified 5 potential toxins with an E-value < 10^{-2}.

All of the toxigenic proteins identified were plant resistance-like proteins that provide protection from pathogenic microorganisms. As resistance proteins exist in the majority of 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 VNT1, AMR3 and BLB2 proteins are not homologous to any biologically relevant toxins.

4.1.2 StmALS

The StmALS protein in BG25 is a modified version of acetolactate synthase (ALS) from *S. tuberosum* (StALS). The protein consist of 659 amino acids and the predicted size of the mature protein is ~65 kDa. StmALS is 99.7% identical to native StALS present in *S. tuberosum,* differing only by two amino acids (W563L and S642I) (Appendix 3).

The native StALS, also known as acetohydroxyacid (AHAS), catalyses the first common step in the biosynthesis pathway of the branched-chain amino acids isoleucine, leucine and valine in plants. 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 (Duggleby and Pang 2000).

ALS-inhibiting herbicides inhibit branched-chain amino acids synthesis by binding to ALS and blocking access to its substrates (McCourt et al. 2006). This causes a deficiency in the amino acids necessary for growth and survival, leading to plant death. Two amino acid substitutions in the native ALS sequence, makes StmALS resistant to ALS-inhibiting herbicides.

The safety of ALS enzymes expressed in crops has been extensively investigated (Mathesius et al. 2009; Chukwudebe et al. 2012; He et al. 2016). Several commercialised crops have herbicide tolerance conferred by alleles of the *Als* gene, e.g. Clearfield canola, Clearfield wheat, Clearfield sunflower, Clearfield lentils (Tan et al. 2005). Additionally, proteins with homologous amino acid sequence to ALS are found in commonly consumed food such as canola, chickpea, barley, and sunflower (Rutledge et al. 1991; Kolkman et al. 2004; Lee et al. 2011; Thompson and Tar'an 2014). No reports of adverse effects due to exposure to ALS enzymes have been reported.

4.1.2.1 Expression of StmALS protein in potato tissues

The level of StmALS expression in BG25 was measured in leaf and tuber tissues, collected from four field locations[14] in representative potato-producing regions of the United States in 2021. Three replicates of samples were collected from each of the four field trial sites, resulting in 12 replicates of samples for each tissue type analysed. Tissues were flash frozen in liquid nitrogen, homogenised and stored frozen until the analysis.

StmALS was extracted from tissues using standard methods and the expression level was quantified in each tissue using immunoblot analysis. A polyclonal antibody for StmALS was

[14] Field trial location in the United States – Michigan, North Dakota, Idaho and Washington.

generated for detecting StmALS in BG25. The antibody is specific for StmALS with minimal cross-reactivity to the endogenous, native StALS. A recombinant form of StmALS protein, derived from *E. coli*, was used as positive control. Protein concentration was quantified using densitometric analysis via the Imagelab software.

The concentration of StmALS in leaf and tuber was calculated using a standard curve. These values were converted to parts per billion (ppb; ng/g) fresh weight (FW) based on the amount of total protein loaded onto the gel.

StmALS expression was detected in BG25 tuber, with a mean of 420 ppb (range: 200 – 570 ppb). StmALS was expressed at a higher level in BG25 leaf, with a mean of 1830 ppb (range: 780 – 6010 ppb). There was variation in the StmALS expression levels amongst samples obtained from various locations. This variation could be attributed to various biotic or abiotic variables, or to the maturity of the sample at the time of collection.

4.1.2.2 Safety of the introduced StmALS

FSANZ has not previously assessed the StmALS protein, however, food derived from several commercially available plants resistant to ALS inhibiting herbicides through modifications to the ALS protein are commercially available and safely consumed (Tan et al. 2005). In addition, FSANZ has assessed and approved homologs of StmALS in four previous assessments (A1006, A1018, A1064 and A1239). These previous assessments did not raise any safety concerns and there have been no credible reports of adverse health effects in humans.

Since the StmALS protein expressed in BG25 is 99.7% identical to the native ALS protein present in *S. tuberosum* (cultivated potato variety) and plants with modifications to the ALS protein are commercially available and safely consumed, bioinformatic analysis is considered sufficient to confirm safety.

4.1.2.3 Bioinformatic analyses of StmALS

Bioinformatic analyses (January 2023) for StmALS that looked for amino acid sequence similarity to known protein allergens and toxins were provided by the applicant. The results did not identify any known allergens or toxins with homology to StmALS.

4.2 Conclusion

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). The VNT1, AMR3, BLB2 and StmALS proteins are derived from potato species and bioinformatic analyses showed that these proteins had no similarity to known allergens or toxins that are of significance or concern.

4.3 Newly expressed dsRNA

4.2.1 Description of the dsRNA

Two cassettes were introduced into BG25 to produce dsRNA. The first cassette contains inverted DNA repeat sequences which targets endogenous *Vlnv* and *Ppo* mRNA and a second cassette containing inverted DNA repeat sequences that targets PVY-CP mRNA from an infecting PVY and inhibits its replication. These sequences include both sense and antisense fragments from either the coding or promoter regions of the target genes.

When the inverted DNA repeat sequences are transcribed, the complementary base pairs of

the sense and antisense strands bind, forming a dsRNA molecule with a hairpin loop. These molecules are then processed by the RNAi post-transcriptional pathway in the cell, forming a RNA-induced silencing complex. This complex binds to the target gene mRNA resulting in its cleavage and degradation. This pathway exists in most eukaryotic organisms, including food-based crops.

Evidence has been provided by the applicant to show that the target genes *Vlnv* and *Ppo*, have been suppressed in the tubers (Section 3.4.6). The PVY resistance assay performed during the development of the BG25 potato line provides evidence that the PVY-CP dsRNA molecule is expressed.

The available data do not indicate these dsRNAs possess different characteristics, or are likely to pose a greater risk, than other RNAi mediators naturally present in potato. A history of safe human consumption of RNAi mediators exists, including those with homology to human genes. The evidence published to date also does not indicate that dietary uptake of such RNA from plant food is a widespread phenomenon in vertebrates (including humans) or, if it occurs, that sufficient quantities are taken up to exert a biologically relevant effect (FSANZ 2013).

5 Compositional analysis

The main purpose of compositional analysis is to determine if, as a result of the genetic modification, an unexpected change has occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analyses can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

The classic approach to the compositional analysis of GM food is a targeted one. Rather than analysing every possible constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors such as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health.

5.1 Key components

The key components to be analysed for the comparison of GM and conventional potato are outlined in the OECD Consensus Documents on Considerations for New Varieties of potato (OECD 2021). There are fewer analytes analysed in this application than in previous GM potato applications. This is due to a recent update to the OECD potato consensus document (OECD 2021), which recommends a reduced number of analytes (12).

The analytes measured include proximates (moisture, carbohydrate, protein, fat, starch and ash), fibre, vitamins, minerals and the anti-nutrient glycoalkaloid. Furthermore, as the RNAi mechanism silences the expression of the *VInv* gene that would impact the hydrolysis of sucrose in fresh and stored potato tubers, the levels of sucrose and reducing sugars (glucose and fructose) were analysed at harvest and after six months of cold storage.

5.2 Study design

BG25 and the control (non-GM Russet Burbank) were grown and harvested from 6 potato growing regions[15] in the Unites Staes in 2021. The agronomic practices and pest control measures used were location-specific and were typical for all aspects of potato cultivation including soil preparation, fertiliser application, irrigation and pesticide-based control methods. The field trials were established in a randomised complete block design with 4 replicates per site.

Tubers from BG25 and the control were obtained at typical harvest maturity. Each sample was composed of 6 randomly selected tubers from each replicate at each site. Fresh tubers from each site were transported at ambient temperature to an analytical laboratory. The long-term storage tubers were transported to an analytical laboratory after 6 months and the tubers were processed within 3 days of receipt.

The compositional analysis included validated methods from either the Association of Official Analytical Collaboration (AOAC), International, the American Oil Chemists' Society (AOCS), or other published scientific methods.

[15] One site from each of Michigan and North Dakota, 2 sites from each of Idaho and Washington.

Homogenised samples were prepared for analysis using 6 whole raw tubers, including the peel. The analytes that were measured in these samples are listed in Figure 4. In total, 16 different analytes were measured. Statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, North Carolina). A linear mixed model analysis of variance was used by combining data from multiple locations. In assessing the significance of any difference between the mean analyte value for BG25 and the control, a P-value of < 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

For analytes where all individual sample values were below the LOQ, a value of half the LOQ was assigned and the P-value and standard deviation were reported as “NA” (not applicable). For analytes where individual analyte values were below LOQ, a value of half the LOQ was assigned when calculating mean.

Total glycoalkaloids (GAs) 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. All samples analysed for ADF were reported to be below the LOQ, and therefore was reported as “NA”.

If a statistically significant difference was observed between BG25 and the control, the value was compared to a combined literature range based on the natural variation of analytes from publicly available data (USDA 2019; OECD 2021; AFSI 2023). The ranges derived from these values account for variability present in non-GM potato varieties due to a wider range of agronomic and environment conditions, as well as different genetic backgrounds.

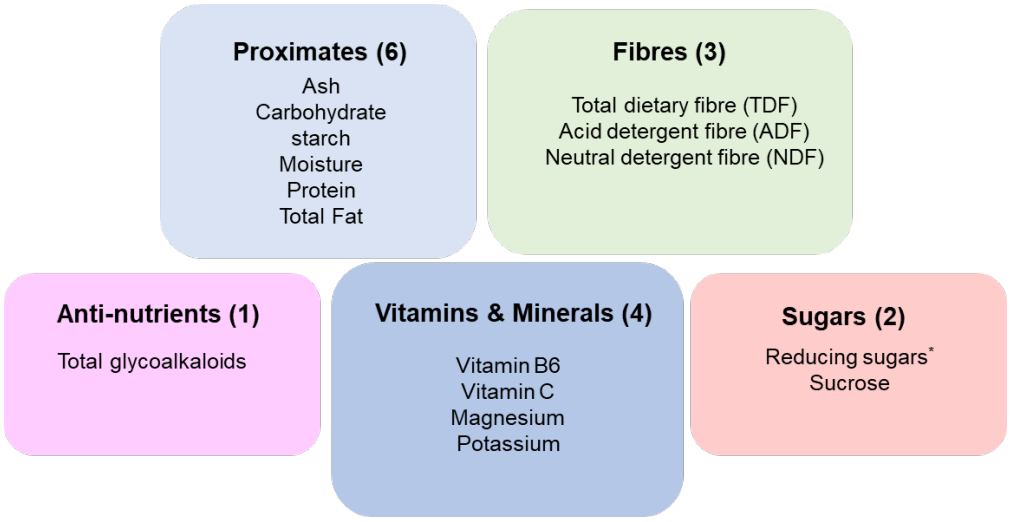

Figure 4. Analytes measured in fresh potato tubers. * Glucose and fructose

5.3 Analyses of key components in tubers

Of the 16 analytes measured, there were 3 analytes for which there was a statistically significant difference in mean values between BG25 and the control; total GAs, reducing sugars and sucrose. A summary of these 3 analytes is provided in Figure 5. For the complete set, including values for the analytes for which no statistically significant differences were found, refer to Application dossier[16] (pages 105 – 107).

[16] https://www.foodstandards.gov.au/food-standards-code/applications/a1289-food-derived-disease-resistant-low-reducing-sugars-and

5.3.1 Glycoalkaloids

The mean total GA level was statistically significantly higher in BG25 tuber compared to the control (Figure 5b). Although the total GA level in BG25 is higher than the control, the observed mean for BG25 falls within the natural variation found across a range of conventional potato lines used for human consumption as represented by the publicly available literature ranges (light grey bar). Therefore, the differences observed here does not raise any safety concern.

5.3.2 Reducing sugars and sucrose

Statistically significantly lower levels of reducing sugars (fructose and glucose), were observed in BG25 tubers at harvest and after six months of cold storage (panels c–d). While the mean levels of sucrose in freshly harvested tubers from BG25 showed no differences, statistically significantly higher levels of sucrose were observed in BG25 tubers after six months of storage compared to the control (panels e–f). These data correlate with the intended silencing the vacuolar invertase gene. Vacuolar invertase would normally facilitate the conversion of sucrose to fructose and glucose during cold storage. For both sucrose and the reducing sugars, the observed mean in BG25 fell well within the natural variability represented by publicly available literature ranges (light grey bar in Figure 5c – f).

Overall, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key constituents in BG25 when compared with conventional non-GM potato varieties already available in agricultural markets. Tubers from BG25 can therefore be regarded as equivalent in composition to tubers from conventional non-GM potato.

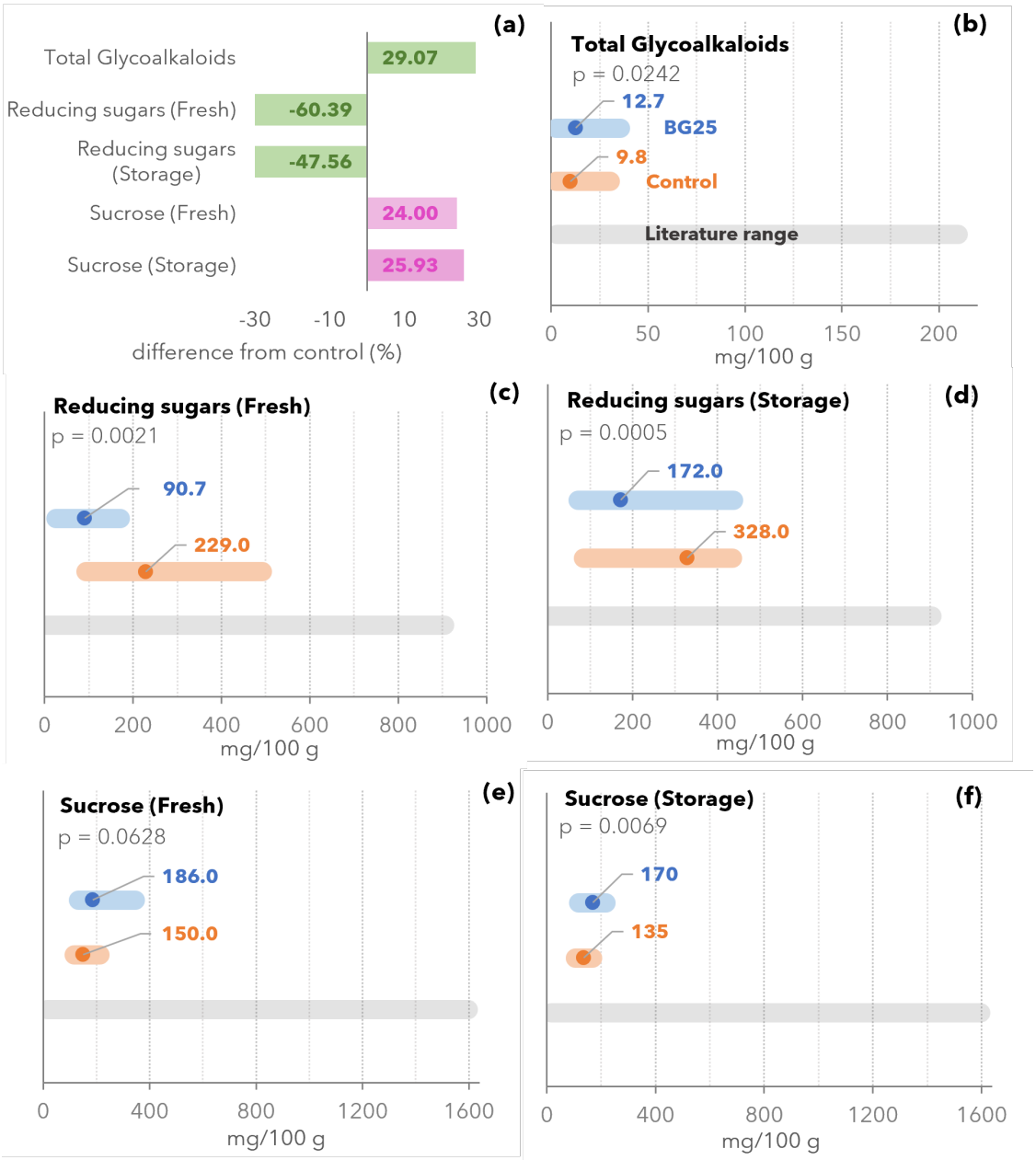

Figure 5. Visual summary of total glycoalkaloids, reducing sugars and sucrose in BG25 and the control (non-GM Russet Burbank). **(a)** Percentage deviation of the mean BG25 value from the mean control value for **(b)** glycoalkaloids as well as for **(c) – (d)** reducing sugars and **(e) – (f)** sucrose (fresh and after storage) for which a statistically significant difference was found (except **(e)**). **(b) – (f)** Measured means (dots) and ranges (coloured bars) for BG25 (blue) and the conventional control (orange) for the 5 analytes as labelled. The light grey bar represent the publicly-available range of values for each analyte. Note that the x-axes vary in scale and unit for each component.

6 Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish the food is nutritionally adequate and will support typical growth and wellbeing. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with a compositional analysis of the food, such as that presented in Section 5.

Where a GM 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 would not be warranted (OECD 2003; Bartholomaeus et al. 2013). If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

Potato line BG25 is the result of genetic modifications to confer resistance to late blight and PVY. In addition, the BG25 line also has low-reducing sugars and reduced browning traits. Compositional analyses of tubers have been undertaken to demonstrate the nutritional adequacy of potato line BG25 and indicate they are equivalent in composition to tubers from conventional potato varieties.

The introduction of food from BG25 into the food supply is therefore expected to have little nutritional impact and, as such, no additional studies, including animal feeding studies, are required.

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Appendix 1

pSIM4363-derived genetic elements

Genetic Element	Source	Relative Position	Size (bp)	Description
Left Border (LB)	Synthetic DNA	1-25	25	Secondary cleavage site releases ssDNA[17] inserts from pSIM4363
LB buffer	*S. tuberosum* var. Ranger Russet.	26-187	162	Buffer for truncations during insertion
Intervening sequence	*S. tuberosum*	188-192	5	Sequence used for DNA cloning
***StmALS* cassette**				
Polyubiquitin promoter (pUbi7)	*S. tuberosum* var. Ranger Russet	193-1,131	939	Drives expression of the *StmAls* gene
StUbi7 gene intron	*S. tuberosum*	1,132-1,700	569	Improves expression of StmALS; is spliced out before translation
Ubiquitin gene monomer	*S. tuberosum*	1,701-1,928	228	Expressed as a fusion with StmALS, then cleaved off
Intervening sequence	Synthetic DNA	1,929-1,934	6	Sequence used for DNA cloning
Modified *Als* gene (*StmAls*)	*S. tuberosum*	1,935-3,914	1,980	Selection marker; modified potato ALS (StmALS)
Intervening sequence	Synthetic DNA	3,915-3,920	6	Sequence used for DNA cloning
Ubiquitin-3 gene terminator (tUbi3)	*S. tuberosum*	3,921-4,275	355	Terminates transcription of *StmAls* gene
Intervening sequence	Synthetic DNA	4,276-4,285	10	Sequence used for DNA cloning
***Rpi-vnt1* cassette**				
Rpi-vnt1 native terminator (tVnt1)	*S. venturii*	4,286-5,210	925	Terminates transcription of *Rpi-vnt1*
Rpi-vnt1 gene coding sequence (*Vnt1*)	*S. venturii*	5,211-7,886	2,676	Expresses the VNT1 protein for late blight protection
Rpi-vnt1 native promoter (pVnt1)	*S. venturii*	7,887-8,595	709	Drives expression of the *Rpi-vnt1*
Intervening sequence	Synthetic DNA	8,596-8,601	6	Sequence used for DNA cloning
***Rpi-amr3* cassette**				
Rpi-amr3 native promoter (pAmr3)	*S. americanum*	8,602-10,519	1,918	Drives expression of the *Rpi-amr3* gene
Rpi-amr3 gene coding sequence (*Amr3*)	*S. americanum*	10,520-13,183	2,664	Expresses the AMR3 protein for late blight protection
Rpi-amr3 native terminator (tAmr3)	*S. americanum*	13,184-13,953	770	Terminates transcription of *Rpi-amr3* gene
Intervening sequence	Synthetic DNA	13,954-13,965	12	Sequence used for DNA cloning
***Rpi-blb2* cassette**				
Rpi-blb2 native terminator (tBlb2)	*S. bulbocastanum*	13,966-16,497	2,532	Terminates transcription of *Rpi-blb2* gene
Rpi-blb2 gene coding sequence (*Blb2*)	*S. bulbocastanum*	16,498-20,387	3,890	Expresses the BLB2 protein for late blight protection
Rpi-blb2 native promoter (pBlb2)	*S. bulbocastanum*	20,388-21,932	1,545	Drives expression of the *Rpi-blb2* gene
Intervening sequence	Synthetic DNA	21,933-21,940	8	Sequence used for DNA cloning
***VInv / Ppo* inverted repeats**				
Granule-bound starch synthase promoter (pGbss)	*S. tuberosum* var. Ranger Russet	21,941-22,626	686	Drives expression of the VInv/Ppo inverted repeat

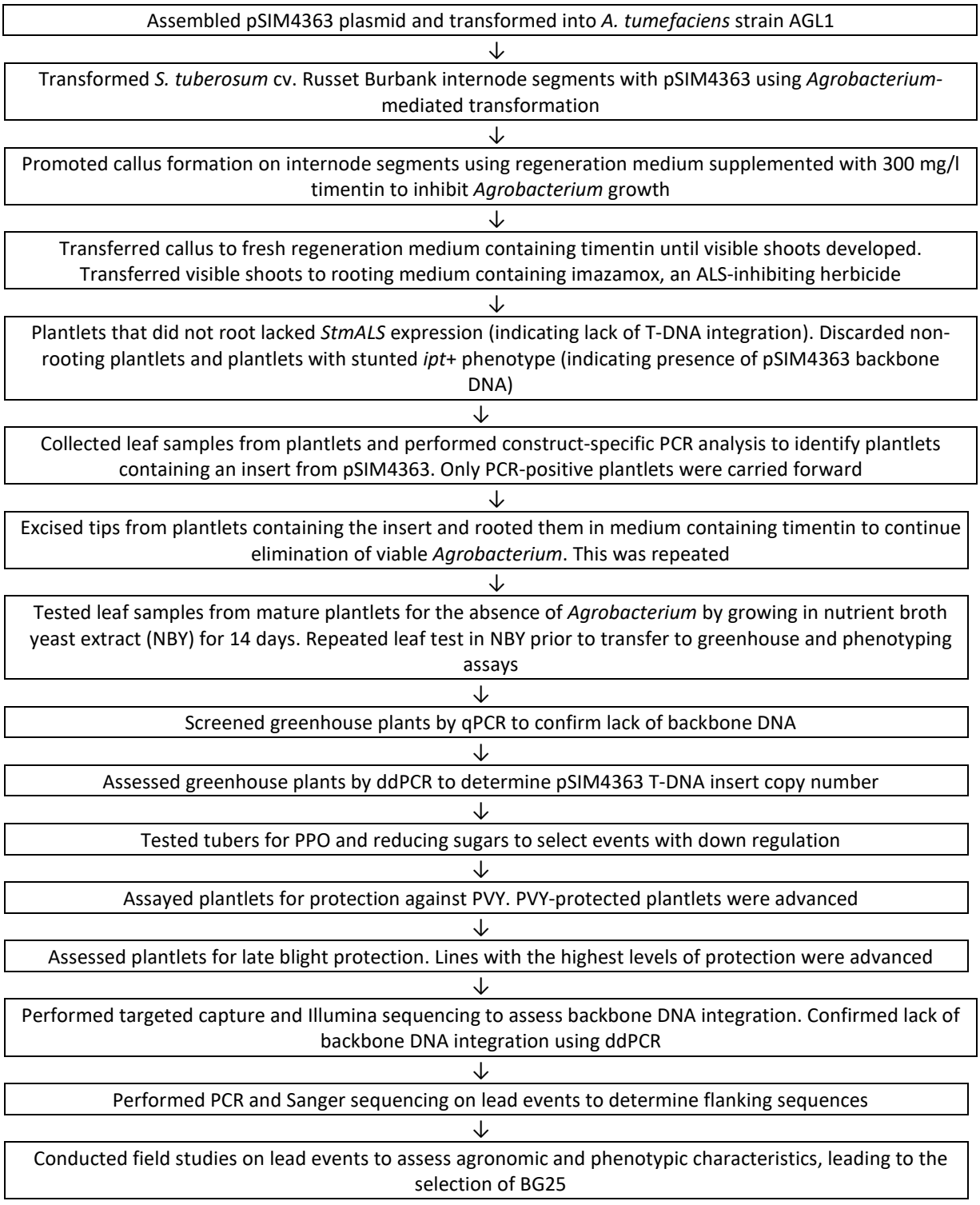
[17] ssDNA: Single-stranded DNA

Genetic Element	Source	Relative Position	Size (bp)	Description
Intervening sequence	Synthetic DNA	22,627-22,633	7	Sequence used for DNA cloning
Vacuolar invertase gene fragment (sense orientation; *VInv*)	*S. tuberosum* var. Ranger Russet	22,634-23,137	504	Generates dsRNA[18] to down regulate *VInv* transcripts
Intervening sequence	Synthetic DNA	23,138-23,143	6	Sequence used for DNA cloning
3'-untranslated region of the polyphenol oxidase gene (antisense orientation; *Ppo*)	*S. verrucosum*	23,144-23,287	144	Forms dsRNA to reduce expression of polyphenol oxidase to reduce black spot
Spacer 1	*S. tuberosum* var. Ranger Russet	23,288-23,450	163	Sequence between the inverted repeats; forms loop in dsRNA transcript
3'-untranslated region of the polyphenol oxidase gene (sense orientation; *Ppo*)	*S. verrucosum*	23,451-23,594	144	Forms dsRNA to reduce expression of polyphenol oxidase to reduce black spot
Intervening sequence	Synthetic DNA	23,595-23,601	7	Sequence used for DNA cloning
Vacuolar invertase gene fragment (antisense orientation, *VInv*)	*S. tuberosum* var. Ranger Russet	23,602-24,099	498	Generates dsRNA to down regulate invertase transcripts
Intervening sequence	Synthetic DNA	24,100-24,105	6	Sequence used for DNA cloning
ADP glucose pyrophosphorylase promoter (pAgp)	*S. tuberosum* var. Ranger Russet	24,106-26,365	2,260	Drives expression of VInv/Ppo inverted repeat
Intervening sequence	Synthetic DNA	26,366-26,377	12	Sequence used for DNA cloning
PVY-CP inverted repeats				
Polyubiquitin promoter (pUbi7)	*S. tuberosum* var. Ranger Russet	26,378-27,316	939	Drives expression of the PVY inverted repeats
StUbi7 gene intron	*S. tuberosum*	27,317-27,885	569	Improves expression of PVY-CP
Ubiquitin gene monomer	*S. tuberosum*	27,886-28,113	228	Expressed as a fusion with PVY-CP, then cleaved off
Intervening sequence	Synthetic DNA	28,114-28,119	6	Sequence used for DNA cloning
Potato virus Y coat protein gene fragment (sense orientation, PVY-CP)	*Potato virus Y* strain N	28,120-28,641	522	Generates dsRNA that target PVY genome for degradation
Spacer 2	*S. tuberosum* var. Ranger Russet	28,642-28,878	237	*Gbss* intron sequence between PVY inverted repeat to form loop in dsRNA
Potato virus Y coat protein gene fragment (antisense orientation, PVY-CP)	*Potato virus Y* strain N	28,879-29,400	522	Generates dsRNA that target PVY genome for degradation
Intervening sequence	Synthetic DNA	29,401-29,406	6	Sequence used for DNA cloning
Ubiquitin-3 gene terminator (tUbi3)	*S. tuberosum*	29,407-29,761	355	Terminator for PVY-CP dsRNA transcript
Intervening sequence	Synthetic DNA	29,762-29,770	9	Sequence used for DNA cloning
Right border (RB) buffer	*S. tuberosum* var. Ranger Russet	29,771-29,931	161	Supports primary cleavage at RB
RB	Synthetic	29,932-29,956	25	Primary cleavage site releases ssDNA insert from pSIM4363

[18] dsRNA: Double-stranded RNA

Appendix 2

Flowchart showing the development process used in the creation of potato line BG25



Appendix 3

Protein sequence alignment between the native full-length potato acetolactase synthase (StALS) and the modified potato ALS (StmALS) in BG25. The StmALS has two amino acid substitutions, **W**563**L** and **S**642**I** (highlighted in red). StALS and StmALS are 99.7% identical.

```
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
              ************************************** ****************
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