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Petition for Extension of Nonregulated Status for V11 Snowden Potatoes with Low Acrylamide Potential and Reduced Black Spot

The J.R. Simplot Company submits this petition under 7 CFR 340.6
to request that the Administrator make a determination
that the article should not be regulated under 7 CFR Part 340.

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J.R. Simplot Company

OECD unique identifier: SPS-∅∅V11-6

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Certification

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioners which are unfavorable to the petition.

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Release of Information

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List of Abbreviations, Acronyms, Definitions, and Commonly Used Terms

Abbreviation	Definition
AGP	Southern blot probe used to detect *Agp* promoter sequence
A. tumefaciens	*Agrobacterium tumefacians*
AP1	Adapter Primer 1
AP2	Adapter Primer 2
APHIS (USDA)	United States Department of Agriculture's Animal and Plant Health Inspection Service
ASN	Southern blot probe used to detect *Asn1* sequence
Asn1	Asparagine synthetase-1 gene
Backbone DNA	DNA associated with construct/vector backbone
bp	Base pair
BRS	Biotechnology Regulatory Services
cwt/A	Unit of measure equal to 100lbs/acre (weight in pounds of harvested tubers per acre divided by 100)
DNA	Deoxyribonucleic acid
DNA insert	The DNA sequence from pSIM1278 located between the LB and RB intended to be integrated into the potato genome
dsRNA	Double-stranded RNA
ETS	Excellence Through Stewardship
FDA	Food & Drug Administration
G0	First generation plants and tuber seed
G1	Plants and tubers resulting from G0 seed
G2	Plants and tubers resulting from G1 seed
G3	Plants and tubers resulting from G2 seed
GBS	Southern blot probe used to detect *Gbs* promoter sequence
IB	Internal bands
ipt	Isopentyltransferase gene – produces cytokinin hormones associated with plant growth and development
JB	Junction band
kb	Kilobase
LB	Left Border (a 25-base pair sequence) similar to *A. tumefaciens* T-DNA border
MOPS	3-(*N*-morpholino)propanesulfonic acid buffer
Non-coding DNA	DNA not coding for translated RNA
OB	Original Endogenous Bands
OECD	Organization for Economic Cooperation and Development
p*Agp*	Promoter of the ADP glucose pyrophosphorylase gene
PCR	Polymerase chain reaction
p*Gbss*	Promoter of the granule-bound starch synthase gene
PhL	Phosphorylase-L gene
PPO	Polyphenol oxidase enzyme
Ppo5	Polyphenol oxidase-5 gene
qPCR	Quantitative / real-time PCR

Abbreviation	Definition
R1	Water dikinase R1 gene
R1	Southern blot probe used to detect the R1 cassette (Chapter 5)
RB	Right Border (a 25-base pair sequence) similar to *A. tumefaciens* T-DNA border
RCB	Randomized complete block design
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantitative PCR
sRNA	All classes of small cellular RNAs
SSC	Saline sodium citrate buffer
T130	Positive control containing backbone
T-DNA	Transfer DNA from *A. tumefaciens* delineated by left and right border sequences
Ubi3	Polyubiquitin 3 terminator
Ubi7	Polyubiquitin 7 promoter
USDA	United States Department of Agriculture

Summary

The J. R. Simplot Company (Simplot) is seeking an extension of deregulated status for the Snowden potato variety event SPS-∅∅V11-6, hereafter referred to as V11. This petition is an extension of Simplot's 13-022-01p petition for the events that were deregulated on November 10, 2014. Simplot is commercializing three of the deregulated events:

- E12—Russet Burbank;
- F10—Ranger Russet; and
- J3—Atlantic.

For the reasons set forth in significant detail below, VII raises no new issues meriting separate review by APHIS under the petition process for nonregulated status of 7 CFR Part 340; rather, based on its similarity to previously deregulated events, VII qualifies for the extension process under 7 CFR 340.6(e).

The rationale for developing V11 potatoes is identical to the rationale for developing the previously deregulated events. Snowden potatoes, the second most popular public variety used for producing potato chips, were transformed in order to provide consumers and the potato industry with potatoes having lower acrylamide potential and reduced black spot. These trait additions would be difficult to achieve through traditional breeding because potato is tetraploid, highly heterozygous, and sensitive to inbreeding depression. Therefore, incorporating desirable traits such as low acrylamide potential and reduced black spot is most efficiently done by transforming each potato variety of commercial interest.

Lowering the acrylamide potential of potatoes is important because acrylamide presents a potential health risk for consumers. Although acrylamide is not present in fresh potatoes, it is formed when the amino acid asparagine and the reducing sugars glucose and fructose in carbohydrate-rich foods are heated at high temperatures. Therefore, reducing the concentrations of free asparagine, glucose, and fructose in potatoes reduces the acrylamide potential of cooked potatoes.

Reducing black spot in potatoes is important because it reduces grower, consumer, and processor waste. Black spot is a post-harvest physiological phenomenon primarily resulting from the handling of potato tubers during harvest, transport, and processing, and refers to the black or grayish color that may form in the interior of damaged potatoes. The enzymatic browning and discoloration is associated with the enzyme polyphenol oxidase (PPO). Enzymatic browning occurs when polyphenol oxidase leaks out from the plastids of damaged potatoes. Reducing the PPO concentrations in potatoes reduces the amount of black spot and therefore potato waste.

V11 was produced by transforming the potato variety Snowden with the same DNA and method that were used for the varieties in the 13-022-01p petition. The phenotype and traits of both V11 and the previously deregulated events are the same, as are the conclusions of the molecular, agronomic, phenotypic, and compositional assessments. The extension process was designed for the approval of new varieties of the same species that incorporate the same genes and traits as previously deregulated events; thus V11 meets the USDA's recommended guidance for extensions.

The genetic construct pSIM1278, used to transform the deregulated Russet Burbank, Ranger Russet, and Atlantic varieties, was used to transform the Snowden potatoes and generate V11. The T-DNA of pSIM1278 contains DNA sequence intended to down-regulate four genes through the mechanism of RNA interference (RNAi):

- *Asn1* (asparagine synthetase) for reduced free asparagine contributing to low acrylamide potential;
- *R1* (water dikinase) for lower reducing sugars contributing to low acrylamide potential;
- *PhL* (phosphoylase-L) for lower reducing sugars contributing to low acrylamide potential; and
- *Ppo5* (polyphenol oxidase-5) for reduced black spot.

As with the previously deregulated events, no novel proteins are expressed in V11. The previously deregulated events and V11 contain no marker genes. Molecular analysis demonstrated that V11 contains a single, intact copy of the pSIM1278 T-DNA with a 14-bp deletion of the left border and 3-bp deletion of the right border. No backbone DNA was inserted, and the DNA insert was shown to be stable across generations. Like the previously deregulated events, V11 contains a stable, well-characterized insert.

V11 has been field tested since 2011 in the U.S. in nine states under field release notifications granted by USDA-APHIS. Comprehensive assessments of phenotype and tubers for V11 were conducted in replicated field studies at a total of seven sites over two growing seasons. The following characteristics were measured:

- Phenotypic: early emergence, final emergence, stems per plant, plant vigor, plant height, vine desiccation; and
- Tuber: total yield, U.S. #1 yield, tubers per plant, size A tubers, size B tubers, oversize tubers, pickout tubers, specific gravities, and total internal defects.

Analysis of these data demonstrated that, similar to the previously deregulated events, V11 is phenotypically comparable to its parental control, Snowden, and other conventional potatoes. In addition, field studies were observed for naturally occurring abiotic and biotic (insect and disease) stressors, and no meaningful differences were observed between V11 and Snowden or other conventional potatoes. A volunteer study was conducted with V11, its parental control, Snowden, and additional conventional varieties. Results of this study demonstrated that V11, like its deregulated counterparts, has no greater potential for overwintering and producing volunteer plants than conventional potatoes. Together, these data support the conclusion that V11 is no more likely to pose a plant pest risk than the previously deregulated events or conventional potatoes, raises no new issues that would merit review under a separate petition for nonregulated status, and qualifies for the extension process under 7CFR 340.6(e).

Extensive nutrient composition analyses of tubers were conducted to compare the composition of V11 to that of its parental control, Snowden. The levels of proximates, vitamins, minerals, total amino acids, and glycoalkaloids were measured. Like the previously deregulated events, V11 is comparable to conventional potatoes with respect to nutrient and glycoalkaloid composition.

Free asparagine and reducing sugars were tested to evaluate trait efficacy in V11 with respect to lower acrylamide potential. Free amino acids and reducing sugars were measured in tubers, and acrylamide was measured in potato chips made from tubers at harvest and after storage. Like the previously deregulated events, chips made from V11 tubers contain less acrylamide than the controls when fried.

As with the previously deregulated events, the propagation of V11 through clonal propagation mitigates concerns about plant pest potential through seed dispersal, survival outside of cultivation, or outcrossing. The Snowden variety produces few flowers and rarely produces seeds. Other factors limiting outcrossing include the tendency for most fertile varieties to be self-pollinated, the relatively low likelihood of attracting honey bees because they lack nectar, and limitation of the pollen transfer range to about 20 meters. Furthermore, true potato seeds are not saved and propagated in the typical farming operation. If potatoes were grown from true potato seed, the offspring would be so diverse that they would not be useful as commercial potatoes. Wild potato varieties are rare in the United States and for the most part geographically isolated from commercial production areas, further reducing concerns about cross-pollination with wild species.

The traits in V11 are intended to enhance the desired quality traits, not the agronomic characteristics of potatoes. Planting, cultivation, management and harvesting techniques are not affected by the incorporated traits. Like the previously deregulated events, V11 is likely to be planted in areas that are already growing potatoes and will not result in a significant expansion of planted acres. Lower levels of asparagine, lower reducing sugars, and reduced black spot are highly unlikely to increase the weediness or invasiveness of potato because they do not influence the fundamental phenotypic characteristics or ecological competitiveness. Field trials over multiple years with V11 did not provide any evidence for altered growth characteristics such as accelerated tuber sprouting, increased plant vigor, increased tuber set, or delayed senescence. Like the previously deregulated events, V11 is unlikely to display enhanced weediness or impact insects and other non-target organisms, weed or disease susceptibility, endangered species, or biodiversity.

In conclusion, based on the data contained herein, Simplot requests that APHIS grant the request for an extension of the determination of nonregulated status to V11 and its progeny.

1.0 Rationale for the Development of V11 Potatoes

V11 (OECD unique identifier SPS-∅∅V11-6) is a new potato event developed by the J. R. Simplot Company (hereafter referred to as Simplot). V11 was generated by transforming the Snowden potato variety with a construct containing DNA potato sequences that confer via RNAi reduced free asparagine and lower reducing sugars, which together contribute to reduced acrylamide potential, and reduced *Ppo5*, which confers reduced black spot (Table 1-1).

The rationale for developing V11 potatoes is identical to the rationale for developing the ten potato events deregulated in Simplot's 13-022-01p petition. Snowden potatoes were transformed in order to provide the potato industry and consumers with lower acrylamide potential and reduced black spot traits. Such multiple trait additions would be difficult to achieve through traditional breeding because potato is tetraploid, highly heterozygous, and sensitive to inbreeding depression. Therefore, producing desirable traits such as low acrylamide potential and reduced black spot is most efficiently done by transforming each potato variety of commercial interest.

Table 1-1. Summary of V11 Genes and Intended Traits

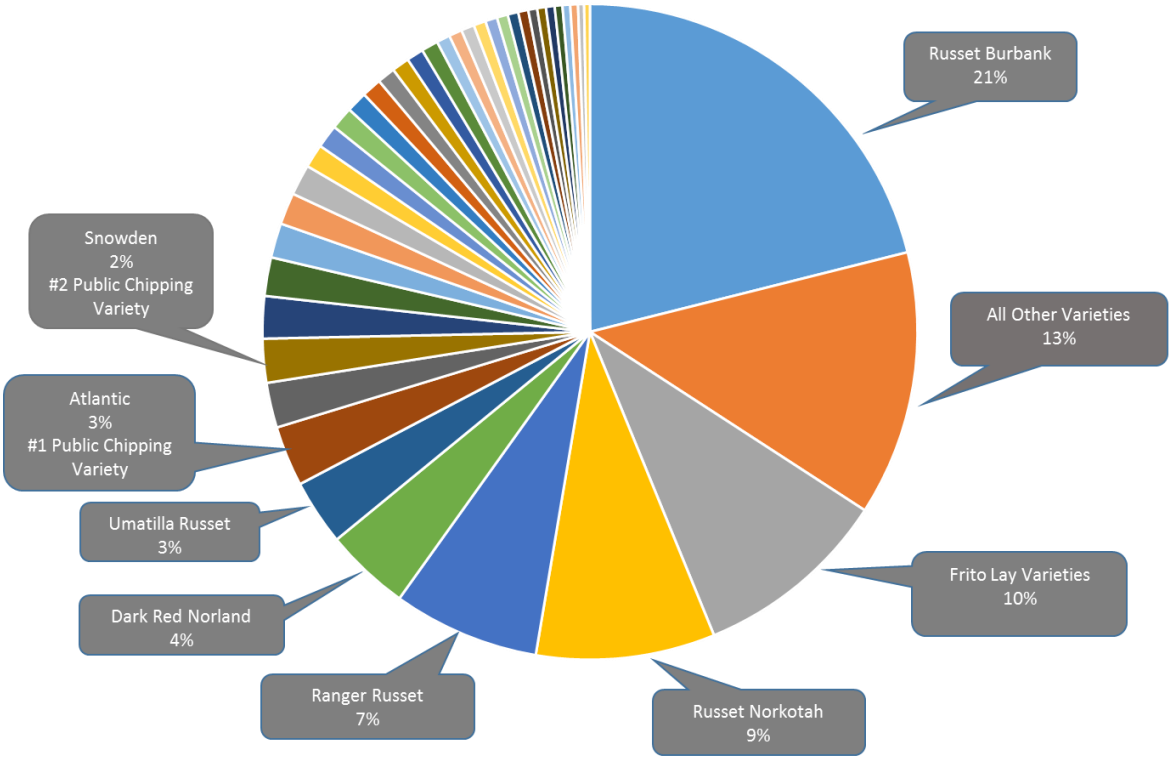
Gene	Intended Function	Intended Trait
Asn1: asparagine synthetase-1	Reduces free asparagine	Contributes to low acrylamide potential
R1: water dikinase	Lowers reducing sugars	Contributes to low acrylamide potential
PhL: phosphorylase-L	Lowers reducing sugars	Contributes to low acrylamide potential
Ppo5: polyphenol oxidase-5	Reduces enzymatic browning	Reduced black spot

Potatoes with lower acrylamide potential may address a possible health concern for consumers and the potato industry, particularly in light of recent toxicology studies (NTP, 2012 and USDA Environmental Assessment, 2014). Although acrylamide is not present in fresh potatoes, it is formed when the amino acid asparagine and the reducing sugars glucose and fructose in carbohydrate-rich foods are heated at temperatures above 120°C (248°F). Based on a recent U.S. Federal Register notice (FDA 2013), FDA has proposed guidance for industry on the reduction of acrylamide levels in food products. In FDA's Draft Guidance for Industry on Acrylamide in Foods, the FDA states that "reducing acrylamide in foods may mitigate potential human health risks from exposure to acrylamide." A rather extensive list of potential mitigation techniques were summarized in the guidance document (FDA 2013) focused primarily on the reducing sugar levels in potatoes. The rationale for the development of potatoes with low acrylamide potential was described on pages 13-15 of the 13-022-01p petition.

The purpose and need for developing potatoes with reduced black spot is reduced grower, consumer, and processor waste. Black spot is a post-harvest physiological phenomenon primarily resulting from the handling of potato tubers during harvest, transport, and processing, and refers to the black or grayish color that may form in the interior of damaged potatoes (USDA Environmental Assessment, 2014). The benefits associated with reducing the severity of black spot in potatoes was discussed on pages 13-15 of the 13-022-01p petition.

The rationale for adding Snowden to the list of potato varieties with low acrylamide potential and reduced black spot is that Snowden is the 2nd most popular public potato variety used for making potato chips (Figure 1-1). Snowden represents significant value to the potato industry because unlike the Atlantic variety, which must be processed right after harvesting, the Snowden variety can be stored for over six months (UNL Crop Watch, 2015). In 2013, Snowden comprised approximately 2% of seed potato acreage in the U.S. (National Potato Council, 2014).

Figure 1-1. 2013 Certified Seed Potato Acres, By Variety



Source: National Potato Council, 2014

1.1 Basis for Determination of Nonregulated Status under 7 CFR §340.6

The Animal and Plant Health Inspection Service (APHIS) and the U.S. Department of Agriculture (USDA) have responsibility under the Plant Protection Act (7 U.S.C. 7701-7772) to prevent the introduction or dissemination of plant pests into or within the United States. Part 340 regulates the introduction of organisms produced or altered through genetic engineering that are or may be considered plant pests. The APHIS regulations at 7 CFR §340.6(e) provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk based on its similarity to previously deregulated events and therefore should no longer be regulated.

Simplot is submitting data for genetically engineered low acrylamide potential and reduced black spot V11 potato and requests a determination from USDA-APHIS that event SPS-∅∅V11-6 and its progeny no longer be considered regulated articles under 7 CFR Part 340.

1.2 Rationale for Using the Extension Process

Section 340.6(e) of the regulations provides that APHIS may extend a determination of nonregulated status to additional regulated articles, upon finding that the additional regulated articles do not pose a potential for plant pest risk, and should therefore not be regulated. Such a finding would be made based on an evaluation of the similarity of the additional regulated articles to an antecedent organism, i.e., an organism that has already been the subject of a determination of nonregulated status by APHIS under Section 340.6, and that is used as a reference for comparison to the regulated article under consideration under the regulations (USDA, 2015).

On November 10, 2014, USDA-APHIS deregulated ten Innate™ potato events in petition 13-022-01p. Simplot is commercializing three of these events:

- E12 —Russet Burbank variety;
- F10 —Ranger Russet variety; and
- J3 —Atlantic variety.

At the present time, Simplot intends to pursue commercialization of the E12, F10, and J3 events. Therefore, for the purposes of this extension, those three events will be considered the antecedent events (hereafter referred to as previously deregulated events), although any or all of the ten previously deregulated events in 13-022-01p would suffice for this purpose.

V11 was produced by transforming an additional variety of potato, Snowden, with the same DNA and method that was used for the antecedent varieties (Table 1-2). The phenotype and traits are also the same for all the events, as are the conclusions of the molecular, agronomic, phenotypic, and compositional assessments. The extension process is suited for the approval of new varieties of the same species that incorporate the same genes and traits as previously deregulated events; thus V11 meets the requirements for an extension, based upon the USDA's recommended guidance.

Table 1-2. Comparison of Previously Deregulated Events to V11

	Deregulated Events E12, F10, and J3	**V11**
USDA Number	13-022-01p	Not yet issued by USDA
Date of Deregulation	November 10, 2014	Not yet deregulated by USDA
Date of USDA Final Environmental Assessment	July 2014	Not yet assessed by USDA
Crop	Potato	Same
Recipient Organism	*Solanum tuberosum*	Same
Varieties	Russet Burbank, Ranger Russet, and Atlantic	Snowden
Transformation Method	*Agrobacterium tumefaciens*	Same
Construct	pSIM1278	Same
Traits	Lower acrylamide potential and reduced black spot	Same
Genes	*Asn1, R1, PhL, and Ppo5*	Same
Gene Functions	*Asn1* down-regulation: reduced free asparagine *R1* down-regulation: lower reducing sugars *PhL* down-regulation: lower reducing sugars *Ppo5* down-regulation: reduced enzymatic browning	Same
Molecular Characterization	Each event is well characterized and contains a stable insert	Same
Agronomic Assessment	Each event is agronomically and phenotypically similar to its parent variety	Same
Compositional Assessment	Each event is compositionally similar to its parent variety, other than the intended changes for efficacy	Same
Plant Pest Characteristics	Each event is similar to its parent variety in terms of reproductive fitness, gene flow, and other plant pest characteristics	Same

1.3 Prior Environmental Release of V11 and Submissions to Other U.S. Regulatory Agencies

As a new transformation event, V11 is regulated by the United States Department of Agriculture-Animal and Plant Health and Inspection Service, Biotechnology Regulatory Services (USDA-APHIS BRS). V11 falls within the scope of the Food and Drug Administration's (FDA) policy statement concerning regulation of food products derived from new plant varieties, including those developed by recombinant DNA techniques. A voluntary safety and nutritional assessment of V11 will be submitted to the FDA's Center for Food Safety and Applied Nutrition (FDA CFSAN).

V11 has been extensively field tested in the U.S. since 2011 in nine states as authorized by the USDA-APHIS permits and notifications listed in Appendix B (Table B-1).

1.4 References

FDA. (2013). Guidance for Industry, Acrylamide in Foods.

NPC. (2013). National Potato Council, 2013 Statistical Yearbook.

NTP. (2012). Technical Report on the Toxicology and Carcinogenesis Studies of Acrylamide in F344/N Rats and B6C3F1 Mice. National Toxicology Program. National Institutes of Health. Public Health Service. U.S. Dept. of Health and Human Services.

UNL Crop Watch. (2015). Snowden Management Profile. (Accessed 2015). http://cropwatch.unl.edu/potato/snowden_characteristics

USDA Environmental Assessment (2014). J.R. Simplot Company Petition (13-022-01p) for Determination of Non-Regulated Status for Innate™ Potatoes with Low Acrylamide Potential and Reduced Black Spot Bruise: Events E12 and E24 (Russet Burbank); F10 and F37 (Ranger Russet); J3, J55, and J78 (Atlantic); G11 (G); H37 and H50 (H). (Accessed 2015) http://www.aphis.usda.gov/brs/aphisdocs/13_02201p_fea.pdf

USDA Guidance on Petitions for Extensions of Non-Regulated Status (2015). (Accessed 2015). http://www.aphis.usda.gov/wps/portal/aphis/ourfocus/biotechnology?1dmy&urile=wcm%3apath%3a%2Faphis_content_library%2Fsa_our_focus%2Fsa_biotechnology%2Fsa_permits_notifications_and_petitions%2Fsa_guidance_documents%2Fct_extensions

2.0 Characterization of the Snowden Variety

The Snowden variety was developed by the University of Wisconsin and released in 1990 (PAA 2009) as a public variety. Plants are tall, semi-erect, and have lightly pigmented stems with slight pubescence. The leaves are open with an olive green color and slightly pubescent. There are few flowers that are medium sized and white with yellow anthers. Under most conditions no seed berries are produced. The tubers are round to oval with buff-colored skin and white flesh. Snowden is a high-yielding variety that produces a high tuber set. Snowden responds well to nitrogen and needs high irrigation. The Snowden variety has high specific gravity, good storability, and short dormancy. Snowden is an excellent chipping

variety from the field as well as at storage. Snowden potatoes have been shown to store for up to nine months at 45°F with low sugar levels (UNL Crop Watch, 2015).

2.1 References

PAA. (2009). Snowden Variety Description. Potato Association of America. (Accessed 2015) http://potatoassociation.org/Industry%20Outreach/varieties/White%20Rounds/snowden.html.

UNL Crop Watch. (2015). Snowden Characteristics. University of Nebraska Extension. (Accessed 2015). http://cropwatch.unl.edu/potato/snowden_characteristics.

3.0 Description of DNA Used for Transformation

The DNA used to transform Snowden to generate V11 is pSIM1278 (Figure 3-1), which is identical to the DNA used to generate the previously deregulated events (described on pp. 27-30 in petition 13-022-01p; Collinge and Clark, 2013).

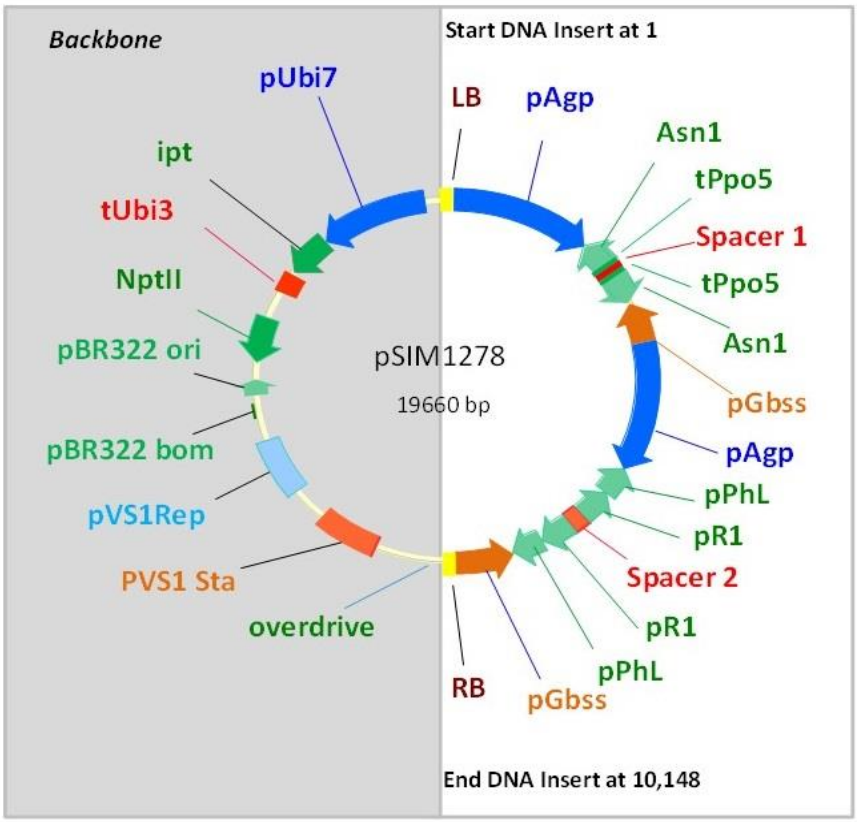
Briefly, pSIM1278 T-DNA contains two silencing cassettes (Figure 3-2 and Table 3-1):

- The first cassette down-regulates expression of the potato asparagine synthetase-1 gene (*Asn1*) and the potato polyphenol oxidase-5 gene (*Ppo5*). This cassette is comprised of two 404-bp inverted repeat fragments of *Asn*1 and two 143-bp inverted repeat fragments of *Ppo5*. The *Asn1* and *Ppo5* fragments are arranged between the two convergent native potato promoters—the potato *Agp* promoter of the ADP glucose pyrophosphorylase gene (*Agp*) and the potato *Gbss* promoter of the granule-bound starch synthase gene (*Gbss*)—that are primarily active in tubers. These promoters drive expression of the inverted repeats to generate double-stranded RNA targeting the native potato genes *Asn1* and *Ppo5* for down-regulation, also known as gene silencing.

- The second cassette lowers reducing sugars by down-regulating the potato phosphorylase-L gene (*PhL*) gene and the potato starch-associated gene (*R1*) by targeting the down-regulation of their promoters. This cassette is comprised of an inverted repeat fragment of the 508-bp promoter region *PhL* and a 531-bp inverted repeat fragment of *R1*. Similar to the first cassette, the *PhL* and *R1* inverted repeat fragments are transcribed by the potato *Agp* and *Gbss* promoters.

The construct backbone (Figure 3-1) contains well-characterized bacterial origins of replication from constructs pVS1 and pBR322, and the *nptII* gene for bacterial resistance to kanamycin. In addition, the backbone contains an expression cassette comprising the *Agrobacterium ipt* gene flanked by the plant's polyubiquitin (*Ubi7*) promoter and polyubiquitin (*Ubi3*) terminator, which was introduced as a 2.6-kb SacII fragment into the vector backbone (Garbarino and Belknap 1994; Collinge and Clark, 2013 - Table 4, page 28). The complete genetic elements of pSIM1278 T-DNA from the left border site to the right border are shown in Figure 3-2.

Figure 3-1. Schematic Diagram of pSIM1278



The vector backbone region, indicated in grey, starts at position 10,149-bp and ends at 19,660-bp (9,512-bp total). The backbone DNA consists mainly of bacterial DNA and is intended only to support maintenance of the DNA insert prior to plant transformation. The DNA insert region, is on the right from 1-bp to 10,148-bp, for a total of 10,148-bp. The DNA insert consists of T-DNA border sequences and potato genomic DNA which are intended for stable integration into the plant genome.

Figure 3-2. T-DNA of pSIM1278 Intended for Insertion

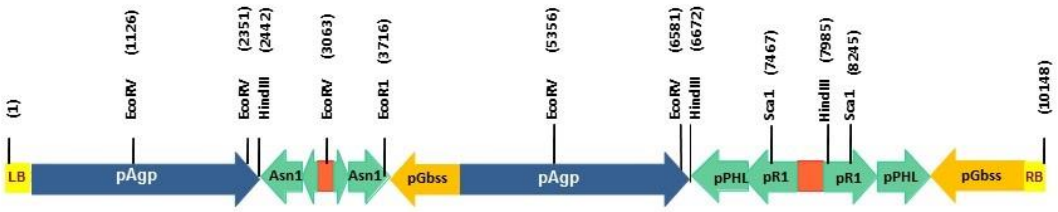


Table 3-1. Genetic Elements of pSIM1278 T-DNA, from Left Border Site to Right Border

Genetic Element	*Origin*	Position (pSIM1278)	Intended Function
1. Left Border (LB) site[1]	Synthetic	1 – 25	Site for secondary cleavage to release single-stranded DNA insert from pSIM1278 (van Haaren et al. 1989)
2. Left Border region sequence including LB	*S. tuberosum* var. Ranger Russet.	26 – 187	Supports secondary cleavage at LB
3. Intervening Sequence	*S. tuberosum*	188 –193	Sequence used for DNA cloning
4. Promoter for the ADP glucose pyrophosphorylase gene (pAgp), 1st copy	*S. tuberosum* var. Ranger Russet	194-2,453	One of the two convergent promoters that drives expression of an inverted repeat containing fragments of Asn1 and Ppo5, especially in tubers
5. Fragment of the asparagine synthetase-1 (Asn1) gene (1st copy antisense orientation)	*S. tuberosum* var. Ranger Russet	2,454-2,858	Generates with (11) double stranded RNA that triggers the degradation of Asn1 transcripts to impair asparagine formation (Chawla et al., 2012[2])
6. 3'-untranslated sequence of the polyphenol oxidase-5 gene (Ppo5) (1st copy, in antisense orientation)	*S. verrucosum*	2,859-3,002	Generates with (9) double stranded RNA that triggers the degradation of Ppo5 transcripts to block black spot development
7. Intervening Sequence	*S. tuberosum*	3,003-3,008	Sequence used for DNA cloning
8. Spacer-1	*S. tuberosum* var. Ranger Russet	3,009-3,165	Sequence between the 1st inverted repeats
9. 3'-untranslated sequence of the polyphenol oxidase-5 gene (Ppo5) (2nd copy, in sense orientation)	*S. verrucosum*	3,166-3,309	Generates with (6) double stranded RNA that triggers the degradation of Ppo5 transcripts to block black spot development
10. Fragment of the asparagine synthetase-1 (Asn1) gene (2nd copy, in sense orientation)	*S. tuberosum* var. Ranger Russet	3,310-3,715	Generates with (5) double stranded RNA that triggers the degradation of Asn1 transcripts to impair asparagine formation (Chawla et al., 2012[2])
11. Intervening Sequence	*S. tuberosum*	3,716-3,721	Sequence used for DNA cloning
12. Promoter for the granule-bound starch synthase (pGbss) gene (1st copy, convergent orientation relative to the 1st copy of pAgp)	*S. tuberosum* var. Ranger Russet	3,722-4,407	One of the two convergent promoters that drives expression of an inverted repeat containing fragments of Asn1 and Ppo5, especially in tubers
13. Intervening Sequence	*S. tuberosum*	4,408-4,423	Sequence used for DNA cloning
14. pAgp, 2nd copy	*S. tuberosum* var. Ranger Russet	4,424-6,683	One of the two convergent promoters that drives expression of an inverted repeat containing fragments of the promoters of PhL and R1, especially in tubers
15. Fragment of promoter for the potato phosphorylase-L (pPhL) gene (1st copy, in antisense orientation)	*S. tuberosum* var. Ranger Russet	6,684-7,192	Generates with (21) double stranded RNA that triggers the degradation of PhL transcripts to limit the formation of reducing sugars through starch degradation

Table 3-1. Genetic Elements of pSIM1278 T-DNA, from Left Border Site to Right Border (continued)

Genetic Element	*Origin*	Position (pSIM1278)	Intended Function
16. Fragment of promoter for the potato R1 gene (pR1) (1st copy, in antisense orientation)	*S. tuberosum* var. Ranger Russet	7,193-7,724	Generates with (20) double stranded RNA that triggers the degradation of R1 transcripts to limit the formation of reducing sugars through starch degradation
17. Intervening Sequence	*S. tuberosum*	7,725-7,730	Sequence used for DNA cloning
18. Spacer-2	*S. tuberosum* var. Ranger Russet	7,731-7,988	Sequence between the 2nd inverted repeat
19. Fragment of promoter for the potato R1 gene (pR1) (2nd copy, in sense orientation)	*S. tuberosum* var. Ranger Russet	7,989-8,520	Generates with (20) double stranded RNA that triggers the degradation of R1 transcripts to limit the formation of reducing sugars through starch degradation
20. Fragment of promoter for the potato phosphorylase-L (pPhL) gene (2nd copy, in sense orientation)	*S. tuberosum* var. Ranger Russet	8,521-9,029	Generates with (16) double stranded RNA that triggers the degradation of PhL transcript to limit the formation of reducing sugars through starch degradation
21. pGbss (2nd copy, convergent orientation relative to the 2nd copy of pAgp)	*S. tuberosum* var. Ranger Russet	9,030-9,953	One of the two convergent promoters that drives expression of an inverted repeat containing fragments of the promoters of PhL and R1, especially in tubers
22. Intervening Sequence	*S. tuberosum*	9,954 – 9,962	Sequence used for DNA cloning
23. Right Border region sequence including RB	*S. tuberosum* var. Ranger Russet	9,963 – 10,123	Supports primary cleavage at RB-Like site
24. Right Border (RB) sequence[1]	Synthetic	10,124 – 10,148	Site for primary cleavage to release single stranded DNA insert from pSIM1278 (van Haaren et al. 1989)

[1]The LB and RB sequences (25-bp each) were synthetically designed to be similar to and function like T-DNA borders from *Agrobacterium tumefaciens*.

[2]ASN1 described as genetic elements 5 and 11 is referred to as StAst1 in Chawla et al. 2012.

[3] GenBank Accession HM36756H is replaced with a citation to GenBank Accession U26831 to properly include four 3' end nucleotides present in the pGbss DNA element of the pSIM1278 construct.

[4] GenBank Accession HM363755 is replaced with a citation to GenBank Accession X83220 to properly include the full pGbss (2nd copy) DNA insert sequence present in the pSIM1278 construct.

[5]GenBank Accession AY566555 was revised to clarify the sources of DNA for the Border regions.

3.1 Activity and Function of the Down-regulated Genes in V11

The activity and function of the down-regulated genes in V11 are identical to those in the previously deregulated events. Transcription of the expression cassettes in pSIM1278 does not lead to protein expression, but instead leads to the production of double-stranded RNA (dsRNA). The dsRNA transcript is derived from DNA sequence from the four target potato genes (*Asn1*, *PhL*, *R1,* and *Ppo5*) that are in turn down-regulated by the resulting transcribed dsRNA sequence through the plant's RNA interference (RNAi) pathway.

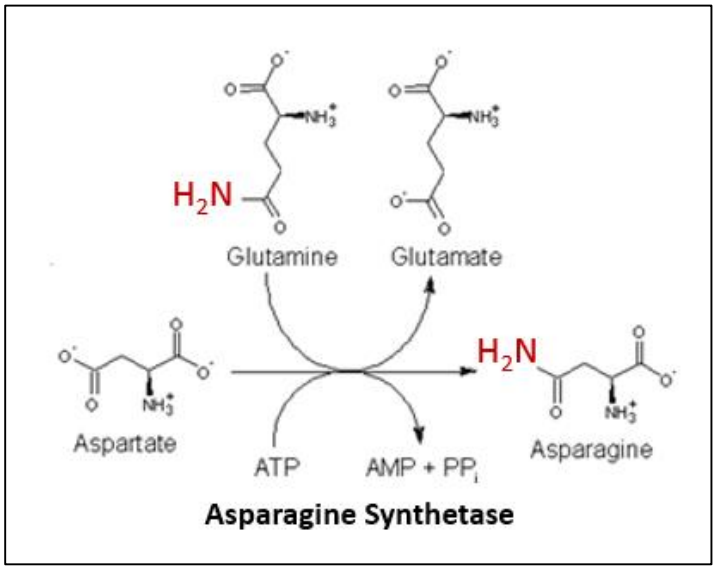
To reduce asparagine, lower reducing sugars, and reduce black spot in the previously deregulated events and V11, four native potato genes were targeted for down-regulation:

1. Asparagine synthetase 1 (*Asn1*) for its biochemical role in asparagine production;
2. Water dikinase R1 (*R1*) for its role in accumulation of reducing sugars;
3. Phosphorylase-L (*PhL*) for its role in accumulation of reducing sugars; and
4. Polyphenol oxidase (*Ppo5*) for its role in production of black spot;

Below is a brief description of the function in the potato of the proteins produced by these genes.

Asparagine Synthetase 1 (*Asn1*)
The expressed protein product of *Asn1*, ASN1, catalyzes the conversion of glutamine to asparagine by transferring the side-chain amine (NH_2) from glutamine to aspartate to form asparagine (Figure 3-3). Asparagine and glutamine are thought to play an important role in the transport and storage of nitrogen (Lehmann and Ratajczak, 2008). While most nitrogen is transported to the potato tuber as glutamine, the majority of glutamine is converted to asparagine by ASN1 (Chawla et al., 2012). Asparagine is the predominant free amino acid in potato tubers and constitutes up to 25% of the total free amino acid pool in tubers (Golan-Goldhirsh, 1986; Koch et al., 2003).

Figure 3-3. Biosynthesis of Asparagine in Plants



It has been shown that down-regulating *Asn1* in tubers decreases the levels of free asparagine while increasing free glutamine levels, without affecting plant growth or tuber phenotype. Furthermore, glutamine does not represent a significant precursor for acrylamide (Stadler, 2005).

Asparagine is a substrate of the Maillard reaction that converts amino acids and reducing sugars to acrylamide during high-temperature processing. This reaction occurs between sugars and amino acids, affecting changes in the color, flavor, functional properties and nutritional value of food (O'brien and Morrissey 1989). Reduction of ASN1 and asparagine levels in potato tubers by down-regulating *Asn1* has been shown to reduce acrylamide levels by as much as 70% in tubers (Collinge and Clark, 2013).

Water Dikinase R1 (*R1*)
Starch is one of the most abundant polymers in nature and is abundant in potato tubers as a storage carbohydrate. In cold storage, the starch in tubers is converted to reducing sugars during a process known as cold storage sweeting. The degradation of starch into reducing sugars has been shown to be due in part by the phosphorylation of the starch molecules in the tuber by R1 (Ritte et al., 2002, 2006).

R1 catalyzes the transfer of phosphates of ATP to α-glucan and water, resulting in phosphorylated starch (Lorberth et al., 1998). Phosphorylation affects the degree of crystalline packing within the starch granule and makes it more accessible to degradation. Thus, loss of R1 activity impairs starch degradation, which reduces accumulation of the reducing sugars glucose and fructose (Ritte et al., 2002, 2006). Down-regulation of this gene can lead to lower concentrations of reducing sugars and help contribute to low acrylamide potential via the Maillard reaction.

α-Glucan Phosphorylase, Starch Phosphorylase L (*PhL*)
The phosphorylase *PhL* gene is also thought to be responsible for degradation of starch to sugar during cold storage (Sonnewald et al., 1995). Phosphorylase L degrades starch by phosphorolytic release of glucose-1-phosphate from glucan chains. Down-regulation of *PhL* in potato and mutation in *Arabidopsis* does not alter total starch levels, but a loss of the enzymatic activity limits reducing sugar accumulation. Down-regulation of this gene can lead to lower concentrations of reducing sugars and help contribute to low acrylamide potential via the Maillard reaction.

Polyphenol Oxidase 5 (*Ppo5*)
Polyphenol oxidase enzymes (PPO) are found in most organisms including animals, plants, fungi and bacteria. Polyphenol oxidases are copper metalloenzymes that oxidize mono- and o-diphenols to o-diquinones by utilizing molecular oxygen (Thipyapong et al., 2004). Typically, PPO activity is latent until the enzyme is released by disruption of the cell structure through forces like wounding and senescence. When cell membranes are damaged, PPO enzyme is released and reacts along with oxygen molecules to produce quinones (Thipyapong et al., 2004). PPO and its role in the production of black and brown quinones are of interest in the post-harvest physiology of many fruit and vegetable crops.

The blackening that occurs after potatoes are peeled or bruised is a phenomenon caused by leakage of polyphenol oxidase from damaged plastids into the potato cell's cytoplasm (Thygesen, 1995). In addition, impacts sustained during harvest and post-harvest activities induce the release of PPO from cell plastids resulting in negative effects on quality and recovery in processing fries and chips, as well as the marketability of fresh potatoes. A family of six genes encoding PPO exists in potato, with one gene (*Ppo5*) being tuber-specific and the remaining five genes responsible for PPO expression in other tissues. By down-regulating *Ppo5*, reduced black spots in cut potatoes have been shown (Collinge and Clark, 2013).

3.2 References

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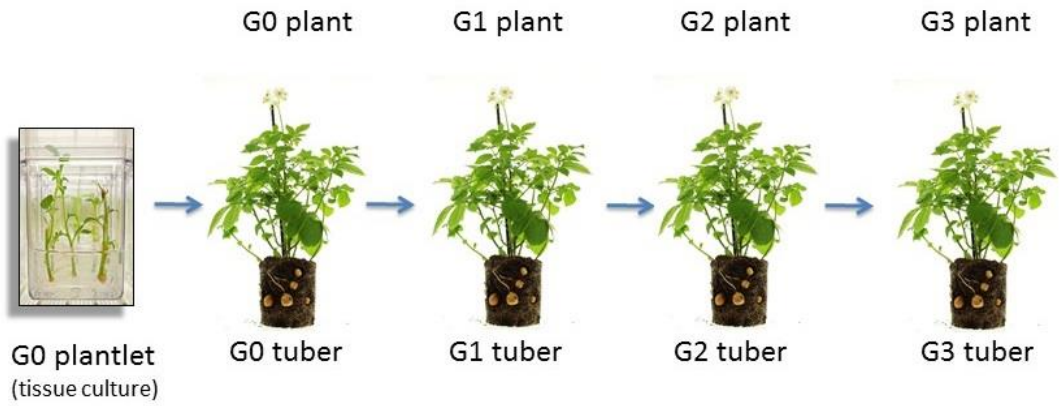
4.0 Development of V11: Description of Marker-Free Transformation

Potato (*Solanum tuberosum* subsp. *tuberosum*) event V11 was developed by *Agrobacterium*-mediated transformation using the same method and genetic material as the previously deregulated events. A description of the transformation methods was provided on pages 24 and 25 of the 13-022-01p petition.

Briefly, transformation was carried out using a modified procedure based on Richael et al., 2008. Potato internode segments of four to six mm were cut from four-week old plants and infected with *Agrobacterium* AGL1 strain carrying pSIM1278. Explants were grown on hormone-free media containing timentin to inhibit *Agrobacterium* growth. Leaf samples from mature G0 plantlets (Figure 4-1) testing positive for the pSIM1278 T-DNA insert were propagated and assayed for the absence of *Agrobacterium*. *Agrobacterium*-free G0 plants were then transferred to greenhouse facilities.

Figure 4-1. Potato Production Generations



Events were analyzed using Southern blots to identify plants containing a single insert of the T-DNA. Based on these results and field trials, V11 was selected and further assessed for insert integrity, copy number, gene silencing and agronomic performance. Subsequent V11 generations were developed through clonal propagation.

Solanum tuberosum varieties are prone to somaclonal variation and even in tissue culture frequently exhibits a degree of heterogeneity (OECD, 1997). Somaclonal variation (genetically dissimilar individuals derived from vegetative propagation, especially common after tissue culture in which a callus stage is included) is mitigated in several steps throughout the event selection process. Initially, a large number of transformation events are produced. The events are carefully observed by trained personnel, and those exhibiting phenotypes with notably poor vigor or off-types are removed. Any potential impact of somaclonal variation would be addressed in replicated field trials where events are grown in several geographic regions and scouted for undesirable anomalies and off-types. This selection process is conducted by experienced agronomists who observe the transformed events and compare with commercial varieties. Further studies to support the lack of meaningful somaclonal variation in V11, including phenotypic evaluations, are discussed in Section 8 of this submission.

4.1 References

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5.0 Genetic Characterization of V11

Like the previously deregulated events, V11 contains an insert derived from transformation with the construct pSIM1278. Molecular analyses demonstrated that V11 contains a single, intact copy of the pSIM1278 insert with a 14-bp deletion of the left border region and a 3-bp deletion of the right border region within the Snowden genome (Figure 5-1). Like the previously deregulated events, V11 contains a stable, well-characterized insert at a single locus with no backbone DNA present. The insert is genetically stable across generations.

Figure 5-1. Structure of the T-DNA Insert Within the Genome of V11

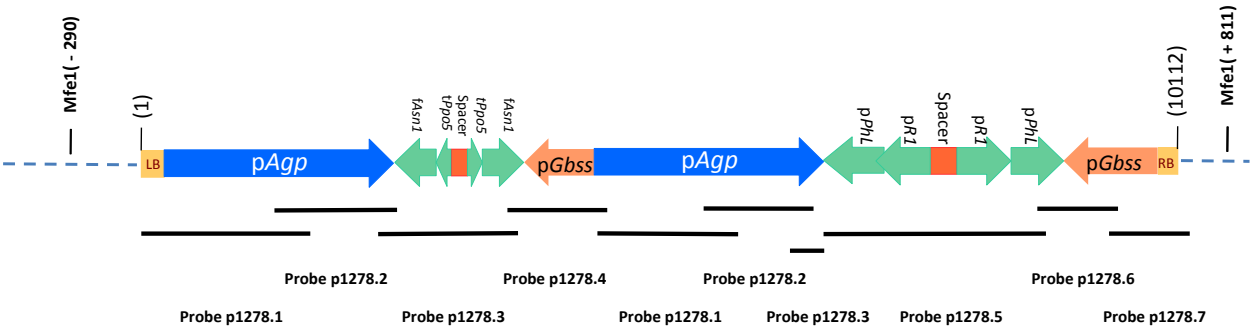
A detailed characterization of V11 included:

(1) Southern blot verification that the pSIM1278 T-DNA insert integrated into a single genomic locus: Section 5.1;
(2) Structural determination of the insert by combining the Southern blot analyses with PCR and sequencing analyses (Appendix A: Molecular Methods): Section 5.2;
(3) Confirmation of absence of pSIM1278 backbone sequence: Section 5.3; and
(4) Confirmation of genetic stability of the insert across generations: Section 5.4.

5.1 Characterization of Insert Number in V11

To assess the number of inserts derived from pSIM1278 in V11, a series of overlapping probes were designed to cover the entire length of the original T-DNA and used to analyze the genome of V11 by Southern blotting. Genomic DNA was digested with the restriction enzyme, MfeI, which does not cut within the T-DNA itself, and thus results in DNA fragments containing an entire insert along with its adjacent plant genomic DNA (Figure 5-2).

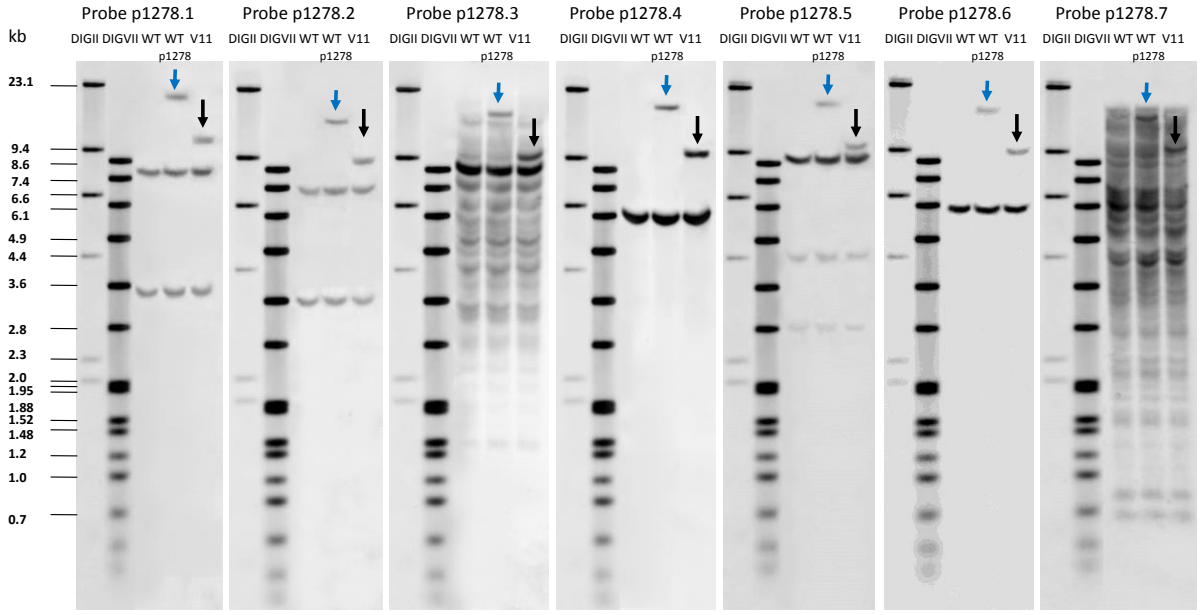
Figure 5-2. Probes and Restriction Sites Used for Insert Analysis of V11



Schematic of pSIM1278 T-DNA along with a set of 7 overlapping probes that cover the entire insert. The flanking regions (dashed lines) and location of MfeI restriction sites are indicated

As shown in the Southern blots presented in Figure 5-3, only a single band of consistent size (~11kb) was observed as specific for V11 using each probe. A copy number and sensitivity control was included by spiking approximately a single genome equivalent of the pSIM1278 construct into a sample of Snowden control DNA (WT p1278). This control ensures the probe sensitivity is capable of detecting a single insert in the genome. The location of the MfeI restriction sites and those described in Figure 5-4 were confirmed by identification and sequencing of the junction regions and flanking sequence using standard molecular approaches (Appendix A: Molecular Methods). Collectively, these data indicate that transformation with pSIM1278 resulted in a single insert flanked by the indicated MfeI sites in the Snowden genome.

Figure 5-3. Southern Blots Showing a Single Insert in the V11 Genome



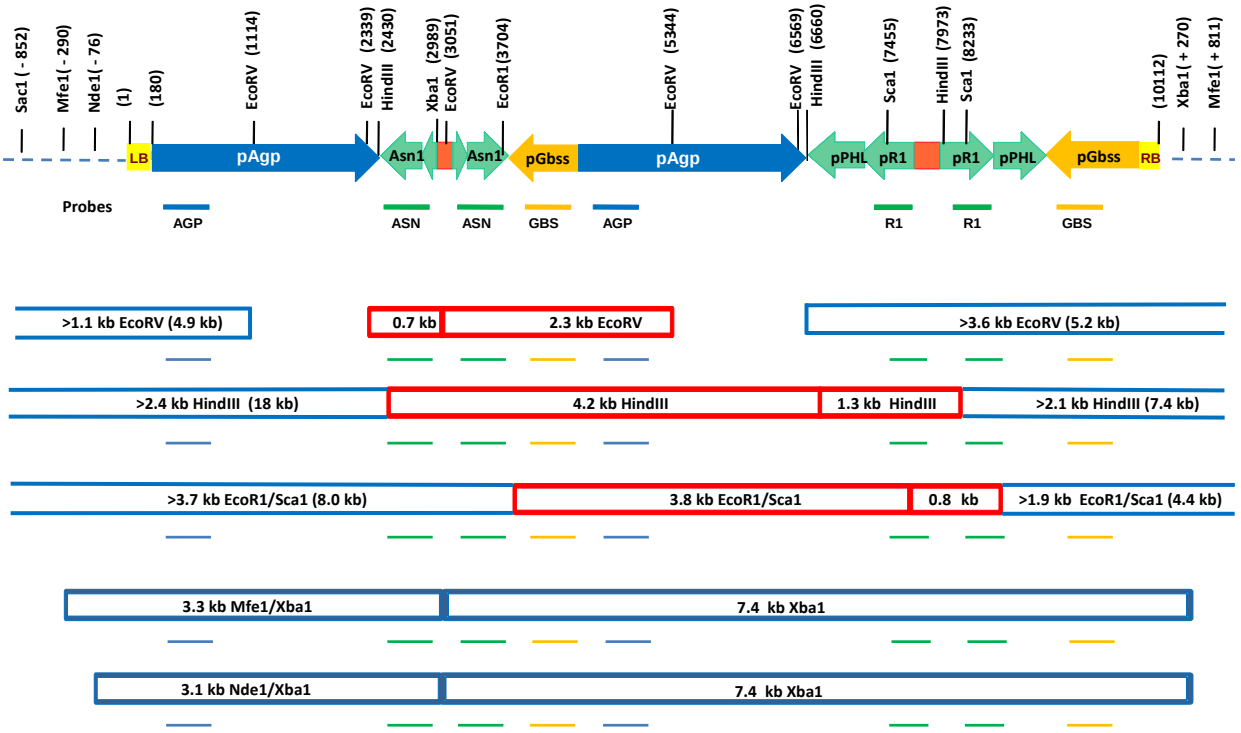
Southern blots of MfeI-digested genomic DNA isolated from Snowden V11 (V11), Snowden control (WT), and Snowden control spiked with pSIM1278-digested plasmid DNA (WT p1278). The restriction enzyme, MfeI, cuts in the flanking region and not within the pSIM1278 T-DNA insert itself. Thus, the entire T-DNA insert is contained within the single band migrating above the 9.4 kb molecular weight marker (black arrows). A copy number control was included by spiking pSIM1278 construct DNA into a Snowden conventional control sample at a concentration of approximately 1 copy / genome prior to digestion (blue arrows). The molecular weight markers, DIGII and DIGVII, are shown in the first two lanes at the left of each gel, respectively. The expected pSIM1278 insert fragment is indicated by a black arrow in each blot. DNA was separated on agarose gels (TAE) using extended electrophoresis to ensure good separation.

5.2 Characterization of Copy Number and Structure of the DNA Insert in V11

The Southern blot analyses presented in this section included a set of probes that hybridize to elements contained within the T-DNA insert, but also recognize endogenous sequence within the plant genome. The probes were used to analyze the copy number and structure by comparing the restriction digest banding patterns of genomic DNA isolated from V11 with the Snowden conventional variety.

The T-DNA insert is summarized in Figure 5-4 along with the probes and their binding sites, pertinent restriction sites, and restriction fragments corresponding to the digests used in the analysis. The bands associated solely with the T-DNA insert (internal bands) are depicted as red boxes whereas bands linking the insert to the flanking region (junction bands) are depicted in blue. The expected size of each band is provided for cross-reference with the Southern blots provided in Figures 5-5 through 5-10.

Figure 5-4. Structure of pSIM1278 DNA Insert Digestion and Hybridization Pattern in V11



The figure represents the structure of the insert associated with the pSIM1278 construct, including designated restriction sites. The digestion pattern for selected enzymes is shown as colored boxes with the digest and fragment size indicated. The probes that are expected to detect each digestion product are indicated below the fragment with a colored line. All expected probe binding sites are indicated by bands, but only the digest/probe combinations necessary to support the model are shown. Red boxes denote internal bands (IB) associated with the original pSIM1278 DNA construct. Blue closed boxes indicate bands of known sizes due to identification of restriction sites within flanking DNA. Open-ended blue boxes indicate junction bands where the second restriction site is unknown. The estimated size of junction bands identified on Southern blots is indicated in parenthesis for all junction bands.

Southern Blot Analysis

As predicted, the expected internal bands were detected by the appropriate probes. The 0.7 kb EcoRV band was uniquely detected by the ASN probe (Figure 5-6), whereas the 2.3 kb EcoRV and 4.2 kb HindIII bands were detected by AGP, ASN, and GBS probes (Figures 5-5, 5-6, and 5-8). The 3.8 kb EcoRI/ScaI band was present in blots detected by AGP, R1, and GBS (Figures 5-5, 5-7, and 5-8, respectively). Lastly, as expected, the 1.3 kb HindIII and 0.8 kb EcoRI/ScaI bands were only detected by the R1 probe (Figure 5-7). These data confirm the internal structure of the pSIM1278 insert within the V11 genome.

Identification of the left and right junction regions allowed us to map a number of additional restriction sites as shown in Figure 5-4. These restriction sites were used to conclusively map the left and right junction regions of the insert using two sets of digests, MfeI/XbaI and NdeI/XbaI. The presence of 3.3 kb MfeI/XbaI and 3.1 kb NdeI/XbaI bands in blots probed with AGP and ASN (Figure 5-9) confirmed the structure of the left side of the insert as both bands connect the already mapped internal bands to the left flanking region. Similarly, the 7.4 kb band produced by XbaI cleavage in both digests connected the internal bands to the right flanking region. This band was detected by all four probes as expected (Figures 5-9 and 5-10). Higher molecular weight fragments have a tendency to migrate slightly faster than expected in DNA isolated from potatoes, which accounts for the slight difference between expected and observed migration of the 7.4 kb band.

Because the flanking regions did not identify restriction sites for EcoRV, HindIII, EcoRI, or ScaI in the neighboring sequence, the actual size of junction bands associated with each of those digests could not be predicted. However, a minimal size was calculated based upon the distance between the known restriction sites and the end of the flanking regions (shown in Figure 5-4 along with estimates of the actual size observed). In all cases, the observed junction bands were greater than the minimal predicated band size, which further supports the structure presented in Figure 5-4. Importantly, only one junction band was identified by the AGP probe associated with the left side of the insert (Figures 5-5 through 5-8). Likewise, a single junction band was identified in each of these digests associated with the right side using probes GBS and R1 (Figures 5-7 and 5-8). These data further support a single insertion model.

A summary of these data is provided in Table 5-1 where the expected and observed band sizes are compared for each digest and associated probe. The number, size, and intensity of observed bands exactly matches what is expected for a single insert of an intact pSIM1278 DNA insert in V11 as shown in Figure 5-1.

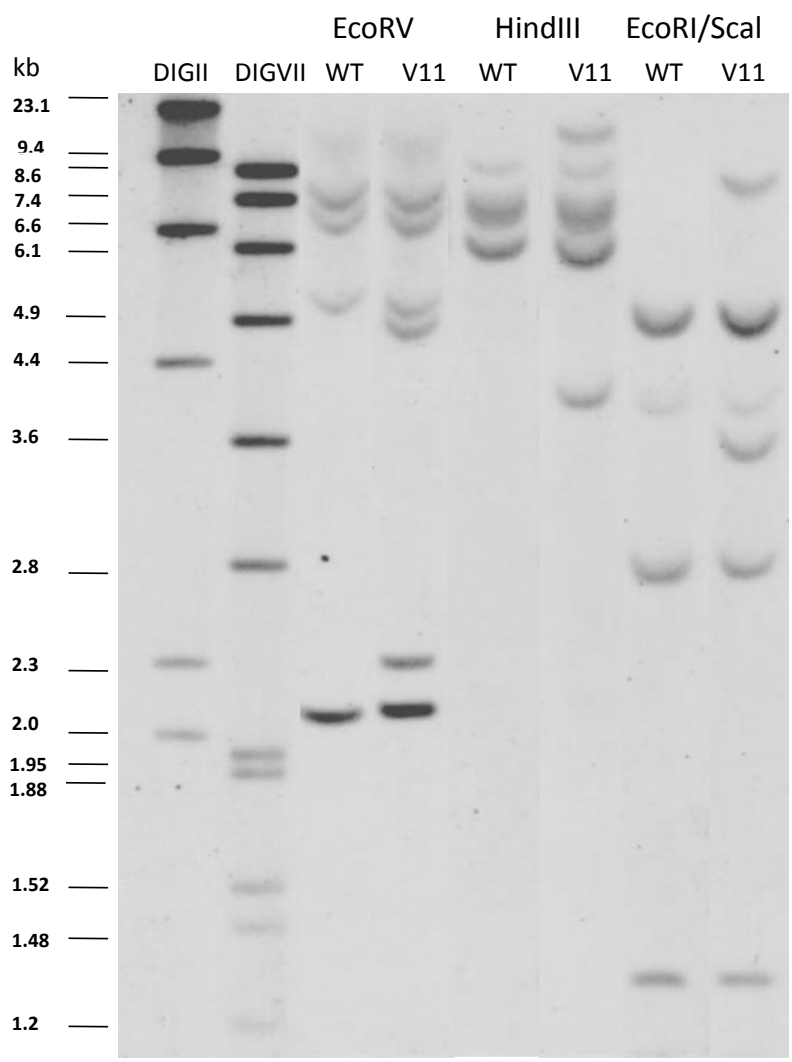
Table 5-1. Predicted and Observed Bands Based on Southern Blots for pSIM1278

Enzyme	Probe	Expected sizes (kb)[1]	Observed sizes (kb)[2]	References
EcoRV	AGP	>1.1, 2.3	4.9, 2.3	Figure 5-5
	ASN	0.7, 2.3	0.7, 2.3	Figure 5-6
	R1	>3.6	5.2	Figure 5-7
	GBS	>3.6, 2.3	5.2, 2.3	Figure 5-8
HindIII	AGP	>2.4, 4.2	18.0, 4.2	Figure 5-5
	ASN	4.2	4.2	Figure 5-6
	R1	>2.1, 1.3	7.4, 1.3	Figure 5-7
	GBS	>2.1, 4.2	7.4, 4.2	Figure 5-8
EcoRI/ScaI	AGP	>3.7, 3.8	8.0, 3.8	Figure 5-5
	ASN	>3.7	8.0	Figure 5-6
	R1	>1.9, 3.8, 0.8	4.4, 3.8, 0.8	Figure 5-7
	GBS	>1.9, 3.8	4.4, 3.8	Figure 5-8
MfeI/XbaI	AGP	>2.9, >7.1	3.3, 7.4	Figure 5-9A
	ASN	>2.9, >7.1	3.3, 7.4	Figure 5-9B
	R1	>7.1	7.4	Figure 5-10A
	GBS	>7.1	7.4	Figure 5-10B
NdeI/XbaI	AGP	>2.9, >7.1	3.1, 7.4	Figure 5-9A
	ASN	>2.9, >7.1	3.1, 7.4	Figure 5-9B
	R1	>7.1	7.4	Figure 5-10A
	GBS	>7.1	7.4	Figure 5-10B

[1] Expected size based upon original T-DNA insert from pSIM1278

[2] Observed size indicates bands identified by Southern blots

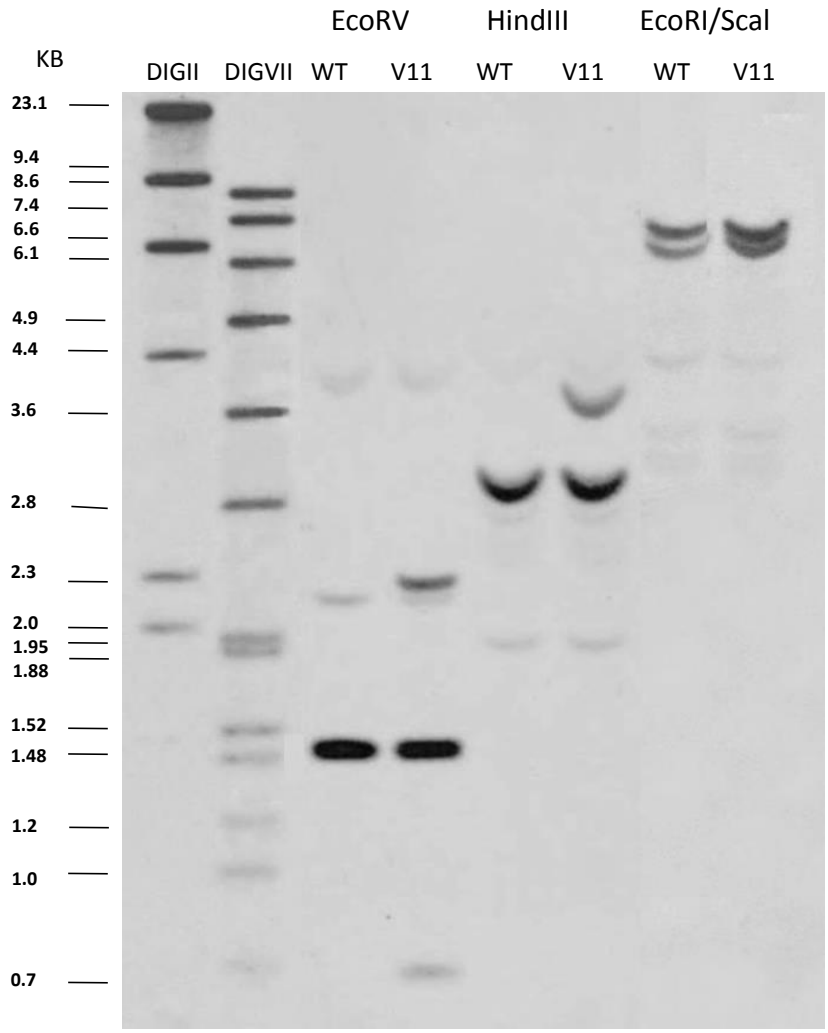
Figure 5-5. Snowden Genomic DNA Hybridization with the AGP Probe



EcoRV	HindIII	EcoRI ScaI
	JB 18	
	OB 9	
OB 8	OB 8	JB 8
	OB 7.2	
OB 7.2	OB 6.4	
		OB 5.3
OB 5.4		
JB 4.9		
	IB 4.2	
		OB 4.1
		IB 3.8
		OB 2.9
IB 2.3		
OB 2.1		
		OB 1.3

Genomic DNA of Snowden control (WT) and V11 was digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. The estimated sizes of bands are summarized in the table and classified into three groups based on the structure of the DNA insert: original endogenous bands (OBs, in green), internal bands (IBs, in red) and the junction bands (JBs, in blue). All molecular weights are presented in kilobases (kb).

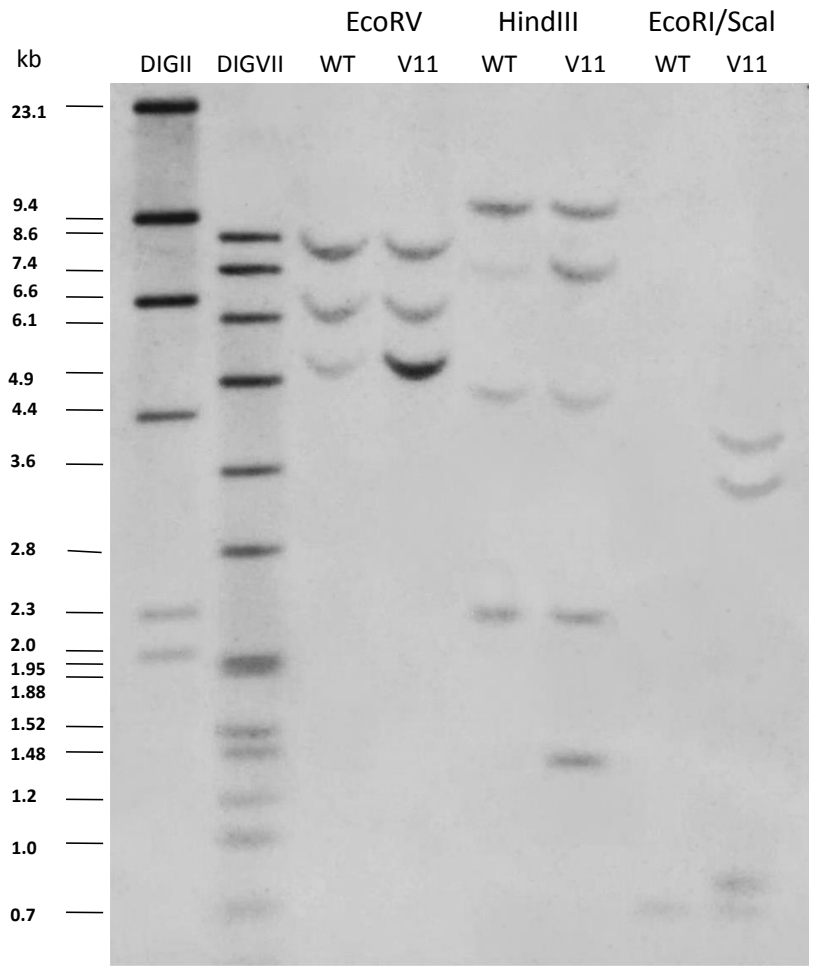
Figure 5-6. Snowden Genomic DNA Hybridization with the ASN Probe



EcoRV	HindIII	EcoRI Scal
		JB 8
		OB 7.4
		OB 7.0
OB 4.3	OB 4.3	OB 4.4
	IB 4.2	
		OB 3.5
	OB 3.2	OB 3.3
	OB 2.8	
IB 2.3		
OB 2.2	OB 2.0	
OB 1.5		
IB 0.7		

Genomic DNA of Snowden control (WT) and V11 was digested with EcoRV, HindIII, and EcoRI/Scal and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. The estimated sizes of bands are summarized in the table and classified into three groups based on the structure of the DNA insert: original endogenous bands (OBs, in green), internal bands (IBs, in red) and the junction bands (JBs, in blue). All molecular weights are presented in kilobases (kb).

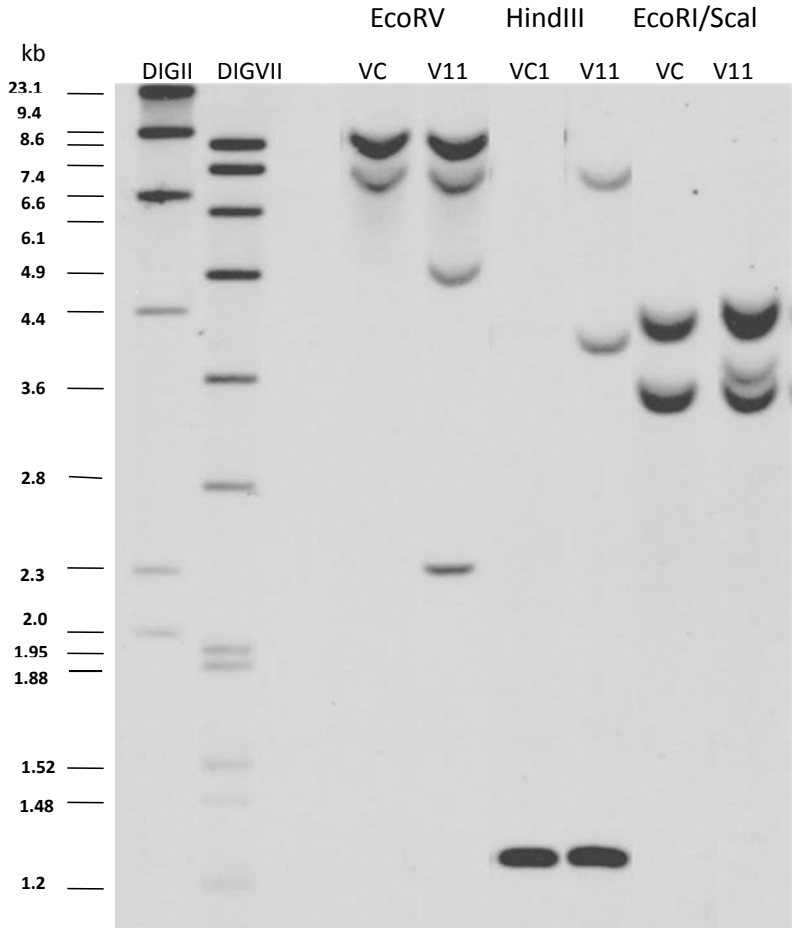
Figure 5-7. Snowden Genomic DNA Hybridization with the R1 Probe



EcoRV	HindIII	EcoRI ScaI
	OB 10	
	OB 7.4	
	JB 7.4	
OB 8.4		
OB 6.6		
OB 5.5		
JB 5.2		
	OB 4.8	
		JB 4.4
		IB 3.8
	OB 2.3	
	IB 1.3	
		IB 0.8
		OB 0.7

Genomic DNA of Snowden control (WT) and V11 was digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. The estimated sizes of bands are summarized in the table and classified into three groups based on the structure of the DNA insert: original endogenous bands (OBs, in green), internal bands (IBs, in red) and the junction bands (JBs, in blue). All molecular weights are presented in kilobases (kb).

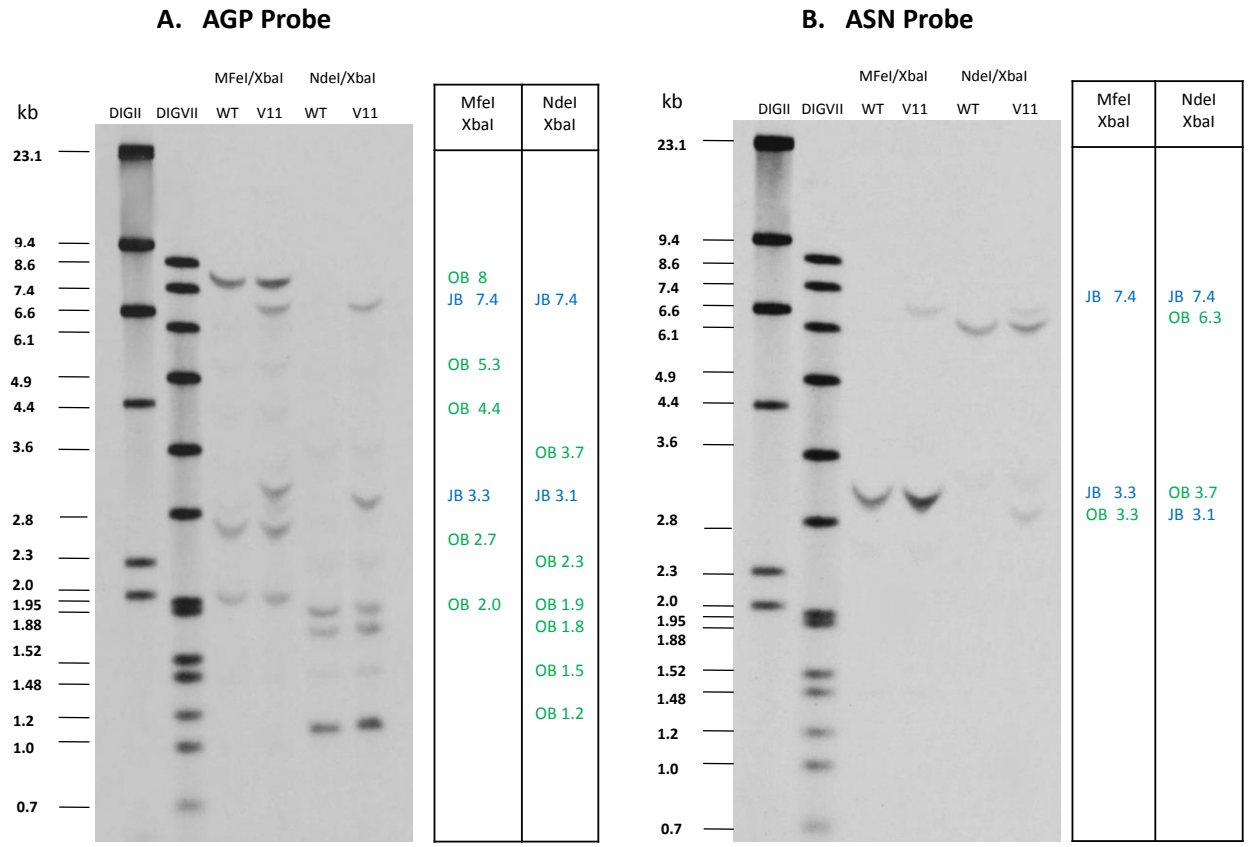
Figure 5-8. Snowden Genomic DNA Hybridization with the GBS Probe



EcoRV	HindIII	EcoRI ScaI
OB 9		
OB 7.4	JB 7.4	
JB 5.2		
	IB 4.2	JB 4.4 OB 4.2 IB 3.8 OB 3.4
IB 2.3		
	OB 1.3	

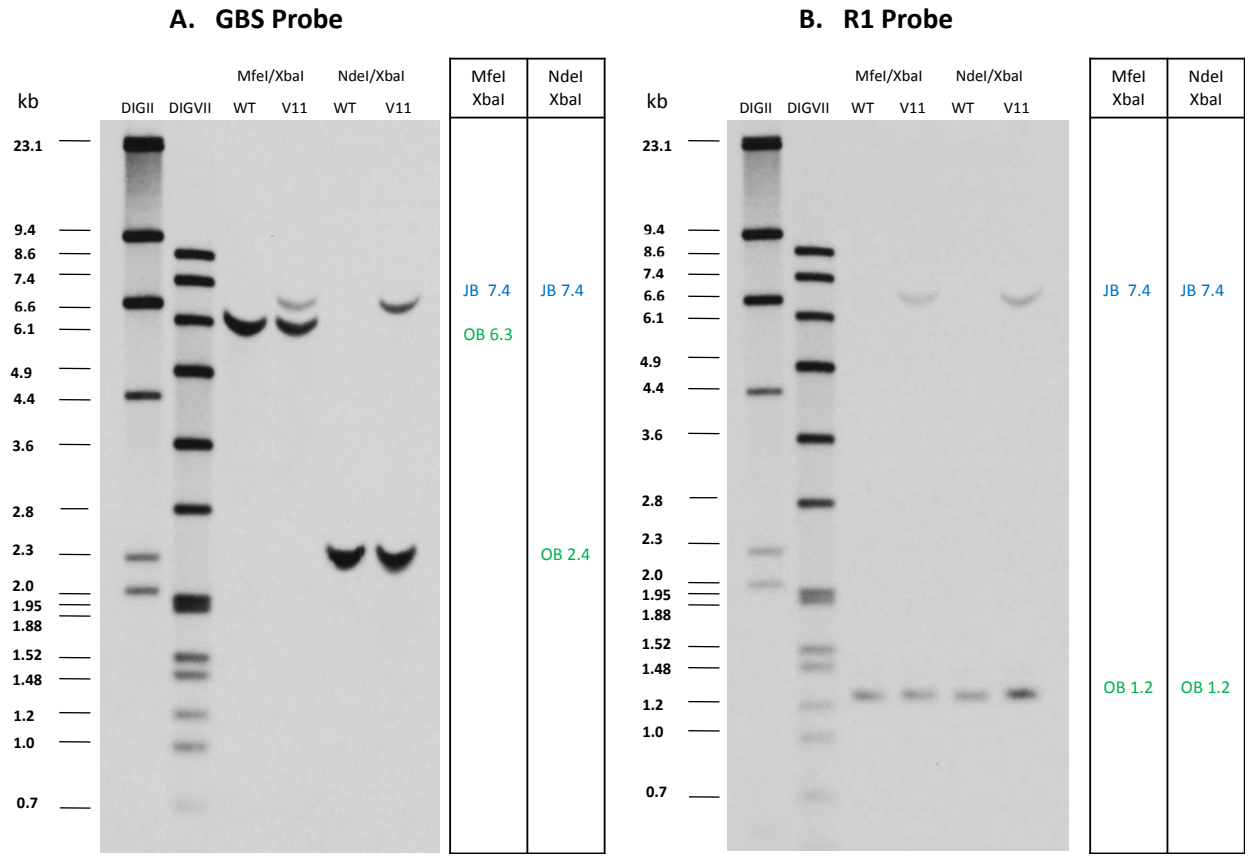
Genomic DNA of Snowden control (WT) and V11 was digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. The estimated sizes of bands are summarized in the table and classified into three groups based on the structure of the DNA insert: original endogenous bands (OBs, in green), internal bands (IBs, in red) and the junction bands (JBs, in blue). All molecular weights are presented in kilobases (kb).

Figure 5-9. Snowden Genomic DNA Hybridization with the AGP and ASN Probes



Genomic DNA of Snowden control (WT) and V11 was digested with MfeI/XbaI and NdeI/XbaI and hybridized with either the AGP or ASN probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. The estimated sizes of bands are summarized in the table and classified into groups based on the structure of the DNA insert: original endogenous bands (OBs, in green) and the junction bands (JBs, in blue). All molecular weights are presented in kilobases (kb).

Figure 5-10. Snowden Genomic DNA Hybridization with the GBS and R1 Probes



Genomic DNA of Snowden control (WT) and V11 was digested with MfeI/XbaI and NdeI/XbaI and hybridized with either the GBS or R1 probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. The estimated sizes of bands are summarized in the table and classified into three groups based on the structure of the DNA insert: original endogenous bands (OBs, in green) and the junction bands (JBs, in blue). All molecular weights are presented in kilobases (kb).

5.3 Absence of pSIM1278 Backbone Sequence in V11

The following methods were used to establish that backbone portions of the pSIM1278 construct were not present:

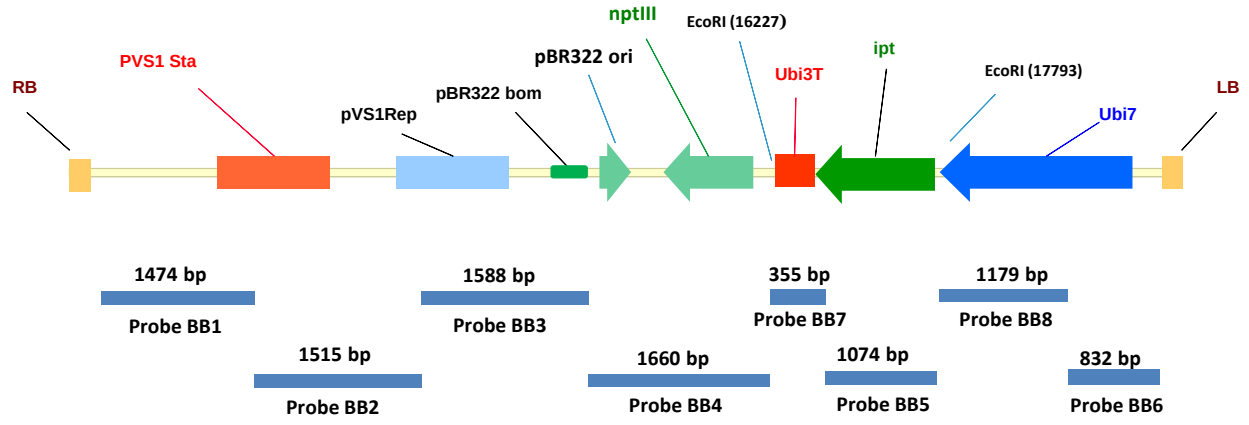
1) Plants with phenotypes associated with the negative selectable isopentenyl isomerase (*ipt*) marker gene in the construct backbone were discarded; and
2) Southern blots were used to confirm absence of the backbone DNA.

As described below, the results demonstrate that, like the previously deregulated events, V11 does not contain backbone sequence.

Agrobacterium-mediated transformation often results in transfer of construct backbone DNA, in addition to the intended region of DNA positioned between the left and right borders (LB and RB) of the plant-derived DNA insert. To reduce the number of events that must be characterized molecularly, a phenotypic screen for plants was employed that contained the *Agrobacterium ipt* gene, which is present in the construct backbone (Richael et al., 2008). When this gene is introduced into potatoes, its overexpression results in stunted growth, abnormal leaves, or the inability to root due to overproduction of cytokinin. Thus, these phenotypes were used to select against plants containing backbone DNA. Like the previously deregulated events, V11 did not present abnormal growth phenotypes and was further characterized using molecular methods to show that it did not contain any backbone sequences integrated into the plant genome.

A series of Southern probes were designed to span the entire construct backbone to detect any backbone DNA in the genome of the transformed potatoes (Figure 5-11). As the absence of DNA is based upon a lack of detection or amplification, a positive control (T130) event was developed, which contains an integrated copy of the entire construct backbone. Control genomic DNA samples from Snowden were included to distinguish between bands associated with the transformation and endogenous bands.

Figure 5-11. Probes for the Backbone of pSIM1278



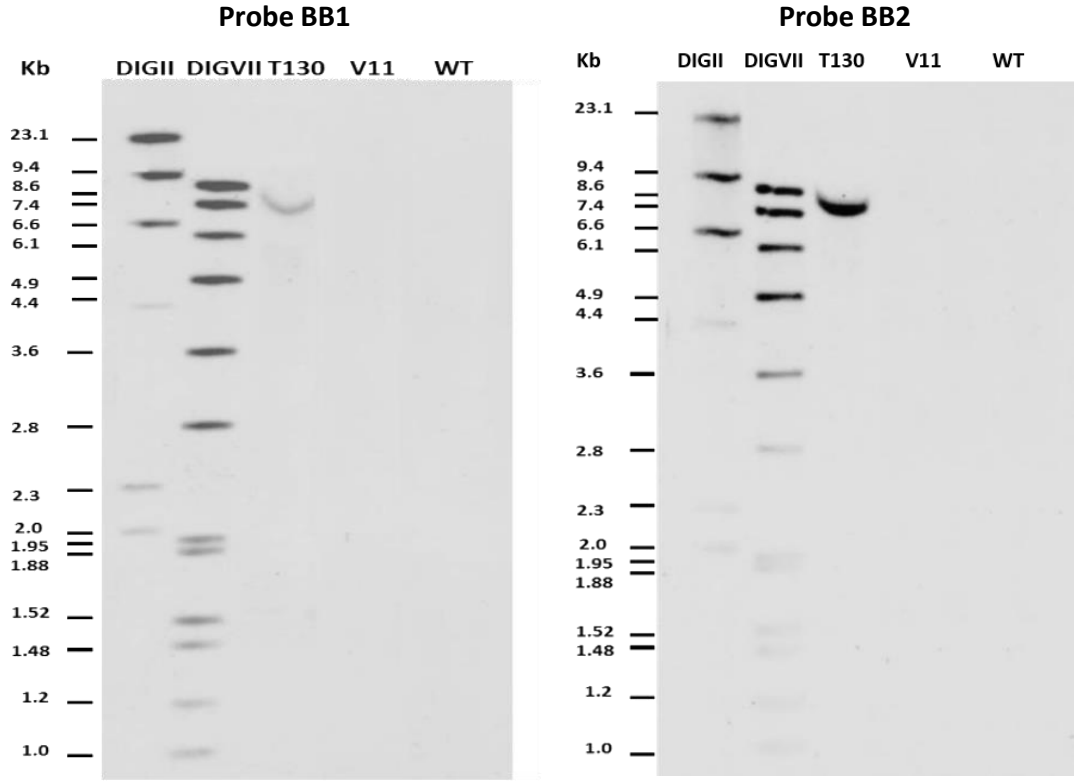
The backbone DNA is spanned by the probes shown as blue rectangles. Probes BB1 - BB5 are specific to the backbone DNA. Probes BB6-BB8 detect both backbone DNA and sequence from the potato genome (Ubi7 promoter, Ubi3 terminator, and Ubi7 promoter, respectively).

Genomic DNA isolated from V11, T130, and Snowden controls (WT) were digested with EcoRI and hybridized with probes BB1-6 (Figure 5-12). Probes BB1-5 were highly specific and only detected bands associated with the T130 positive control, whereas probe BB6 detected a pair of endogenous bands. These bands were expected since the region of the construct detected by this probe is derived from the potato Ubi7 promoter.

Similarly, the regions of the construct detected by probes BB7 and BB8 are derived from potato DNA, Ubi3 terminator and Ubi7 promoter, respectively. The Southern blot for probe BB7 was analyzed similar to probes BB1-6. However, to simplify the banding patterns when using probe BB8, genomic DNA was digested with both EcoRI and ScaI. In addition, the positive control for these blots consisted of Snowden genomic DNA spiked with pSIM1278 construct DNA. The probes detected three endogenous bands, in addition to the positive control in each sample. A review of all Southern blots with eight probes demonstrated that only the positive control, T130, contained unique backbone bands that hybridized with the probes (Figures 5-12 through 5-15).

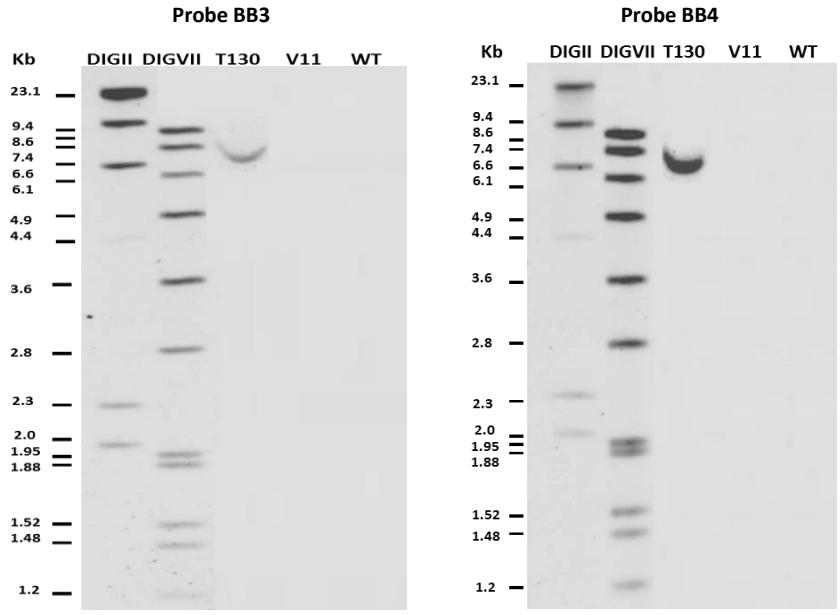
Collectively, the Southern blot analyses showed the genome of V11 does not contain sequence from the backbone of construct pSIM1278. This finding is consistent with the previously deregulated events.

Figure 5-12. Southern Blot Analysis of Construct Backbone DNA using Backbone Probes BB1 and BB2



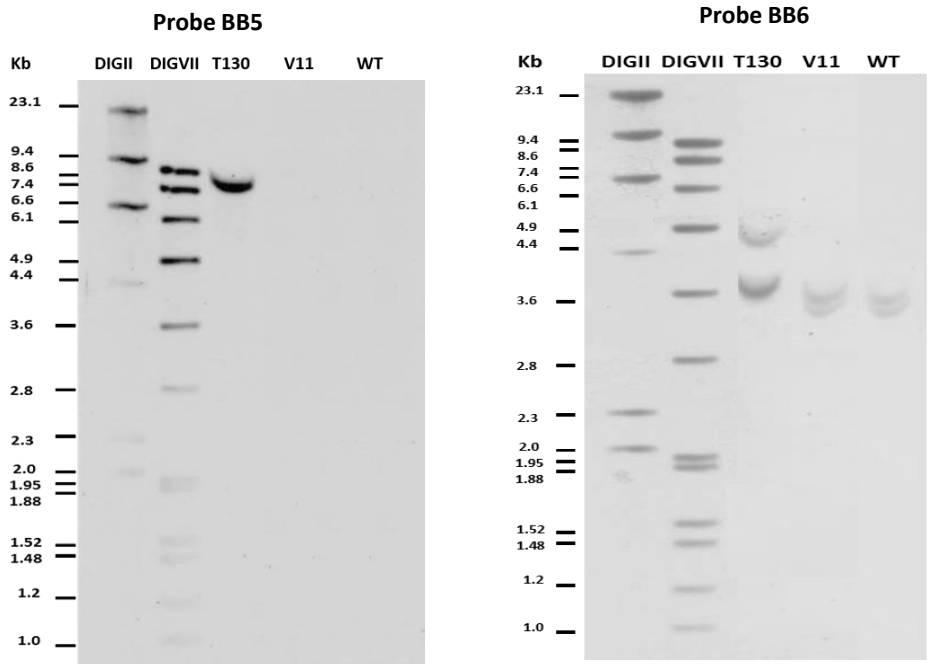
Genomic DNA was digested with EcoRI and analyzed by Southern blot using probes BB1 and BB2. WT = Snowden control, V11 = Event V11, T130 = positive control containing backbone DNA. Lanes 1 and 2 are molecular weight markers (DIG II, and DIGVII, respectively) with sizes indicated next to gel in kilobases (kb).

Figure 5-13. Southern Blot Analysis of Construct Backbone DNA using Backbone Probes BB3 and BB4



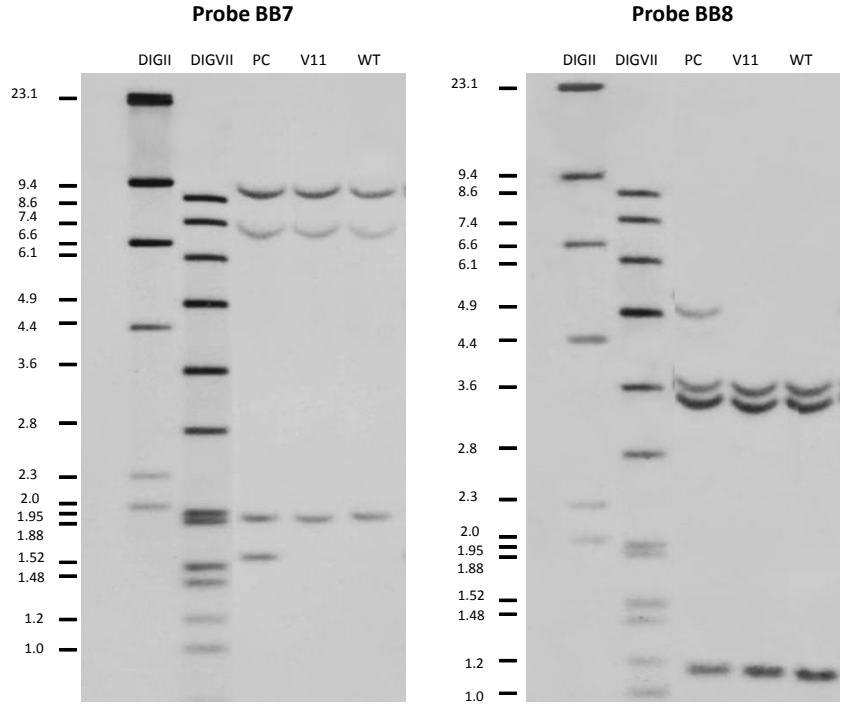
Genomic DNA was digested with EcoRI and analyzed by Southern blot using probes BB3 and BB4. WT = Snowden control, V11 = Event V11, T130 = positive control containing backbone DNA. Lanes 1 and 2 are molecular weight markers (DIG II, and DIGVII, respectively) with sizes indicated next to gel in kilobases (kb).

Figure 5-14. Southern Blot Analysis of Construct Backbone DNA using Backbone Probes BB5 and BB6



Genomic DNA was digested with EcoRI and analyzed by Southern blot using probes BB5 and BB6. WT = Snowden control, V11 = Event V11, T130 = positive control containing backbone DNA. Lanes 1 and 2 are molecular weight markers (DIG II, and DIGVII, respectively) with sizes indicated next to gel in kilobases (kb).

Figure 5-15. Southern Blotting Analysis of Construct Backbone DNA using Backbone Probes 7-8



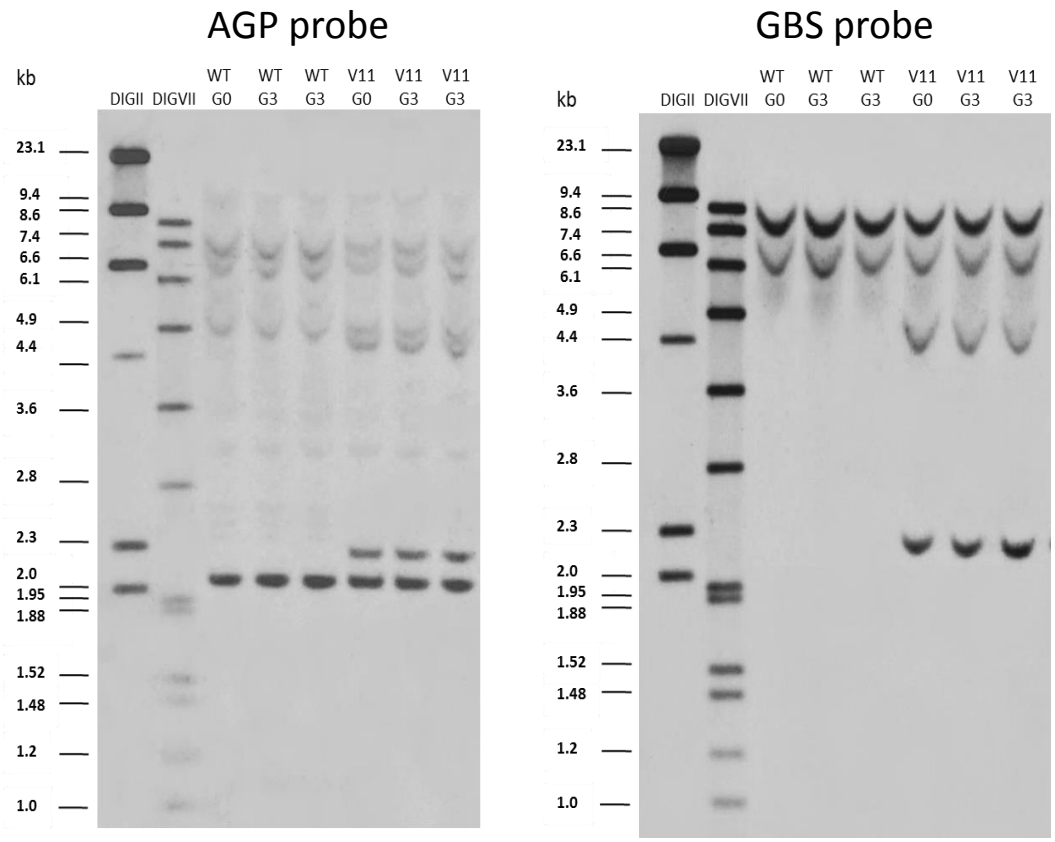
Genomic DNA was digested with either EcoRI (probe 7) or EcoRI/ScaI (probe 8) and analyzed by Southern blot. PC = Snowden genomic DNA spiked with pSIM1278 construct DNA, WT = Snowden control, V11 = Event V11. Lanes 1 and 2 are molecular weight markers (DIG II, and DIGVII, respectively) with sizes indicated next to gel in kilobases (kb).

5.4 Genetic Stability of the Insert Across Generations

T-DNA can be unstable in a transformed host where the instability rate ($0.5\text{-}5.9\times10^{-4}$) is associated with meiosis and meiotic recombination (Müller et al*.,* 1987; Conner et al*.,* 1998). Since potatoes are reproduced vegetatively and do not undergo meiosis, the T-DNA insert is expected to be genetically stable.

Genetic stability of the T-DNA insert in V11 was assessed by analyzing the structure using Southern blot analysis of genomic DNA isolated from G0 and G3 plants. Southern blots were performed on EcoRV-digested DNA as this digest produces independent bands corresponding to each end of the insert (e.g. 4.9 and 5.1 kb) along with an internal band (e.g. 2.3kb) as described in Figure 5-4 and Table 5-1. The AGP and GBS probes were used because, collectively, they hybridize to each of the three predicted bands, which includes the ends of the T-DNA insert. As expected, the banding pattern on Southern blots of EcoRV-digested DNA analyzed with the AGP and GBS probes was the same between G0 and G3 plants generated through clonal propagation (Figure 5-16). Thus, the V11 insert is genetically stable, similar to the previously deregulated events.

Figure 5-16. Southern blot of EcoRV digestion with AGP probe



Southern blots of genomic DNA (3 µg) following digestion with EcoRV and probed for AGP (left) or GBS (right) sequence. Each blot compares DNA from the indicated generation to DNA from the initial transformant (G0). Snowden untransformed controls (WT), Submitted event (V11). Genetic stability is established by the consistent digestion pattern between the original transformant (G0) and the G3 generation.

5.5 Summary of the Genetic Characterization of V11

Molecular analysis demonstrated that V11 contains a single, intact copy of the pSIM1278 insert with a 14-bp deletion of the left border region and 3-bp deletion of the right border region within the Snowden genome. The T-DNA insert consisted solely of sequence targeted for insertion and did not contain any detectable construct backbone DNA. The structure of the DNA insert was intact as in the original construct and was shown to be stable across generations. Given the demonstrated DNA insert stability in V11 over generations, it is likely that stability will be maintained during subsequent cycles of vegetative propagation. The molecular characterization of V11 is consistent with the previously deregulated events.

5.6 References

Conner, A.J., Mlynárová, L., Stiekema, W.J., and Nap, J.P. (1998). Meiotic Stability of Transgene Expression is Unaffected by Flanking Matrix-associated Regions. Molecular Breeding *4,* 47-58.

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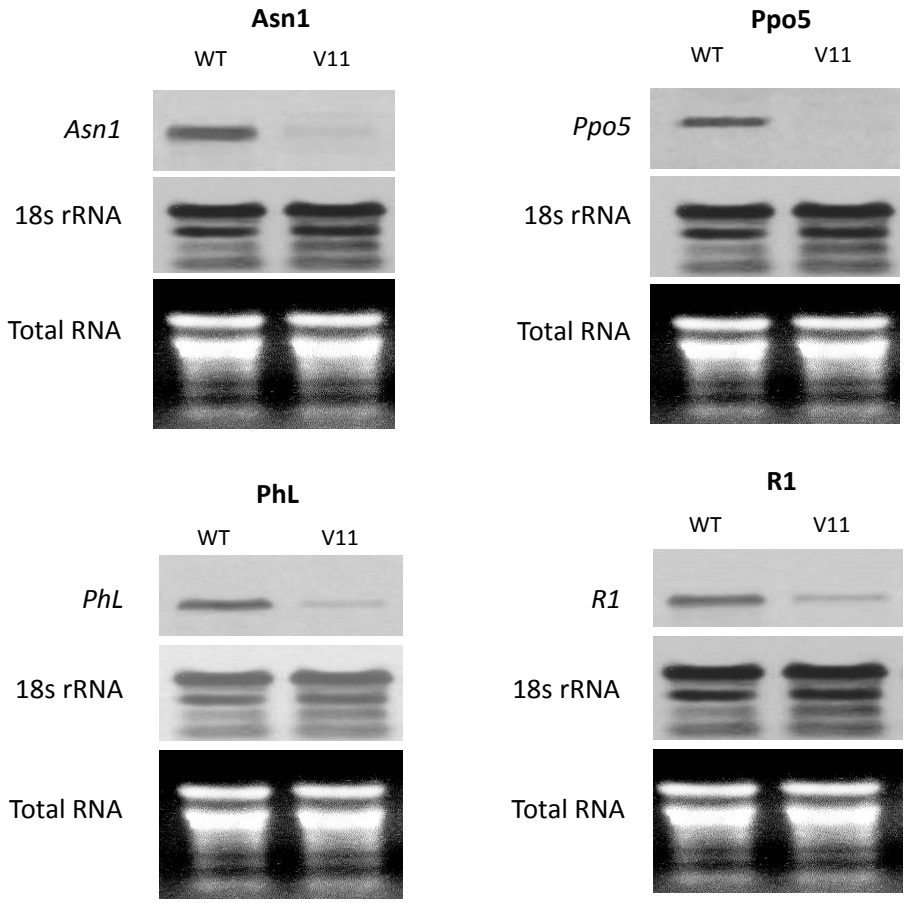
Richael, C.M., Kalyaeva, M., Chretien, R.C., Yan, H., Adimulam, S., Stivison, A., Weeks, J.T. and Rommens, C.M. (2008). Cytokinin Vectors Mediate Marker-free and Backbone-free Plant Transformation. Transgenic Research *5*, 905–917.

6.0 Gene Down-regulation in V11

Similar to the previously deregulated events, V11 was characterized using northern blot analysis to determine the effectiveness of the down-regulation of the four target genes: *Asn1*, *R1*, *PhL*, and *Ppo5*. The expression level of each target transcript was determined using RNA isolated from V11 and Snowden control (WT) tubers (see Appendix A for methods).

In tubers, the primary target tissue, robust down-regulation of *Asn1* and *Ppo5* and partial down-regulation of *PhL* and R1 was observed (Figure 6-1). Similar down-regulation of the four genes in tubers of V11 occurred in the previously deregulated events (Collinge and Clark 2013). Gene down-regulation was assessed by northern blot analysis in other tissues (leaves, stems, flowers, and roots; data not shown) and is summarized in Table 6-1.

Figure 6-1. Target Gene Expression in Tubers



Northern blot analysis of total RNA (20 µg) isolated from tubers of field-grown plants for V11 and the Snowden control (WT). Blots were hybridized with probes specific to the *Asn1, Ppo5, PhL, or R1* transcripts (upper panels). A probe specific to the internal control 18s rRNA (middle panels) and ethidium bromide stained total RNA (lower panels) were used as internal and loading controls.

Table 6-1. Gene Down-regulation in V11

	Tubers	Leaf	Stems	Roots	Flowers
Asn1	Yes	Yes	Yes	No	No
R1	Yes	No	No	No	No
PhL	Yes	No	No	No	No
Ppo5	Yes	No	No	No	No

6.1 References

Collinge, S. and Clark, P. (2013). Petition for Determination of Nonregulated Status for Innate™ Potatoes with Low Acrylamide Potential and Reduced Black Spot Bruise: Events E12 and E24 (Russet Burbank); F10 and F37 (Ranger Russet); J3, J55, and J78 (Atlantic); G11 (G); H37and H50 (H). J.R. Simplot Company Petition JRS01 (USDA Petition 13-022-01p).

7.0 Comparative Assessment of V11 Potato

As was done for the previously deregulated events in 13-022-01p, phenotypic and compositional comparative assessments were conducted to determine the safety of V11 relative to conventional potatoes. These assessments used conventional potatoes for comparison to V11 and established the safety of V11 relative to potato varieties that have a long history of safe use in the environment and as food and feed.

To ensure accurate evaluations of V11, proper selection of comparator varieties was important. For V11, the most relevant comparator is Snowden, its parental variety. The only difference between V11 and Snowden is that V11 underwent transformation and contains a pSIM1278 insert. Statistical analysis was used to determine whether V11 was different from its parental control, Snowden.

Other important comparators include additional varieties of conventional potatoes, which were grown in typical potato-growing regions. These represent a wide range of potato varieties that are planted commercially and were used to assess the normal range of phenotypic and compositional variation. In some cases, the data obtained from the comparator varieties was used to generate a statistical tolerance interval. Scientific literature was used to generate a range of values for compositional analytes of potatoes.

A summary of how the comparative assessment data were interpreted after statistical analysis is as follows:

- When p-values were available and the p-value indicated no statistical significance, it was unlikely that there was a difference that was meaningful, and the assessment was considered complete.
- If the p-value indicated statistical significance or if a p-value was not present, the mean value of the event was compared to the tolerance interval, conventional variety range, or the combined literature range. If the mean value for V11 was within any of those, we concluded that V11 was within the natural variation of potatoes and that the difference was unlikely to be meaningful.
- If the mean value of the event was outside the ranges, further consideration was given to the difference in the context of phenotype or composition equivalence.

The phenotype and compositional comparative assessments of V11 are discussed further below. The analyses indicate that V11 was comparable to its parent variety, Snowden, and other conventional potatoes with respect to the characteristics measured. Overall, these analyses indicate that V11 is as safe and nutritious as conventional potato varieties and poses no more risk than conventional potato varieties in food, feed, and the environment.

8.0 Phenotypic Performance and Field Observations

The purpose of the agronomic trials was to confirm that V11 had an equivalent phenotype compared to the control Snowden, when grown at multiple locations representing the major areas for potato production in the U.S.

Observations throughout the growing season allowed for a thorough assessment of:

1. Agronomic/phenotypic characteristics;
2. Tuber characteristics;
3. Biotic and abiotic stress susceptibility; and
4. Volunteer potential.

These assessments demonstrate that the addition of the DNA insert in V11 did not result in unintended effects associated with weediness or pest-like characteristics. In addition, the phenotypic comparability between V11 and Snowden also supports the conclusion of lack of meaningful somaclonal variation in V11.

8.1 Field Trial Locations

During 2012 and 2013, V11 and its parental control, Snowden, were grown at multiple locations representing the major production areas for potatoes (Table 8-1). At some locations, additional conventional varieties were also grown. The agronomic practices and pest control measures used were location-specific and were typical for potato cultivation. They were recommended by both regional potato extension specialists and agronomists and they related to all aspects of soil preparation, fertilizer application, irrigation, and pesticide-based control methods.

All field trials were conducted in accordance with applicable USDA APHIS notification requirements at 7 CFR Part 340.3 (Appendix B). Additional details for the methods used to conduct the field portion of the phenotypic and compositional comparative assessments can be found in Appendix C.

Table 8-1. Field Trial Locations

Year	State	County	Material Tested[1]	Trial Design[2]	Rows x Planted Tubers/ Row	Seed Type	Regional Specifics
2012	FL	St. Johns	V11 Snowden Control	RCB, 3 reps	4x20	Mini-tubers	Typical for Florida, which produces almost 8 million cwt/year, mainly for the chip and fresh potato industry, with harvests in spring.
2012	WI	Adams	V11 Snowden Control	RCB, 3 reps	3x20	Mini-tubers	Typical for Wisconsin, which produces about 20 million cwt/year, for both the chip and fresh potato industry, with harvests in fall. Large areas are dominated by muck soils.
2013			V11 Snowden Control Gala Purple Majesty C0095051-7W Norkotah	RCB, 4 reps	4x20	Tubers	
2012	MI	Montcalm	V11 Snowden Control	RCB, 3 reps	4x20	Mini-tubers	Typical for Michigan, which produces about 15 million cwt/year, for both the chip and fresh potato industry, with harvests in fall. The climate is characterized by mild temperatures and ample rain.
2013			V11 Snowden Control Gala Purple Majesty C0095051-7W Norkotah	RCB, 4 reps	4x20	Tubers	
2013	WA	Grant	V11 Snowden Control Gala Purple Majesty C0095051-7W Norkotah	RCB, 4 reps	4x20	Tubers	Typical for Washington, which produces about 85 million cwt/year, mainly for the fry industry, with harvests in fall. Ideal growing conditions give rise to very high yields per acre.
2013	PA	Berks	V11 Snowden Control Gala Purple Majesty C0095051-7W Norkotah	RCB, 4 reps	4x20	Tubers	Typical for Pennsylvania, which produces about 2 million cwt/year, mainly for the chip industry, with harvests in fall.

[1]The conventional varieties used were selected because they represent a range of common potato varieties that are currently planted commercially.
[2]RCB=Randomized Complete Block design

8.2 Phenotypic and Tuber Assessment Results

Summaries of phenotypic and tuber characteristics (yield and grading) of V11 and the Snowden control grown in 2012 and 2013 are shown in Tables 8-2 and 8-3, respectively. Explanations of the scoring characteristics can be found in Appendix C.

The phenotypic characteristics of V11 and the control are shown in Table 8-2. There were no statistical differences for any of the traits measured.

The yield and grading characteristics of V11 and the control are shown in Table 8-3. There were no statistical differences for total yield, U.S.#1 yield, tubers per plant, size A tubers, size B tubers, oversize tubers, pickout tubers, and specific gravity. Compared to its parental control, Snowden, V11 had fewer total internal defects (24.9% vs. 18.7%). This could be related to the efficacy provided by the PPO down-regulation and the absence of color in bruises or defects in V11. However, the mean of V11 for total internal defects fell within the conventional variety range. Fewer total internal defects would be considered a positive outcome and would not indicate increased plant pest potential.

Overall, the results demonstrate there are no major differences between V11 and its parental control, Snowden. These data support the conclusion that V11 is unlikely to have increased plant pest potential when compared to the control.

Table 8-2. Phenotypic Characteristics

Characteristic	Variety	N	Mean	P-Value[1]	Standard Deviation	Conventional Variety Range[2]
Early Emergence (%)	Control	24	62.5	0.8243	35.4	3.75-96.3
	V11	25	61.0		34.2	
Final Emergence (%)	Control	24	90.8	0.9818	13.2	56.3-106
	V11	25	90.9		12.6	
Stems Per Plant (#)	Control	24	2.6	0.4804	1.75	1-5.95
	V11	25	2.8		1.74	
Plant Vigor (1-5 Scale)[3]	Control	24	3.1	0.1856	1.04	1.33-4.00
	V11	25	3.4		0.978	
Plant Height (cm)	Control	24	56.7	0.3267	20.2	16.4-81.1
	V11	25	59.1		17.8	
Vine Desiccation (%)	Control	22	60.6	0.7981	36.1	0-99.8
	V11	22	58.7		32.6	

[1]Underlined P-values indicate statistically significant differences.
[2]The range of mean values of conventional varieties.
[3]Plant vigor was assessed on a 1 to 5 scale where 1 = severely less than the varietal average, 2 = noticeably less than varietal average, but not severe, 3 = plants are similar to the varietal average, 4 = slightly more than varietal average, 5 = obviously more than the varietal average, based on the principal investigator's professional experience which includes knowledge of potato growth and development for their specific geography.

Table 8-3. Tuber Characteristics

Characteristic	Variety	N	Mean	P-Value[1]	Standard Deviation	Conventional Variety Range[2]
Total Yield (cwt/A)	Control	21	314	0.2216	150	89.2-554
	V11	22	342		144	
U.S.#1 Yield (cwt/a)	Control	21	274	0.4144	153	62.7-522
	V11	22	295		143	
Tubers Per Plant (#)	Control	21	8.10	0.199	2.88	3.67-18.2
	V11	22	9.02		3.64	
Size A Tubers (%)	Control	21	75.3	0.981	8.40	28.0-83.3
	V11	22	75.4		7.98	
Size B Tubers (%)	Control	21	15.7	0.875	8.86	6.00-70.5
	V11	22	15.0		8.44	
Oversize Tubers (%)	Control	21	8.55	0.8095	9.52	0-23.8
	V11	22	9.25		9.99	
Pickout Tubers (%)	Control	21	0.474	0.9221	1.47	0-17.3
	V11	22	0.294		0.894	
Specific Gravity	Control	21	1.076	0.1008	0.0080	1.05-1.09
	V11	22	1.078		0.0064	
Total Internal Defects (%)	Control	21	24.9	<u>0.0471</u>	33.6	0-93.8
	V11	22	18.7		27.5	

[1]Underlined P-values indicate statistically significant differences.
[2]Range of mean values of conventional varieties.

8.3 Insect, Disease, and Abiotic Stressor Assessments

Naturally occurring biotic (insect and disease) and abiotic stressors were observed and recorded by the principal investigators with expertise in potato cultivation. The stressor observations provided an opportunity to assess V11 across a broad range of stressors and locations at several points during the growing season and observe potential environmental interactions. Recorded stressors varied depending on which stressors were present or expected to be present. Even if no stressors were present, zeroes were recorded because the stressors were looked for and comparisons can be made between V11 and the its parental control, Snowden. Examples of common potato disease and insect symptoms can be found in Appendix C, Table C-4.

Stressors were rated at early season, midseason, and late season on a 0 to 3 scale, where:

- 0 = no symptoms observed
- 1 = slight symptoms were observed, but not interfering with plant development,
- 2 = moderate symptoms were present, intermediate between slight and severe, and,
- 3 = severe symptoms were observed that interfered with plant development.

The insect, disease, and abiotic stressor evaluations for V11 and the control are shown in Table 8-4. Stressor evaluations were intended to be categorical and were not statistically analyzed. The range of ratings for V11 and Snowden were compared for each observation, and a difference occurred when the range of V11 did not overlap with the range of Snowden. In total, no differences were observed for 148 out of 155 insect, disease, or abiotic stressors. The seven differences that were observed varied across sites and years.

- One difference was noted between V11 and Snowden during 41 individual observations of seven abiotic stressors.
- Three differences were observed between V11 and Snowden during 57 individual observations for 12 diseases.
- Three differences were observed between V11 and Snowden during 57 individual observations for nine insects.

The small number of observed differences between V11 and Snowden and the lack of trends across sites supports a conclusion of no altered environmental interactions of V11 compared to its parental control, Snowden.

Table 8-4. Abiotic and Biotic Stressor Observations

Stressor	Total Observations	Observations Without Differences	Observations With Differences	Differences[1]
Abiotic Stressors				
Cold Stress	2	2	0	-
Compaction	1	1	0	-
Drought	4	4	0	-
Hail	1	1	0	-
Heat Stress	14	14	0	-
Water Stress	12	12	0	-
Wind Damage	7	6	1	Adams Co., WI 2012 Obs. 1: V11 = 0-1; Ctrl = 0-0; Ref = 0-1
Total	41	40	1	-
Disease Stressors				
Bacterial Wilt	1	1	0	-
Blackleg	1	1	0	-
Botrytis	3	2	1	Adams Co., WI 2012 Obs. 2: V11 = 0-1; Ctrl = 1-2; Ref = N/A
Early Blight	17	17	0	-
Late Blight	14	14	0	-
Leaf Roll Virus	2	1	1	Adams Co., WI 2012 Obs. 2: V11 = 0-2; Ctrl = 1-2; Ref = 0-0
Powdery Mildew	1	1	0	-
Rhizoctonia	7	7	0	-
Sclerotinia	3	3	0	-
Stem Rot	1	1	0	-
Verticillium Wilt	2	1	1	Grant Co., WA 2013 Obs. 3: V11 = 1-1; Ctrl = 0-0; Ref = 0-3
White Mold	5	5	0	-
Total	57	54	3	-

Table 8-4, Continued. Abiotic and Biotic Stressor Observations

Stressor	Total Observations	Observations Without Differences	Observations With Differences	Differences[1]
Insect Stressors				
Aphid	14	13	1	Adams Co., WI 2012 Obs. 3: V11 = 1-2; Ctrl = 2-2; Ref = 0-0
Armyworm	1	1	0	-
Colorado Potato Beetle	18	16	2	Adams Co., WI 2013 Obs. 1: V11 = 0-1; Ctrl = 0-0; Ref = 0-2 Montcalm Co., MI 2012 Obs. 2: V11 = 0-2; Ctrl = 0-0; Ref = 0-2
Flea Beetle	3	3	0	-
Japanese Beetle	1	1	0	-
Leaf Beetle	2	2	0	-
Leaf Hopper	14	14	0	-
Looper	3	3	0	-
White Flies	1	1	0	-
Total	57	54	3	-

[1]The range of values observed in conventional reference varieties (Ref).
N/A means a reference range was unavailable.
Obs. 1 = early season. Obs. 2 = mid-season. Obs. 3 = late season.

8.4 Volunteer Potential

In an agricultural setting, volunteers are plants that grow from tubers dropped or left behind during planting, harvest, and other field operations, sometimes in a subsequent growing season. This volunteer potential study was intentionally planted with a known quantity of tubers to simulate tubers left behind after harvest. The objective was to evaluate the potential of V11 potatoes to overwinter and produce volunteer plants compared with its parental variety, Snowden.

The study was conducted at multiple sites to include a range of environmental conditions. Tubers of V11, Snowden, and conventional references were planted in the fall of 2012 at two sites (Table 8-5). The sites were monitored from planting until conditions were too cold for plant growth and again during the following spring when the soil warmed until approximately July 15, 2013. Volunteer plants were counted, removed, and devitalized approximately every two weeks. This assessment compared the total number of volunteer plants for V11 and Snowden over the observation period and found no differences (Table 8-6).

Table 8-5. Volunteer Potential Field Trial Locations

State	County	Variety	Trial Design[1]	Rows x Planted Tubers/ Row[2]	Regional Specifics
ID	Canyon	V11 Snowden Ranger Russet Norkotah Shepody, Atlantic Russet Burbank	RCB 4 reps	3x10	Typical for Southwest Idaho, which produces about 120 million cwt/year, mainly for the fry industry.
WA	Grant	V11 Snowden Ranger Russet Norkotah Umatilla Pacific, Atlantic Russet Burbank	RCB 4 reps	3x10	Typical for Washington, which produces about 85 million cwt/year, mainly for the fry industry, with harvests in fall. Ideal growing conditions give rise to very high yields per acre.

[1]RCB=Randomized Complete Block design. Number of blocks was equal to the number of reps.
[2]30 total tubers per rep.

Table 8-6. Mean Total Field Volunteers Observed For Each Variety

Variety	N[1]	Mean Total Volunteers[2]	Standard Deviation	Conventional Variety Range
Atlantic[1]	8	0.0	0.0	0.0-0.1
Russet Burbank[1]	8	0.0	0.0	
Ranger Russet[1]	8	0.1	0.2	
V11[1]	8	0.0	0.0	
Snowden[1]	8	0.0	0.0	

[1]N=number of sites (2) times the number of replications per site (4)
[2]Mean total volunteers = total number of volunteers counted during observation period divided by N

Most varieties tested, including V11 and Snowden, produced no volunteer plants. Low numbers of volunteers were seen in some other varieties. These results indicate that winter conditions were harsh enough to devitalize almost all tubers at both sites. The few volunteers observed may be explained by random variations in seed size or planting depth. Larger tubers and deeper planting depth would both provide greater insulation during winter and increase the chance of volunteers being produced. While the study attempted to control both of these factors, some variation is to be expected. The study was designed to give tubers a better chance to produce volunteer plants than they would in a commercial cropping system. For example, both whole and cut tubers were planted and covered with soil. A tuber dropped during harvest and exposed to winter weather would almost certainly be devitalized by freezing in most major potato growing areas. The lack of volunteer plants in V11 and the Snowden control indicates there is likely no altered volunteer potential in V11. All available data and evidence supports that V11 has no pest potential and is not weedy.

8.5 Conclusions for Agronomic Performance and Field Observations

Results of these agronomic trials confirmed that, like the previously deregulated events, V11 is phenotypically and agronomically similar to its parental control, Snowden, when grown at multiple locations representing the major areas for potato production in the U.S. Observations throughout the growing season demonstrated no meaningful differences in phenotypic and agronomic characteristics, tuber characteristics, biotic and abiotic stress susceptibility, and volunteer potential. No phenotypes that could indicate enhanced weediness, survivability, or plant pest potential were noted for V11.

9.0 Compositional Assessment

As with the previously deregulated events, a compositional analysis of V11 was conducted to evaluate the levels of key nutrients (proximates, vitamins, amino acids, and minerals) and glycoalkaloids compared to the parental control, Snowden. In addition, concentrations of free amino acids, sugars, and acrylamide were evaluated in V11 and Snowden to measure efficacy of the low acrylamide potential and lowered reducing sugars traits. The U.S. FDA will review the details of the compositional analyses as a component of the food and feed safety assessment of V11 potatoes.

Tubers for the compositional assessment were generated in the same field studies as described for the phenotypic testing (Table 8-1). Briefly, tubers were collected from seven sites in 2012 and 2013 with 3-4

replications per site. Samples were obtained by randomly selecting six mid-sized tubers (at harvest) from each site and rep. Samples (whole tubers, including skin) were powdered in an industrial blender with liquid nitrogen and stored at -70°C until analysis. Analytical testing was conducted by Covance Laboratories, Inc. Additional details for the composition and statistical methods can be found in Appendix C.

Results of the composition studies demonstrate that, like the previously deregulated events, V11 is comparable to conventional potatoes with respect to nutrient and glycoalkaloid composition. As expected, and similar to the previously deregulated events, the levels of reducing sugars (glucose and fructose), free asparagine, and acrylamide are lower in V11 than in Snowden.

9.1 Compositional Nutrient Analysis

These analyses were conducted to confirm that composition of V11 remained within the normal levels for potato and would have equivalent food quality, feed quality, and safety when compared to its parental control, Snowden, and conventional potatoes. The compositional assessments determined the concentrations of:

1) proximates, vitamins, and minerals (Table 9-1)
4) total amino acids (Table 9-2)
5) glycoalkaloids (Table 9-3)

Table 9-1. Proximates, Vitamins, and Minerals in V11 and Its Parental Control, Snowden

Compound	Variety	Mean	P-value[1]	N	Standard Deviation	Range		Tolerance Interval[2]		Combined Literature Range[3]	
						Min	Max	Min	Max	Min	Max
Moisture (%)	V11	78.5	0.1064	22	1.89	76	83	71.7	87	63.2	86.9
	Control	79.2		21	1.83	76.3	83.2				
Protein (%)	V11	2.34	0.9048	22	0.259	1.99	2.91	0.83	3.48	0.7	4.6
	Control	2.33		21	0.24	2.01	2.82				
Fat (%)	V11	0.166	0.8899	22	0.053	0.1	0.3	0	0.5	0.02	0.2
	Control	0.162		21	0.061	0.1	0.33				
Ash (%)	V11	1.03	0.6646	22	0.105	0.82	1.2	0.5	1.37	0.44	1.9
	Control	1.01		21	0.107	0.803	1.2				
Crude Fiber (%)	V11	0.475	0.3731	22	0.086	0.34	0.63	0.197	0.83	0.17	3.5
	Control	0.503		21	0.102	0.353	0.7				
Carbohydrates (%)	V11	17.9	0.1296	22	1.87	13.5	20.5	9.3	25.4	13.3	30.5
	Control	17.3		21	1.81	13.4	20.4				
Total Calories (kcal/100g)	V11	82.5	0.1161	22	7.70	64	93.2	48.8	111	80	110
	Control	79.9		21	7.29	64.2	92.1				
Vitamin B_3 (Niacin) (mg/100g)	V11	2.19	0.0984	22	0.259	1.62	2.64	0.794	2.68	0.09	3.1
	Control	2.05		21	0.201	1.68	2.32				
Vitamin B_6 (mg/100g)	V11	0.11	0.9855	22	0.011	0.097	0.14	0.064	0.19	0.11	0.34
	Control	0.11		21	0.011	0.096	0.14				
Vitamin C (mg/100g)	V11	26.9	**0.005**	22	2.45	22.1	32	12.1	34.4	1	54
	Control	24.1		21	4.10	15.2	30.4				
Copper (mg/100g)	V11	0.08	0.9679	22	0.023	0.05	0.12	0.011	0.16	0.02	0.7
	Control	0.08		21	0.024	0.05	0.12				
Magnesium (mg/100g)	V11	22.6	0.232	22	3.77	17.9	31	11.3	31	11.3	55
	Control	21.8		21	3.51	17.4	29.4				
Potassium (mg/100g)	V11	488	0.1021	22	43.0	426	605	240	587	350	625
	Control	473		21	39.2	405	557				

[1]P-values indicating significant differences with controls are bold and underlined.

[2]TI = 99% Tolerance Interval, 95% confidence.

[3]Literature ranges are from Lisinska and Leszczynski (1989), Rogan et al., (2000), Horton and Anderson (1992), Talburt and Smith (1987).

Table 9-2. Total Amino Acids in V11 and Its Parental Control, Snowden

Compound	Variety	Mean	P-value[1]	N	Standard Deviation	Range		Tolerance Interval[2]		Combined Literature Range[3]	
						Min	Max	Min	Max	Min	Max
Alanine (mg/100g)	V11	70.9	**0.0067**	22	5.62	60.4	82.9	22.4	105	39.2	95.2
	Control	64.2		21	4.99	56.7	76.1				
Arginine (mg/100g)	V11	142	**0.0056**	22	29.4	109	204	15.8	188	70.0	138
	Control	123		21	21.6	89.4	169				
Aspartic Acid (mg/100g)	V11	300	**<.0001**	22	35.0	249	377	44.2	799	339	738
	Control	519		21	62.9	414	627				
Glutamic Acid (mg/100g)	V11	495	**<.0001**	22	79.3	327	653	128	581	292	604
	Control	350		21	44.4	283	428				
Glycine (mg/100g)	V11	72.7	**0.0103**	22	7.89	59.3	89.3	8.60	110	1	97.5
	Control	65.4		21	6.67	56.8	81.7				
Histidine (mg/100g)	V11	36.0	0.1944	22	5.74	30.1	49.1	11.5	52.5	13.3	46.9
	Control	34.3		21	5.14	27.5	45.7				
Isoleucine (mg/100g)	V11	82.2	**0.0085**	22	9.05	67.7	101	20.0	123	52.5	95.3
	Control	75.5		21	8.37	63.8	94.5				
Leucine (mg/100g)	V11	138	**0.0026**	22	13.0	114	167	3.60	225	68.5	138
	Control	124		21	11.5	109	153				
Lysine (mg/100g)	V11	118	0.0534	22	11.4	99.8	143	36.6	173	68.7	137
	Control	111		21	8.76	102	132				
Methionine (mg/100g)	V11	39.2	0.1648	22	4.03	31.8	46.6	11.3	59.7	9	128
	Control	36.9		21	3.52	30.2	42.9				
Phenylalanine (mg/100g)	V11	96.6	0.0638	22	10.7	75.9	121	11.7	154	55.2	109
	Control	91.2		21	9.73	76.6	114				
Proline (mg/100g)	V11	78.9	0.3559	22	16.1	55.8	111	0	155	35.5	146
	Control	72.3		21	13.7	51.9	95.3				
Serine (mg/100g)	V11	82.7	**0.0049**	22	10.2	63.2	103	10	130	50.0	102
	Control	74.7		21	7.30	62.0	90.9				
Threonine (mg/100g)	V11	85.6	**0.0027**	22	8.91	70.3	105	11.5	129	43.6	85.5
	Control	77.7		21	7.53	68.6	97.1				
Tryptophan (mg/100g)	V11	20.9	0.2731	22	4.66	13.9	32.2	7.20	36.3	11.4	28.2
	Control	20.1		21	4.47	11.5	27.6				
Tyrosine (mg/100g)	V11	85.9	**0.0020**	22	10.2	72.0	108	17.3	124	45.7	94.2
	Control	76.1		21	8.83	66.1	94.3				
Valine (mg/100g)	V11	109	**0.0225**	22	13.0	90.0	133	43.3	159	75.2	145
	Control	102		21	12.2	82.6	123				

[1]P-values indicating significant differences with controls are bold and underlined.

[2]99% Tolerance Interval, 95% confidence.

[3]Literature ranges are from Talley et al., (1984), Rogan et al., (2000).

No statistical differences were found between V11 and Snowden for most of the proximates, vitamins and minerals measured (Table 9-1). Vitamin C content of V11 was greater than its parental control, Snowden, but was within the tolerance interval and literature range.

A significant difference between V11 and its parental control, Snowden, was noted for several total amino acids (Table 9-2): alanine, arginine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, serine, threonine, tyrosine, and valine. Aspartic acid was expected to be lower and glutamic acid was expected to be higher in V11 than in Snowden because of the down-regulation of the *Asn* gene (Figure 3-3). In each case, the mean for V11 was within the tolerance interval and/or the combined literature range, so V11 was considered equivalent to conventional potatoes.

9.2 Glycoalkaloids

Glycoalkaloids are toxins commonly found in Solanaceous crops, including potato. 95% of the total glycoalkaloids in potato tubers consists of α-solanine and α-chaconine (OECD, 2002). Today, the widely accepted safety limit for total glycoalkaloids in tubers is 20mg/100gm fresh weight (Smith et al., 1996).

The mean concentration of glycoalkaloids in V11 was not statistically different from its parent control, Snowden, and was within the generally accepted safety limit.

Table 9-3. Glycoalkaloids in V11 and Its Parental Control, Snowden

Compound	Variety	Mean	P-value[1]	N	Standard Deviation	Range		Tolerance Interval[3]	
						Min	Max	Min	Max
Glycoalkaloids[2] (mg/100g)	V11	9.70	0.3878	22	4.10	5.00	19.4	0	20.4
	Control	10.8		21	7.21	5.04	38.9		

[1]P-values indicating significant differences with controls are bold and underlined.
[2]Total of α-solanine and α-chaconine
[3]99% Tolerance Interval, 95% confidence

9.3 Traits Affecting Composition

An assessment of trait efficacy of V11 for low acrylamide potential and lowered reducing sugars consisted of the following analyses:

1) free amino acids (Table 9-4)
2) reducing sugars (Table 9-5)
3) acrylamide (Table 9-6)

Free amino acid analysis demonstrated that, as expected, down-regulation of *Asn1* was effective in reducing free asparagine in tubers. The results show that V11 tubers contained statistically less free asparagine and statistically more free glutamine than Snowden tubers (Table 9-4). However, the mean concentrations of free asparagine and free glutamine for V11 were still within the tolerance intervals and therefore considered within the normal range for potatoes.

Table 9-4. Free Amino Acids in Tubers at Harvest

Compound	Variety	Mean	P-value[1]	N	Standard Deviation	Range		Tolerance Interval[2]	
						Min	Max	Min	Max
Asparagine (mg/100g)	V11	79.4	**<.0001**	22	21.6	35.5	128	0	520
	Control	312		21	51.4	212	407		
Aspartic Acid (mg/100g)	V11	53.7	0.3054	22	35.0	33.8	77.8	4.20	71.4
	Control	51.5		21	62.9	35.8	74.0		
Glutamine (mg/100g)	V11	222	**<.0001**	22	62.2	71.2	322	0	298
	Control	125		21	36.0	55.9	181		
Glutamic Acid (mg/100g)	V11	66.5	0.2872	22	13.5	37.9	90.2	4.40	96.4
	Control	61.8		21	11.5	41.9	78.4		

[1]P-values indicating significant differences between V11 and control are in bold and underlined.
[2]99% Tolerance Interval, 95% confidence.

A review of the biosynthetic pathway for asparagine and glutamine in Figure 3-3 illustrates how a reduction in asparagine could lead to increases in glutamine. Through the activity of ASN1, the side-chain amine from glutamine is transferred to aspartate to form asparagine and glutamate. Down-regulation of *Asn1* would favor increased glutamine and reduced asparagine levels. Because of the down-regulation of the *Asn1* gene in V11, increased free glutamine and reduced asparagine were expected.

The V11 event contains expression cassettes designed to lower levels of reducing sugars fructose and glucose in tubers. A down-regulation cassette for the promoters of the starch-associated gene (*R1*) and the phosphorylase-L gene (*PhL*) was introduced in V11. These traits should function by slowing the conversion of starch to the reducing sugars glucose and fructose. In V11, partial down-regulation of *R1* and *PhL* resulted in slightly lower levels of reducing sugars at 0 (fresh) and 3 months after harvest, though the results were not statistically significant (Table 9-5). After nine months of storage, reducing sugars increased in both the V11 and its parental control. There were no differences in sucrose between V11 and its control.

Table 9-5. Tuber Sugars at Harvest and Stored at 50°F

Timing	Variety	Mean	P-value[1]	N	Range		Tolerance Interval[2]	
					Min	Max	Min	Max
Fructose + Glucose (mg/100g)								
Fresh	V11	26.7	0.7689	22	5.50	108	1	435
	Control	35.1		21	5.20	145		
Month 3	V11	53.5	0.2127	6	11.5	204	1	435
	Control	151		5	26.7	319		
Month 6	V11	39.4	0.945	3	11.5	95.0	1	435
	Control	14.7		3	11.1	19.1		
Month 9	V11	92.3	0.997	3	80.9	99.1	1	435
	Control	105		3	84.2	125		
Sucrose (mg/100g)								
Fresh	V11	197	0.8569	22	114	424	1	443
	Control	194		21	116	432		
Month 3	V11	147	0.4911	6	131	170	1	443
	Control	179		5	127	262		
Month 6	V11	98.0	0.7371	3	55.0	169	1	443
	Control	74.2		3	62.4	82.1		
Month 9	V11	171	0.9867	3	146	209	1	443
	Control	145		3	143	148		

[1]P-values indicating significant differences between V11 and control are in bold and underlined.
[2]99% Tolerance Interval, 95% confidence.

Lowered asparagine, fructose and glucose levels led to an overall reduction of acrylamide in processed potato products because they are reactants in the formation of acrylamide. In order to demonstrate the lower potential acrylamide trait, field-grown tubers of V11 and Snowden at harvest and after 3, 6, and 9 months of storage at 50°F were made into chips, and the acrylamide concentration of the chips was measured (Table 9-6).

At the time of harvest, potato chips made with V11 tubers contained 64.3% less acrylamide than chips made with Snowden (Table 9-6). When potatoes were stored throughout 3 months at 50°F, acrylamide concentrations in V11 were 48.9% lower than the control. Acrylamide concentrations in V11 chips were numerically but not statistically lower than Snowden after tuber storage at 50°F for 6 and 9 months. The significantly lower acrylamide levels at 0 and 3 months after storage were expected from down-regulation of the *Asn1, R1 and PhL* genes, thus reducing the reactants free asparagine and reducing sugars. Similar reductions in reducing sugars and acrylamide were reported by Zhu et al., 2014.

Snowden is recommended for 3 to 6 months storage (USPB 2014, UNL Crop Watch 2015), so lower acrylamide potential in tubers stored for up to 6 months after harvest will provide value throughout the typical storage time.

Table 9-6. Acrylamide in Chips Made From Potatoes at Harvest and After Storage at 50°F

Timing	Compound	Variety	Mean	P-value[1]	Percent Reduction	N	Range		Tolerance Interval[2]	
							Min	Max	Min	Max
Fresh	Acrylamide (ppb)	V11	262	**<.0001**	64.3	22	112	540	10	1,185
		Control	734			21	239	1540		
Month 3	Acrylamide (ppb)	V11	289	**0.0066**	48.9	6	125	582	10	1,185
		Control	566			5	399	857		
Month 6	Acrylamide (ppb)	V11	306	0.6386	47.9	3	279	335	10	1,185
		Control	587			3	337	717		
Month 9	Acrylamide (ppb)	V11	708	0.9839	15.6	3	499	1080	10	1,185
		Control	839			3	530	1030		

[1]P-values indicating significant differences between V11 and control are in bold and underlined.
[2]99% Tolerance Interval, 95% confidence.

9.3 Compositional Assessment Conclusions

Like the previously deregulated events, a thorough compositional assessment was conducted on V11 and its parental control, Snowden. Two types of analyses were conducted:

- compositional nutritional assessment, for those analytes important to general potato nutrition; and
- traits affecting composition, for those specific analytes related to gene down-regulation and trait efficacy.

The nutritional assessment, evaluating proximates, vitamins, minerals, amino acids, and glycoalkaloids demonstrated that, like the previously deregulated events, V11 is compositionally equivalent to

Snowden and is as safe and nutritious for food and feed as conventional potatoes that have a long history of safe consumption.

The efficacy assessment, evaluating free amino acids and reducing sugars as well as acrylamide concentrations in chips demonstrated that, like the previously deregulated events, V11 has lower levels of free asparagine, lower levels of reducing sugars, and lower acrylamide potential in potato chips than Snowden.

9.4 References

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10.0 Environmental Safety Assessment

Like the previously deregulated events, the environmental safety of V11 is supported by extensive testing including phenotypic performance, trait efficacy, genetic characterization, and compositional assessment. Information on V11 has been reviewed to determine the potential risk to the environment using the following five criteria:

(1) Potential to become a weed of agriculture or to be invasive of natural habitats;
(2) Potential for gene flow to sexually compatible plants;
(3) Potential to become a plant pest;
(4) Potential impact on non-target species including humans; and
(5) Potential impact on biodiversity.

10.1 Potential to Become Weedy, Invasive, or a Plant Pest

Weediness is a term used to describe the ability of a plant to become a weed (survive and thrive) outside of cultivation. Like the previously deregulated events, multiple field trials with V11 did not provide any evidence for altered growth characteristics such as accelerated tuber sprouting, increased plant vigor, increased tuber set, delayed senescence, volunteer potential, or other key phenotypic characteristics associated with weediness, invasiveness, or survival outside of cultivation.

Potato is a poor competitor and does not thrive in a non-cultivated environment (Love 1994). Due to modern agricultural practices it is unlikely that potatoes would persist in a field from one crop cycle to the next, particularly since potatoes are typically grown as a rotational crop. Also, much like Canada, in the northern U.S. most of the production areas experience deep frost penetration in the soil, minimizing the likelihood of over-winter survival (CFIA 1996). Results of the phenotypic assessment demonstrate no differences between V11 and its parental control with respect to survivability, and thus it is unlikely that V11 possesses increased potential to become a weed of agriculture, be invasive of natural habitats, or be a plant pest.

10.2 Potential for Gene Flow to Sexually Compatible Plants

Like the previously deregulated events, gene flow from V11 is expected to be minimal due to agricultural practices and biological characteristics of the Snowden variety. A thorough treatment of the weediness potential, hybridization risk, gene transfer, impact to non-target species, and implications for biodiversity was provided in the USDA petition number 13-022-01p on pages 56 through 60.

Generally, the potential for gene transfer in any potatoes through outcrossing within the species is minimal for several reasons:

1) a high percentage of fertile potatoes are self-pollinated and are not frequented by honeybees due to a lack of nectar;
2) pollen transfer between plants is limited to about 20 meters (Conner and Dale 1996) making transfer between commercial-scale fields unlikely;
3) it is unlikely that true potato seeds produced through outcrossing would grow into mature potatoes since potato seeds are not saved and propagated in a typical farming operation; and
4) potatoes are almost always clonally propagated using seed potatoes, thus removing the potential for further propagation of seed produced through outcrossing.

In the unlikely event that outcrossing was to occur between V11 and other potatoes, the impact would be negligible because tubers and not true potato seeds are harvested for future plantings. If seedlings did arise from an outcrossing event, establishment would be nearly impossible since potatoes are grown in rotation and are poor competitors compared to other crop and weed species (Love 1994; CFIA 1996). If seedlings were to establish after harvest, they would be easily identified and eliminated as part of the standard agricultural practices in potato production. In either case, seedlings could be identified and eliminated to prevent them from entering the commercial stream.

10.3 Potential Impact on Non-Target Species and Biodiversity

Like the previously deregulated events, V11 has no impact on non-target organisms. V11 does not express any traits with pesticidal activity; thus interactions with other species in the environment are, by definition, non-target. Observational data and field studies noted in the phenotypic assessment support the conclusion that no adverse impacts to non-targets occurred and no evidence of altered plant-disease interactions were noted. The mechanism of action for the reduced black spot and lower reducing sugar traits is down-regulation of endogenous potato genes. No novel proteins are produced. In addition, V11 is compositionally equivalent to conventional potatoes. Therefore, V11 does not possess any mechanism to harm non-target organisms or biodiversity and does not pose any risk to threatened or endangered species or humans when compared to commercially grown conventional potato varieties.

10.4 References

CFIA. (1996). Biology Document BIO1996-09: The Biology of *Solanum tuberosum* (L.) (Potatoes). Canadian Food Inspection Agency. http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-94-08/biology-documents/solanum-tuberosum-l-/eng/1330982063974/1330982145930

Conner, A.J., and Dale, P.J. (1996). Reconsideration of Pollen Dispersal Data from Field Trials of Transgenic Potatoes. Theoretical and Applied Genetics *92*, 505-508.

Love, S. (1994). Ecological Risk of Growing Transgenic Potatoes in the United States and Canada. American Potato Journal *71*, 647-658.

11.0 Stewardship of V11 Snowden Potatoes

Simplot is committed to the responsible introduction and stewardship of Innate™ potatoes, including the previously deregulated events and V11. The Simplot Stewardship Program for Innate™ potatoes is based on industry best practices for managing the development, production, sale, distribution and utilization of seed improved using biotechnology.

Simplot Plant Sciences is a member of Excellence Through Stewardship® (ETS) (BIO 2007). ETS is an industry-coordinated program that provides guidance for stewardship over the entire lifecycle of a biotech product—from early testing through commercial introduction and discontinuation, when appropriate. In 2015, Simplot Plant Sciences completed its first ETS Global Stewardship Audit which verifies that appropriate stewardship programs and comprehensive quality management systems are in place to manage both regulated materials and deregulated products.

11.1 Stewardship for Commercial Products

After regulatory authorizations, Innate™ potatoes will be managed by Simplot and its licensees in accordance with the Innate™ Stewardship Program. This commercial stewardship program has been designed to direct Innate™ seed and harvested potatoes only to Simplot-authorized growers, packers, processors and marketers who agree to abide by crop stewardship requirements (Table 11-1). Innate™ seed multiplication will be controlled by Simplot. Seed multiplication will be contracted only by Simplot and only with selected licensees who agree to transfer Innate™ seed under the direction of Simplot to its licensed growers. The Simplot requirement for stewardship will help to direct Innate™ potatoes in the marketplace and facilitate separation between Innate™ and non-Innate™ crops.

Table 11-1. Key Requirements of Innate™ Stewardship Program

- Innate™ Limited Use License and Stewardship Agreement
- Compliance training, annual
- Seed and crop segregation in storage and isolation of fields
- Identification of seed and crop
- Equipment and conveyance cleaning before and after use
- Secure shipment and storage
- Wastes and off-grade transferred only to approved users
- Volunteer monitoring and control
- Stewardship incident reporting and management procedures
- Implementation of corrective actions and process improvements
- Compliance auditing by Simplot or its designee
- Seed and crop transfers to Simplot-authorized third-parties, only
- No unlicensed seed multiplication (e.g., no grower-saved seed)

11.2 Regulatory Clearances in Foreign Markets and Plan for U.S. Commercialization

To help facilitate trade between the U.S. and other nations, international regulatory approvals will be sought from key trading partner countries. Simplot has conducted a U.S. trade and market assessment which will be used to guide the regulatory and commercialization strategies for Innate™ potatoes, including V11.

11.3 References

BIO. 2007. Product Launch Stewardship Program. Biotechnology Industry Organization.

12.0 Summary of Environmental Assessment Considerations

USDA recently completed a thorough environmental assessment of the previously deregulated events in 13-022-01p in July 2014 (USDA, 2014a). Because V11 was generated using the same genetic material and transformation method as the previously deregulated events, has the same traits and gene functions, has been thoroughly characterized molecularly, and is comparable phenotypically and compositionally to conventional potatoes, V11 presents no new or different environmental issues from those previously considered. There are no additional environmental issues from the Snowden variety that are different from the Russet Burbank, Ranger Russet, and Atlantic varieties previously assessed.

Table 12-1 provides a summary of environmental safety conclusions for the previously deregulated events and a comparison with the environmental assessment considerations for V11 Snowden.

Table 12-1. Summary of Environmental Assessment Considerations for V11

Attribute/Measure	Antecedent Events[a]	Determination of Non-regulated Status for V11
Meets purpose, need, and objectives	Yes	
Unlikely to pose a plant health risk	Satisfied – risk assessment (USDA, 2014b)	Will be satisfied with USDA risk assessment
Acreage and areas of potato production	Total acreage dedicated to potato is unlikely to change, but adoption of Simplot's biotech potato may reduce acreage dedicated to conventional potatoes.	Same
Management Practices		
Agronomic practices	Agronomic practices will remain the same as used currently.	Same
Pesticide use	Pesticides are currently used to control insects, nematodes, fungi, and weeds.	Same
Potato seed production	Potato seed results from cutting tubers grown specifically for the purpose of generating seed stocks.	Same
Organic potato production	Organic potato growers use practices and standards for production, cultivation, and product handling and processing to ensure that their products are not pollinated or comingled with conventional or GE crops.	Same
Environment		
Water resources	The primary cause of agricultural non-point source pollution is increased sedimentation from soil erosion, which can introduce sediments, fertilizers, and pesticides to nearby lakes and streams. Agronomic practices such as crop nutrient management, pest management, and conservation buffers help protect water quality from agricultural runoff. Water usage for irrigation would be expected to continue to increase.	Same
Soil quality	Agronomic practices such as crop type, tillage, and pest management can affect soil quality. Growers will adopt management practices to address their specific needs in producing potatoes. Erosion potential may continue to increase.	Same
Air quality	Agricultural activities such as burning, tilling, harvesting, spraying pesticides, and fertilizing, including the emissions from farm equipment, can directly affect air quality. Aerial application of herbicides may impact air quality from drift, diffusion, and volatilization of the chemicals, as well as motor vehicle emissions from airplanes or helicopters.	Same
Climate change	Agriculture-related activities are recognized as both direct sources of greenhouse gases (GHGs) (e.g. exhaust from motorized equipment) and indirect sources (e.g. agriculture-related soil disturbances, fertilizer production.	Same
Animal communities	Animals consuming tubers from deregulated events may be exposed to increased levels of glutamine, but this is not expected to be detrimental.	Same

Table 12-1, Continued. Summary of Environmental Assessment Considerations for V11

Attribute/Measure	Antecedent Events	Determination of Non-regulated Status for V11
Plant communities	In the unlikely event of hybridization of the deregulated varieties with conventional potatoes, resulting progeny may contain lowered polyphenol oxidase levels. However, this is not expected to be detrimental. The deregulated events are no weedier than conventional potatoes.	Same
Gene flow	Traits in the deregulated events are not expected to increase weediness in potato.	Same
Soil microorganisms	Abundance and diversity of soil microorganisms in and around potato fields is expected to remain as it is currently.	Same
Biological diversity	The biological diversity in potato fields is lower than in the surrounding habitats.	Same
Human and Animal Health		
Risk to human health	Glycoalkaloid and patatin exposure would continue. For humans consuming high-temperature cooked potatoes, acrylamide levels could be reduced by up to ~60-70%, which would benefit human health.	Similar. Data in this petition demonstrate acrylamide levels in V11 could be reduced by up to ~49-64%.
Risk to animal feed	Glycoalkaloids would continue to pose a risk to livestock if potato stems and foliage are fed to them, which is not likely.	Same
Socioeconomic		
Domestic economic environment	Because of its potential human health benefits (lower acylamide) and potential reduced wastage (lower black-spot) the deregulated potatoes may comprise a larger share of the domestic potato market, and may result in increased revenues.	Same
Trade economic environment	The foreign trade impacts associated with a determination of nonregulated status of the events are similar to the no action alternative. Import of each specific trait requires separate application and approval by the importing company. If the deregulated traits are approved by importing countries, it may make up a larger percentage of potato import markets.	Same

[a]Source: USDA, 2014a.

12.1 References

USDA. (2014a). Final Environmental Assessment. (Accessed 2015)
http://www.aphis.usda.gov/brs/aphisdocs/13_02201p_fea.pdf

USDA. (2014b) Plant Pest Risk Assessment. (Accessed 2015)
http://www.aphis.usda.gov/brs/aphisdocs/13_02201p_ppra.pdf

13.0 Conclusions: Extension of Nonregulated Status for V11

As with the previously deregulated events, the potato industry and consumers will benefit from introduction of Snowden potatoes with lower levels of asparagine, reducing sugars, and black spot. Considering that the Snowden variety, like other potatoes, is difficult to breed, biotechnology applications are ideally suited for simultaneously incorporating multiple traits. The propagation of commercial potatoes through cloning mitigates concerns such as seed dispersal, survival outside of cultivation, or outcrossing that could contribute to increased plant pest potential. Based on multi-year field trials evaluating composition, phenotypic performance, and insect/disease stressors, as well as the basic understanding and familiarity of potato biology, V11 poses no significant risk of persistence in the environment or altered environmental interactions as a result of weediness or increased plant pest potential.

The data presented here demonstrate that introduction of V11 Snowden potatoes will have a similar environmental impact as the previously deregulated events and conventional potatoes, and poses no increased risk to the environment. The unconfined introduction and cultivation of these potatoes is not expected to cause any adverse environmental, biological impacts, or detrimental effects on plant health.

Simplot now seeks an extension of the deregulation of events in 13-022-01p and requests nonregulated status for V11 based on the weight of evidence demonstrating that these potatoes are unlikely to pose a plant pest risk. We respectfully submit that V11 and its progeny should not be classified as “regulated articles” as defined under 7 CFR Part 340.

Appendix A: Molecular Methods

1.0 Genetic Characterization

The following methods were used to generate the molecular data presented in this petition for V11. Methods are included for characterization of the pSIM1278 insertion, including insert structures, absence of vector backbone, identification of flanking regions, and genetic stability.

1.1 Characterization of Insert Structures

Plant Material. Snowden plants used for characterization of insert DNA were grown in Sunshine mix-1 (www.sungro.com) in two-gallon pots in a greenhouse that was controlled for temperature (18°C minimum/27°C maximum) and light (16-h photoperiod with an intensity of ~1500 µmol/m2/s). Snowden control plants were also grown in the greenhouse under the same conditions and used as a background control. After one to two months of growth, leaf materials were taken from V11 and Snowden control plants for genomic DNA isolation.

DNA Isolation. DNA was isolated from leaf material using a modified CTAB protocol. Briefly, 1 g leaf tissue was ground under liquid nitrogen then suspended in extraction buffer containing 50 mM EDTA, 0.1 M Tris-HCl, pH 8.0, and 0.35 M Sorbitol. Tissue was pelleted by centrifugation, rinsed with extraction buffer, and resuspended in 2 ml extraction buffer with 100 µg/mL RNase A. An equal volume of lysis buffer containing 0.2 M Tris-HCl, pH 8.0, 50 mM EDTA, 2M NaCl, 2% CTAB, and 0.8 ml 5% Sarkosyl was added and incubated 20 min. at 65°C. DNA was extracted with two rounds of 24:1 chloroform: isoamyl alcohol followed by precipitation in Isopropanol, centrifugation, and a single wash with 70% Ethanol. Purified genomic DNA was resuspended in TE, pH 8.0 and quantified using a Qubit 2.0 Fluorometer (Molecular Probes) with the dsDNA Broad Range Assay kit (Molecular Probes).

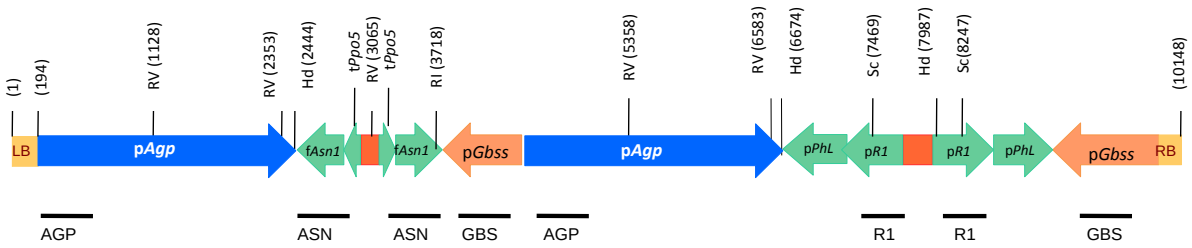
DNA Gel (Southern) Blot Analysis. 3 µg of plant DNA was digested overnight in 400 µl final volume with at least 5 µl (10 units/µl) restriction enzyme (Invitrogen) at 37^{o}C. Digested DNA was concentrated by ethanol precipitation (40 µl of 3 M NaOAc, pH5.3 and 1 ml ethanol) at -20^{o}C for 30 min followed by a wash with 70% ethanol. The DNA pellet was dissolved in 20 µl 1X TE followed by addition of 5 µl DNA gel loading buffer, which consists of 40% sucrose and 0.35% Orange G (Sigma) in water.

Membrane Preparation. Digested plant DNA was loaded on a large 0.7% agarose gel (170 ml) containing 0.5X tris-borate-EDTA (TBE) buffer and 3-5 µl ethidium bromide (10 mg/ml) and run at 30 volts for 18 hrs. The gel was photographed using a gel documentation system from Alpha Innotech (Santa Clara, California), and then depurinated by submerging it into 0.25 N HCl for 20 min. After subsequent denaturation in 0.5 M NaOH /1.5 M NaCl for 2 X 15 min and neutralization in 1.5 M NaCl and 0.5 M Tris-HCl, pH7.5, for 2 X 15 min on a shaker at room temperature the gel was equilibrated with 10X SSC for 10 min. The transfer of DNA to the nylon membrane was carried out using 10X SSC according to a standard capillary transfer method.

Probe Preparation. The labeling of the PCR-derived probe was achieved using Hotmaster *Taq* enzyme and buffer (Fisher BioReagents) according to Roche's DIG labeling instructions. A standard 50 µl reaction consisted of 5 µl of 10x Hotmaster *Taq* Buffer, 2-5 µl of 10 uM forward primer, 2-5 µl of 10 µM reverse primer, 5 µl DIG-labeled dNTP (Roche), 10 ng construct template, 0.75 µl Hotmaster *Taq* polymerase, and water. The PCR amplification conditions were dependent on each DIG-labeled probe. PCR with regular dNTP instead of DIG labeled dNTP was used as control. Quality of the DIG-labeled probe was

assessed by running a small amount of the probe on 1% agarose DNA gel (it always ran slower than control PCR product). The probe was denatured before use by incubating the probe at 100°C for 5 min, placing on ice for 2 min. For the exact position of probes in the DNA insert, see Figure A-1.

Figure A-1. DNA Insert Probes



RV=EcoRV, R1=EcoRI, Sc=ScaI, Hd=HindIII,
LB = Left Border like region containing 25-bp Left Border and 162-bp flanking sequence.
RB = Right Border like region containing 25-bp Right Border and 161-bp flanking sequence.

Hybridization. The nylon membrane carrying transferred DNA was prehybridized in 40 ml pre-warmed DIG Easy Hybridization solution (Roche) at 42°C for 1-4 hours in a bottle in a standard hybridization oven (Amerex Instruments Inc.) at 20-25 rpm. Hybridization was carried out by replacing the prehybridization buffer with a fresh amount of the same preheated solution, now containing 25-50 µl denatured DIG labeled probe, and continuing the incubation at 42°C, 20-25 rpm for about 16 hrs. The hybridization solution could be store at -20°C and reused up to 3 times. The reused hybridization solution was heated at 68°C for 10 minutes before use.

Detection. The hybridization solution was removed and replaced by 100 ml washing solution I (2X SSC/0.1% SDS). The membrane was washed twice in washing solution I for 10 min at room temperature. This low stringency buffer was poured off and preheated high stringency washing solution II (0.5X SSC/0.1% SDS, 65°C) was added immediately. The membrane was washed twice in washing solution II at 65°C for 20 min each at 25-30 rpm. This was followed by a brief rinse with 2X SSC to remove SDS. The membrane was rinsed with 150 ml of 1X DIG Washing Solution (Roche) in a tray for 2 min and incubated in 1X Blocking solution (Roche) for 0.5-3 hrs on a low-speed shaker. The blocked membrane was incubated with DIG antibody solution (1:10,000 dilution of Anti-DIG-alkaline phosphate conjugate with 1X Blocking solution) for 30 min on a shaker. The membrane was washed twice (15 min each) with 1X DIG Washing Solution (Roche) and equilibrated with 1X detection buffer. The detection reaction was carried out with 2 ml CDP-Star solution (1:100 diluted stock of CDP-Star with 1X detection buffer) for 5 min. The membrane was wrapped in a plastic film and exposed to the Z-ray film in the dark. Depending on the experiment, multiple exposures were taken from 30 sec to 30 min. The films were developed with Konica SRX-101A Z-ray film developer. The developed films were scanned to obtain the final images.

1.2 Characterization of Vector Backbone Sequences

Plant material. Plants used for DNA gel blot analysis were grown for two months in Sunshine mix-1 (www.sungro.com) in two-gallon pots in a greenhouse controlled for temperature (18°C minimum/27°C maximum) and light (16-h photoperiod with an intensity of ~1500 µmol/m^2/s).

DNA Isolation. Genomic DNA was isolated from the leaves of greenhouse-grown plants as described in section 1.1 for use in the following assays.

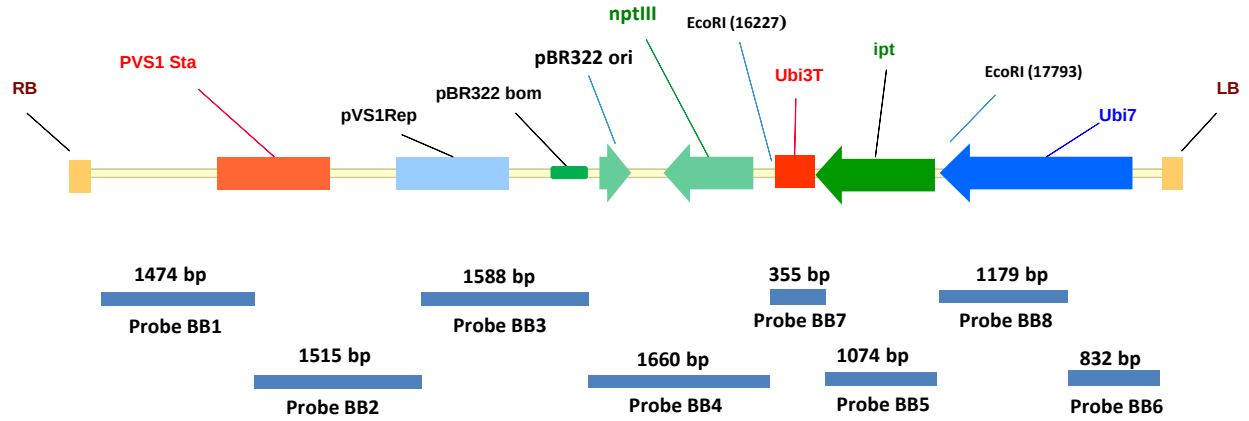
Southern Blot - Gel Preparation. Digested DNA was electrophoresed on a large 0.7% agarose gel (170 ml) containing 0.5X Tris-borate-EDTA (TBE) buffer and 3-5 µl ethidium bromide (10 mg/ml) for 18 hrs using 30 volts. The gel was photographed using a gel documentation system from Alpha Innotech (Santa Clara, California), and then depurinated by submersion in 0.25N HCl for 20 min. After subsequent denaturation in 0.5 M NaOH / 1.5 M NaCl for 2 x 15 min and neutralization in 1.5 M NaCl / 0.5 M Tris-HCl (pH 7.5), for 2 x 15 min on a shaker at room temperature, the gel was equilibrated with 10X SSC for 10 min. The transfer of DNA to the nylon membrane was carried out using 10X SSC using capillary transfer.

Southern Blot - Probe Preparation. Nylon filters cross-linked with DNA digested with *Eco*RI were hybridized independently with eight different probes, which covered entire vector backbone (see Table A-1 for primer sequences and Figure A-2 for the linear arrangements of the probes described in Table A-1). Untransformed potato varieties were used as negative controls, and the T130 event carrying the entire backbone of pSIM1278 vector provided positive controls.

Table A-1. Backbone Probes for pSIM1278

Backbone probe	Size (bp)	pSIM1278 Coordinates	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
BB1	1,474	10,149-11,622	ACTAGTTGTGAATAAGTCGCTGTG	ATCGGAATCGACTAACAGAACAT
BB2	1,515	11,591-13,105	CCGGGGCCGATGTTCTGTTAG	GCTCGCCGGCAGAACTTGAG
BB3	1,588	13,054-14,641	GCCGCGTGTTCCGTCCACAC	CCTGTCGGGTTTCGCCACCT
BB4	1,660	14,614-16,273	CAAGTCAGAGGTGGCGAAAC	CTTTATGCTCATTGGGTTGAGTA
BB5	1,074	16,590-17,663	AGTCCACCCGAAATATAAACAAC	GGTATGGACCTGCATCTAATTTTC
BB6	832	18,827-19,658	GCTCTAATATAGCGCATTTCAAG	GCTTCCAGCCAGCCAACAGCTC
BB7	355	16,232-16,586	CTATTTTTTTACTATATTATACTCAAC	TTTTAATGTTTAGCAAATGTCTTATC
BB8	1,179	17,668-18,846	GATCCACCTCCACGTAGACGGAG	GAAATGCGCTATATTAGAGCATA

Figure A-2. Probes for pSIM1278 construct backbone testing



[1]Numbering system based on pSIM1278 (Chapter 4: Donor Genes and Regulatory Sequences, Table 4-1 and Figure 4-1). The backbone DNA is spanned by the probes shown as blue rectangles.

The labeling of PCR-derived probes were achieved using Hotmaster Taq enzyme and buffer (Fisher BioReagents) according to Roche's DIG labeling protocol. A standard 50 µl reaction consisted of 5 µl of 10X Hotmaster Taq Buffer, 2-5 µl of 10 µM forward primer, 2-5 µl of 10 µM reverse primer, 5 µl of DIG labeled dNTPs (Roche), 10 ng construct template, 0.75 µl Hotmaster Taq polymerase, and water. The PCR amplification conditions were optimized for each DIG-labeled probe. PCR with regular dNTPs instead of DIG labeled dNTPs was used as positive control. Quality of the DIG labeled probe was assessed by analyzing a fraction of the product on a 1% agarose gel alongside control (unlabeled) PCR product. The probe was denatured before use by incubating the probe at 100°C for 5 min, and then quenched on ice for 2 min.

Southern Blot – Hybridization and Exposure. The nylon membrane carrying transferred DNA was prehybridized in 40 ml pre-warmed DIG Easy Hybridization solution (Roche) at 42°C for 1-4 hrs in a hybridization oven (Amerex Instruments Inc.) rotating at 20-25 rpm. Hybridization was carried out by replacing the prehybridization buffer with a fresh amount of the same preheated solution containing 25-50 µl denatured DIG labeled probe, and continuing the incubation with rotation (20-25 rpm) at 42°C for about 16 hrs. The probe-containing hybridization solution was stored (-20°C) and reused up to 3 times. The reused hybridization solution was heated at 68°C for 10 minutes before use. The hybridization solution was removed and replaced by 100 ml washing solution I (2X SSC/0.1% SDS). The membrane was washed twice in washing solution I for 10 min at room temperature. This low stringency buffer was poured off and preheated high stringency washing solution II (0.5X SSC/0.1% SDS, 65°C) was added immediately. The membrane was washed twice in washing solution II at 65°C for 20 min each at 25-30 rpm. This was followed by a rinse with 2X SSC to remove SDS. The membrane was rinsed with 150 ml of 1X DIG Washing Solution (Roche) in a tray for 2 min and incubated in 1X Blocking solution (Roche) for 0.5-3 hours on a low-speed shaker. The blocked membrane was incubated with DIG antibody solution (1:10,000 dilution of Anti-DIG-alkaline phosphate conjugate with 1X Blocking solution) for 30 min on a shaker. The membrane was washed twice (15 min each) with 1X DIG Washing Solution (Roche) and equilibrated with 1X detection buffer. The detection reaction was carried out with 2 ml CDP-Star solution (1:100 diluted stock of CDP-Star with 1X detection buffer) for 5 min. The membrane was wrapped in plastic film and exposed to the Z-ray film in the dark. Depending on the experiment multiple

exposures were taken from 30 sec to 30 min. Films were developed with a Konica SRX-101A Z-ray film developer. The developed films were scanned to obtain the final images.

PCR-Based Identification of Vector Backbone sequences. A PCR assay was used to verify the absence of sequence containing the junction between backbone and the DNA insert from pSIM1278. As diagrammed in Figure A-3, each junction was tested using two sets of PCR primers. The standard 30 µl PCR reaction mixture consisted of 3 µl of 10X PCR buffer, 0.6 µl of 10 mM dNTPs, 0.6 µl of 10 µM forward primer, 0.6 µl of 10 µM reverse primer, 100 ng of genomic DNA template, with 0.3 µl of HotMaster Taq polymerase (Fisher BioReagents). The PCR was carried out under the following amplification conditions: 1 cycle of 3 min at 95°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 55°C, 30 sec at 68°C, and finishing with 10 min at 68°C. Primers were designed to amplify fragments indicative of (1) junctions between DNA insert border regions and flanking backbone DNA or (2) regions entirely within the backbone DNA that flank the DNA insert. The primer pair JY725-JY726 amplified a 377-bp fragment comprising the junction at the Right Border region and flanking backbone, and primers JY915-JY749 were used to amplify a 215-bp backbone fragment flanking the Right Border of both pSIM1278 (see Figure A-3 and Table A-2). The primer pair JY718-JY719 amplified a 587-bp fragment comprising the junction at the Left Border region and flanking backbone of pSIM1278, and primers JY1029-JY1030 were used to amplify a 503-bp backbone fragment flanking the Left Border of pSIM1278 (see Figure A-3 and Table A-2**).**

Figure A-3. PCR primers for detecting backbone adjacent to left and right borders of pSIM1278

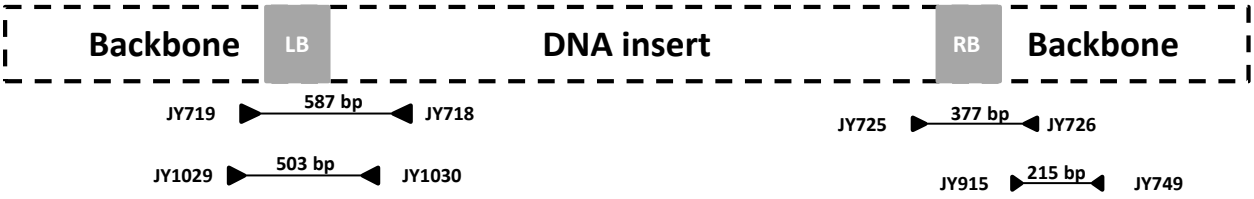
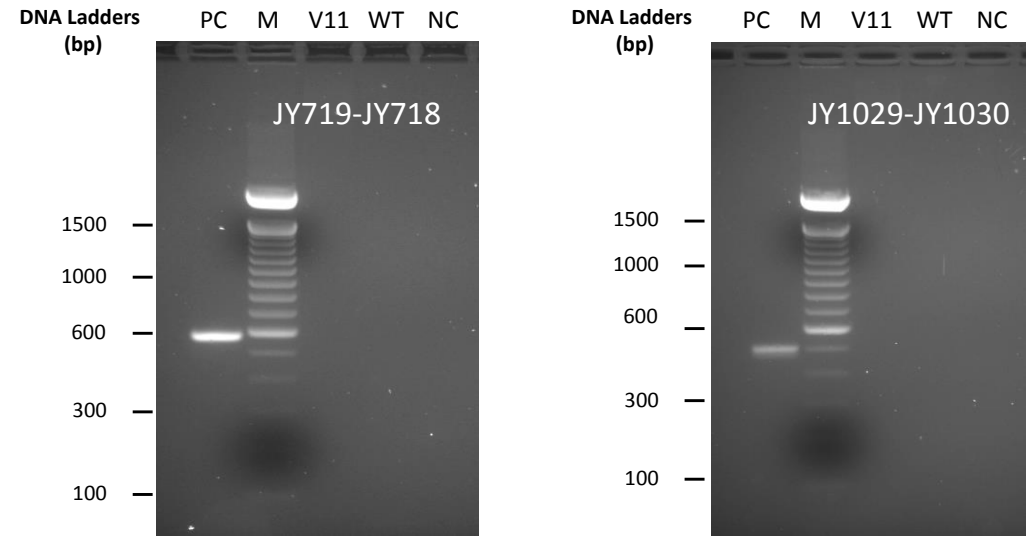
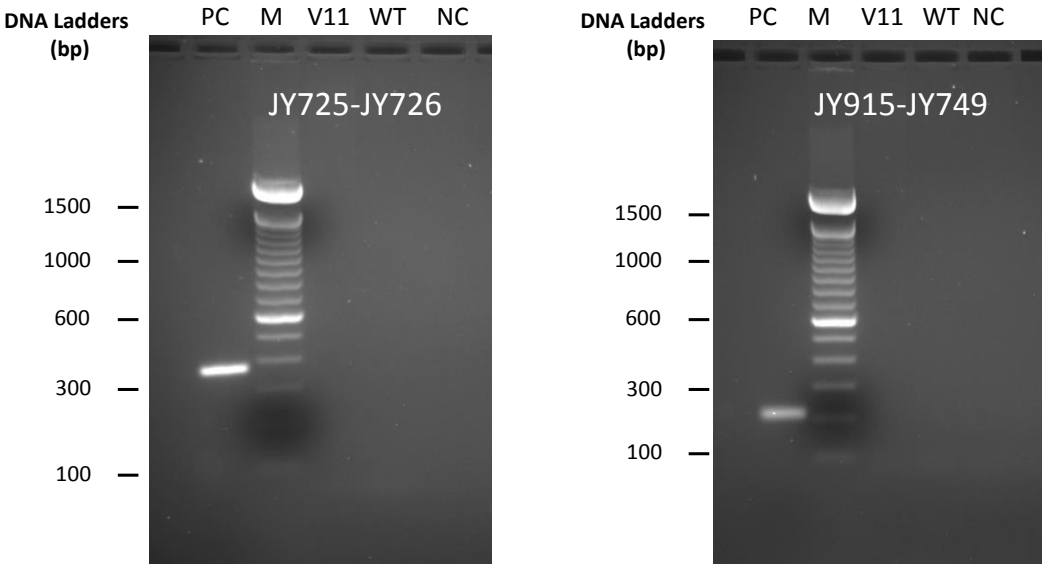


Figure A-4. Confirmation of the Absence of Backbone DNA Junctions

Detection of Left Border of the DNA insert and flanking backbone of pSIM1278



Detection of Right Border of the DNA insert and flanking backbone of pSIM1278



Ethidium bromide stained agarose gels for the PCR reactions using the primer sets indicated. PC = positive control plasmid, M = 100-bp DNA marker (Invitrogen), V11 = Submitted Snowden event, WT = Snowden wild type control, NC = Negative control.

None of the PCR reactions amplified junction regions in either V11 or WT samples, whereas in each case positive controls amplified as expected. Consistent with the Southern data, PCR failed to identify the presence of any backbone DNA in V11. The absence of backbone DNA adjacent to the DNA insert is further supported by the lack of backbone DNA within the insert flanking sequences (Section 5.3).

Table A-2. Primer Sequences and PCR Products for Detection of Backbone Adjacent to Left and Right Borders of pSIM1278

PCR Primers for Detecting Backbone Adjacent to Left Border of pSIM1278				
Name	**Sequence (5′ to 3′)**	**Location in pSIM1278**	**Product length (bp)**	**Backbone (bp)**
JY719	GAGCTGTTGGCTGGCTGGAAG	19,637-1,9657 (backbone)		
JY718	GTTGGAAATCAATTATCACTGAG	541-563 (AGP promoter)	587	24
JY1029	CCGTTCTTCCGAATAGCATC	19,507-19,526 (backbone)		
JY1030	CGGGTTATCGGTTCTTAACG	330-349 (AGP promoter)	503	154
PCR Primers for Detecting Backbone Adjacent to Right Border of pSIM1278				
Name	**Sequence (5′ to 3′)**	**Location in pSIM1278**	**Product length (bp)**	**Backbone (bp)**
JY725	GCTTCCCGTATACAACATAACATG	9,813-9,836 (GBSS promoter)		
JY726	GATCTCAAACAAACATACACAGCG	10,166-10,189 (backbone)	377	41
JY1029	CCGTTCTTCCGAATAGCATC	19,507-19,526 (backbone)		
JY1030	CGGGTTATCGGTTCTTAACG	330-349 (AGP promoter)	215	215

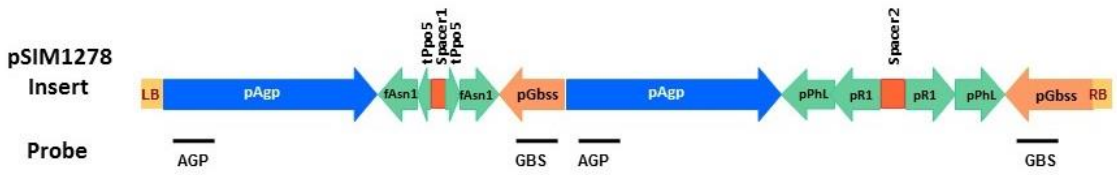
1.3 Analysis of Genetic Stability

Materials. DNA insert stability was demonstrated in the originally transformed material (G0) by extracting and evaluating DNA from leaves of plants that had been propagated *in vitro* and never planted in soil. For G1 and G2 analyses, leaves of two plants from each event and one plant from each control were collected from field trial. For the G3 analyses, leaves of two plants from each event and one plant from each control were collected from plants grown in a greenhouse. The G2 tubers of unmodified Snowden control and V11 were harvested from Hancock, Wisconsin, Florida, and Michigan State University field trial sites and were used for the catechol assay.

DNA Isolation. Genomic DNA was isolated from the leaves of greenhouse-grown plants as described in Section 1.1 for use in the following assays.

Southern Blot Analysis. Extracted genomic DNA from leaves was digested with *Eco*RV and hybridized with two probes (GBS and AGP). The probes used in the Southern blot analysis are depicted in Figure A-5. 3 µg of DNA was digested overnight in 400 µl final volume with at least 5 µl (10 units/µl) restriction enzyme (Invitrogen) at 37°C. Digested DNA was concentrated by ethanol precipitation (40 µl of 3M NaOAc, pH 5.3 and 1 ml ethanol) at -80°C for 10 min followed by a wash with 70% ethanol. The DNA pellet was dissolved in 20 µl 1X TE followed by addition of 2 µl DNA gel loading buffer, which consists of 40% sucrose and 0.35% Orange G (Sigma) in water.

Figure A-5. Probes Used for the Southern Blot Stability Analysis



Membrane Preparation. Digested DNA was loaded on a large 0.7% agarose gel (170 ml) containing 0.5X TBE buffer and 3-5 µl ethidium bromide (10 mg/ml) and run at 30 volts for 18 hrs. The gel was photographed using a gel documentation system from Alpha Innotech (Santa Clara, California), and then depurinated by submerging it into 0.25 N HCl for 20 min. After subsequent denaturation in 0.5 M NaOH /1.5 M NaCl for 2 X 15 min and neutralization in 1.5 M NaCl and 0.5 M Tris-HCl, pH7.5, for 2X15 min on a shaker at room temperature and equilibrated with 10X SSC for 10 min. The transfer of DNA to the nylon membrane was carried out using 10X SSC according to a standard capillary transfer method (Sambrook and Russell 2001).

1.4 Northern Blot Methods

Plant material. Gene expression levels were determined by carrying out RNA gel blot analyses on tubers, stolons, roots, stems, leaves, and flowers of greenhouse-grown plants of V11. Three replications per event were used in the analysis.

RNA Isolation. RNA was extracted from 1 g of tuber and root tissue using Plant RNA reagent (Invitrogen, Carlsbad, California), whereas Trizol reagent (Invitrogen) was used to extract RNA from leaves, flowers and stems. The concentration of isolated RNA was measured spectrophotometrically at 260 nm, and RNA quality was confirmed by running a sample on a 1% agarose gel with 1X MOPS buffer (200 mM MOPS, 50 mM NaOAc , 20 mM EDTA , pH7.0) for 30-60 min at 90 volts.

RNA transfer. 20-25 µg of RNA was denatured in RNA loading dye (Sigma) for 10-min at 650 C followed by incubation on ice for 5-min. Denatured RNA was loaded onto an RNA gel containing 1% agarose, 1X MOPS, 0.1-0.25 µg/ml ethidium bromide and 2% formaldehyde (5.5 ml 36.5% stock in 100 ml solution). The gel was run at 80-85 volts for 2-3 hrs and then photographed using the gel documentation system from Alpha Innotech (Santa Clara, California). The gel was soaked twice in 10X SSC for 15-min to remove the formaldehyde. RNA was transferred from the gel to a positively charged nylon membrane (Roche, Indianapolis) by capillary blotting with 10X SSC for 16-18 hrs, and the transferred RNA was stabilized onto the filter by UV cross-linking (UVP, Upland, California). The RNA-containing membrane was stored at 40 C.

Preparation of DIG labeled Probe. A PCR based method was used to prepare DIG labeled probes for four target transcripts and an internal control of 18s rRNA. The primers used for PCR amplification and the lengths of probes are listed in Table 2. A typical 50 µl labeling reaction consisted of 5 µl HotMaster Taq Buffer (Fisher BioReagents), 2-5 µl of 10 µM forward primer, 2-5µl of 10 µM reverse primer, 5µl

DIG-labeled dNTP (Roche, Indianapolis), 5-30 ng plasmid template, 0.50-0.75 µl HotMaster Taq polymerase, and dH2O for a total volume of 50 µl. PCR conditions were specific for each DIG-labeled probe. The DIG-labeled probe was checked on 1% agarose gel and always ran slower than the control. **PCR product.** The probe was denatured before use by incubating for 5 min at 100° C and then transferring to ice. Hybridization. Nylon membranes containing transferred RNA were pre-hybridized in 40 ml pre-warmed DIG Easy Hybridization solution (Roche, Indianapolis) for at least 1-4 hrs at 20-25 rpm in a hybridization oven set at 42° C (Amerex Instruments). The hybridization solution was replaced by a mix of 40 ml fresh pre-warmed hybridization solution and 25-50µl of denatured DIG-labeled probe, and the membrane was incubated in this mix for 3-16 hrs at 42° C. The hybridization solution can be store at -20° C and reused. The reused hybridization solution was heated at 68° C for 10 minutes before use.

2.0 Sequence Analysis

Potato material. Leaves from greenhouse-grown event V11 and untransformed Snowden plants were used to extract DNA for characterization of the insert junctions.

DNA Isolation. Genomic DNA was isolated from the leaves of greenhouse-grown plants as described in section 1.1 for use in the following assays.

Adapter ligation-mediated PCR Junction fragments were amplified by PCR using digested DNA ligated with adapter primers AP1 and AP2 as described by O'Malley et al., 2007. Briefly, 200 ng genomic DNA was digested for 3-5 hours with a restriction enzyme for which an adapter had been designed (EcoRI, HindIII, BamHI, AseI/NdeI.). The digested DNA was ligated with its respective oligonucleotide adapter in a reaction with 1X T4 Ligation Buffer, 1.5 units T4 DNA Ligase, 64ng digest DNA fragments, 0.3 mM ATP, and the adapter to a final concentration of 0.1 mM. The ligation reaction was used as template for the primary PCR, carried out with a DNA insert-specific primer and AP1 with Hot Master Taq polymerase (Fisher BioReagents) under the following amplification conditions: 1 cycle of 3 min at 95°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C, 4min at 68°C, and finishing with 10 min at 68°C. A 1 µl aliquot of the primary PCR product was used for secondary reactions (1 cycle of 3 min at 95°C; 35 cycles of 30 sec at 94°C, 30 sec at 62°C, 2.5 min at 68°C; 1 cycle of 10 min at 68°C) with a nested DNA insert-specific primer and AP2. This protocol was performed for untransformed control plants along with V11. Products of the secondary PCR were run on 1% agarose in TAE buffer. Bands unique to V11 were gel-extracted using a Qiagen QIAquick Gel Extraction kit, cloned into pGEM-T Easy vector (Promega, Madison WI), and sequenced. Primers spanning the junction between DNA insert and chromosomal flanking DNA were designed and used to confirm sequences in genomic DNA.

3.0 References

O'Malley R.C., Alonso, J.M., Kim, C.J., Leisse, T.J., Ecker, J.R. (2007). An Adapter Ligation-mediated PCR Method for High-throughput Mapping of T-DNA Inserts in the *Arabidopsis* Genome. Nature Protocols *2*, 2910-2917.

Sambrook J, Russell DW (2001) Southern Hybridization. In: Molecular Cloning: A Laboratory Manual. (Ed. Argentine J) Cold Springs Harbor Laboratory Press, Cold Springs Harbor, NY, pp. 6.33-6.38.

Appendix B: V11 USDA Notifications and Permits

Since 2011, field trials of V11 have been conducted in nine states in the continental U.S. Table B-1 lists past plantings (2011-2014 growing seasons) and USDA notifications in place for the upcoming 2015 growing season.

Table B-1. V11 USDA Release Notifications and Planted Acreage Details*

Year of Planting	Permit Name	Permit Valid Through	State	Number of Counties Where V11 was Planted	Acreage (Whole Plot)
2011	11-063-103n	4/4/2012	ID	1	0.1
2012	11-356-101n	1/23/2012	FL	1	0.15
	12-066-102n	4/2/2013	MI	1	1.5
			WI	1	0.18
			NE	1	0.12
	12-249-103n	9/28/2013	ID	1	0.74
			WA	1	0.74
	12-073-103n	4/9/2013	ID	1	4
2013	13-072-112n	4/2/2014	WA	1	1.2
			PA	1	2
	13-074-121n	4/1/2014	WI	1	0.41
	13-079-108n	4/16/2014	MI	1	1.5
	13-095-107n	4/22/2014	MI	1	0.09
	13-079-102n	4/10/2014	WI	1	0.09
	13-098-106n	4/29/2014	NE	1	0.8
2014	14-066-105n	3/20/2015	WA	1	0.28
			ID	1	0.65
			PA	1	0.27
	14-066-106n	3/19/2015	MI	2	1.5
			NY	1	0.74
	14-076-104n	3/25/2015	MI	1	4.3
	14-089-101n	4/4/2015	WI	1	2.1
	14-105-105n	5/1/2015	NE	1	24
			OR	1	5.46
	14-084-101n	4/15/2015	MI	1	0.88
	14-273-102n	10/31/2015	FL	1	0.5
2015 (in progress)	15-060-101n	4/3/2016	NE	2	TBD
			MI	1	TBD
			WI	2	9.4
			NY	1	TBD
	15-051-107n	4/1/2016	ID	1	2.1

*Plantings through April 10, 2015 are listed. The 2015 planting season is still underway.

Appendix C: Phenotypic Methods

Varieties grown in field trials are described in Table C-1. In 2012, test and control varieties were grown at all sites. In 2013, test, control, and reference varieties were grown at all sites. Reference varieties are commercially-available varieties that provide a range of values common to conventional potatoes.

For the 2012 evaluations, nutrient film technology (NFT)-produced mini-tubers for V11 and the control variety were planted. This NFT seed was grown at CSS Farms in Colorado City, Colorado. G0 plants from tissue culture were used to grow mini-tubers using nutrient film technology, in which seed is propagated hydroponically using water enriched with dissolved nutrients. In this system, a large number of small seed can be produced because multiple seed harvests from each plant are possible.

For the 2013 evaluations, field-grown G1 seed tubers from each event and the control variety were used to plant the field trials. This seed was grown on a seed farm in Cody, Nebraska. Field-grown G1 seed is typically larger than NFT mini-tuber seed and resembles typical sized potatoes.

Table C-1. Varieties in Field Trials

Variety	Type	Genotype	Seed Type	Seed Source
2012				
Snowden	Control	N/A	G0 mini-tubers	CSS Farms - Colorado City, CO
V11	Test	pSIM1278		
2013				
Snowden	Control	N/A	Field-grown G1-tubers	CSS Farms – Cody, NE
V11	Test	pSIM1278		
Gala	Reference	N/A		
Purple Majesty	Reference	N/A		
C0095051-7w	Reference	N/A		
Norkotah	Reference	N/A		

N/A = not applicable

The experiments were established in a randomized complete block design (RCB). The RCB is typical for the evaluation of new potato varieties and events. In 2012, each plot consisted of four rows (except the site in Adams Co. WI. 2012 which had 3 rows) approximately 20 feet long, each containing 20 mini-tubers. There were three replicates at each site. In-row seed spacing was approximately 12 inches. The mini-tubers were either mechanically or hand planted to a depth of 3-6 inches. In 2013, the sites had plots that consisted of four rows. Each row was approximately 20 feet long, each containing 20 field-grown G1 seed pieces. There were four replicates at each site. In-row seed spacing was approximately 12 inches. The tubers were either mechanically or hand planted to a depth of 3-6 inches.

The agronomic practices and pest control measures used were location-specific and were typical for potato cultivation. They were recommended by both regional potato extension specialists and agronomists and they related to all aspects of soil preparation, fertilizer application, irrigation, and pesticide-based control methods. An example of typical inputs for Snowden potato production is given in Table C-2. V11 and untransformed varieties received identical inputs and treatments within each site.

The trial sites selected for the phenotypic evaluations were different agricultural zones and represented the main production areas for potatoes in the U.S.

Table C-2. Example of Agronomic Inputs for Snowden Potato Varieties

Planting Date	April 1 to May 10
Vine maturity	110-120 days after planting
Planting Rate	15,000 - 18,000 seed pieces or 17 – 23 cwt/A
Row Spacing	34-36" between rows
Seed Spacing	Approximately 12" within row
Fertilizer	For 500 cwt/A yields and optimum soil test levels: 200 lb N; 100-180 lb P_2O_5; 60 lb K_2O/ acre
Yield/Acre	300-500 cwt/A

The phenotypic characteristics evaluated are listed in Table C-3.

Tubers were harvested during early fall except in St. John's County, FL where tubers were harvested in late spring. At harvest, all tubers from one row of each plot were transported to Michigan State University for testing. The grading methods employed were similar to those used to grade commercial potatoes intended for the production of chips.

The specific gravity was determined by using a weight in air/weight in water measurement. Sub-samples of tubers were first weighed in air and then weighed submerged under water at room temperature. From the two measurements, specific gravity was calculated using the following formula:

specific gravity = weight in air/ (weight in air - weight in water).

Specific gravity is the industry standard for measuring solids and is thus an important characteristic to compare V11 to its parental control, Snowden.

Table C-3. Characteristics Evaluated

Characteristic measured	Evaluation timing[1]	Data description	Scale
Early Emergence	Early season	# of plants emerged out of 20 seed pieces planted, scored in middle two rows of each plot at approximately 50% emergence	0-100%
Final Emergence	Early season	# of plants emerged out of 20 seed pieces planted, scored in middle two rows of each plot at approximately complete emergence	0-100%
Stems per Plant	Early season	Number of stems of 10 non-systematically selected plants in the middle rows of each plot	Number of stems per plant
Plant Vigor	Midseason	Visual estimate of relative vigor	1 to 5 point scale[2]
Plant Height	Midseason	Measured from the soil surface at the top of the hill, to the top of the uppermost leaf of 10 non-systematically selected plants in the middle rows of each plot	cm
Vine Desiccation	Late season	Visual estimation of the percent of vines desiccated in the middle rows of each plot	0-100%
Total Yield	After harvest	Weight of one of the center two rows, scaled to weight per unit area	Cwt/acre
U.S. #1	After harvest	Total tuber weight minus the weight of oversize and pickout tubers	Cwt/acre
Tubers per plant	After harvest	Total number of tubers in a single-row divided by the total number of plants in the same row	Tubes/plant
Grade A	After harvest	Tubers 2 to 3.25 in. diameter	% of tubers by weight
Grade B	After harvest	Tubers <2 in. diameter	% of tubers by weight
Oversize (unusable)	After harvest	Tubers >3.25 in. diameter	% of tubers by weight
Pickout (unusable)	After harvest	Unmarketable tubers based on visual physiological defects	% of tubers by weight
Specific gravity	After harvest	Tuber sample weight in air/(weight in air - weight in water)	Numeric specific gravity value
Total internal defects	After harvest	Sum of internal defects such as hollow heart, vascular necrosis, internal discoloration, internal brown spot, and nematode or insect damage	% of tubers affected by any internal defect

[1]Early season observations were made within approximately 45 days after emergence. Midseason observations were made during the early bloom stage. Late season notes were taken during the crop senescence stage prior to chemical or mechanical vine desiccation.

[2] 1 to 5 scale for vigor:

1 = severely less than the varietal average; 2 = noticeably less than varietal average, but not severe; 3 = plants are similar to the varietal average; 4 = slightly more than varietal average; 5 = obviously more than the varietal average.

Table C-4. Common Potato Disease and Insect Symptoms[1]

Insect or Disease Agent	Symptom
Emposasca fabae (Potato Leafhopper)	Leaf feeding damage
Epitrix species (Flea Beetle)	Shot-holes in leaves
Leptinotarsa decemlineata (Colorado Potato Beetle)	Defoliation
Limonius californicus (Wireworm)	Bored holes in tubers and shoots
Ostrinia nubilalis (European Corn Borer)	Severe vine wilting above point of injury
Bactericera (Paratrioza) cockerelli (Potato Psyllid)	Yellows
Phthorimaea operculella (Tuberworm)	Foliar and tuber damage
Various aphid spp.	Leaf suckling damage
Aster Yellows MLO	Purple top disease
Potato Leafroll Virus	Rolling of leaves and net necrosis
Potato Spindle Tuber Viroid	Potato spindle tuber disease
Potato Virus A,M, X, Y	Mosaic symptoms
Tobacco Rattle Virus	Stem mottling
Erwinia carotovora	Blackleg, aerial stem rot and tuber soft rot
Corynebactium sepedonicum	Bacterial ring rot
Ralstonia solanacearum	Brown rot
Phytophthora infestans	Late blight
Phytophthora erythroseptica	Pink rot
Verticillium spp.	Early dying
Sclerotinia sclerotiorum	Sclerotinia stalk rot
Rhizoctonia solani	Canker
Streptomyces scabies	Scab
Fusarium spp.	Dry rot
Pythium ultimum	Water rot, shell rot, *Pythium* leak
Alternaria solani	Early blight
Botrytis cinerea	Gray mold

[1]All stressors shown here were not necessarily observed at all sites or observation timings. This table is meant to give the reader an accurate list of insects and diseases that may impact potatoes.

Volunteer Potential Methods

Tubers harvested from V11 and control plots in the phenotypic trials were used to plant the volunteer potential study. Conventional reference varieties were provided by the grower from commercial sources.

The experiments were established in a randomized complete block (RCB) design with four replicates at each site. Each plot consisted of three rows approximately 10 feet long. In-row seed spacing was approximately 12 inches and each row contained 10 tubers. The tubers were hand planted to a depth of 3-6 inches.

After tubers were planted in the fall of 2012, the field was monitored for volunteers approximately every two weeks until weather conditions became too cold for plant growth. In the spring, when the weather became suitable for the emergence of volunteers, the field was monitored approximately every two weeks for volunteers until July 2013. The number of volunteers found in each plot was noted in the study notebook and the volunteers were removed and devitalized.

Statistical Analysis

The statistical analysis for phenotypic, grading, and stressor data was performed by Simplot using SAS 9.3. All data were subjected to analysis of variance using the following linear mixed model:

$$Y_{ijkl} = \alpha_i + \beta_j + \gamma_{k(j)} + (\alpha\ \beta)_l + \varepsilon_{ijkl}$$

- α = mean of treatment (fixed)
- β = effect of site (random)
- γ = rep[site] (random)
- ε = residual random error

Where α_i denotes the mean of the i^{th} treatment (fixed effect), β_j denotes the effect of the j^{th} site (random effect), $\gamma_{k(j)}$ denotes the random rep effect (within site), $(\alpha\ \beta)_{ik}$ denotes the interaction between the i^{th} treatment and random k^{th} site effect, and ε_{ijkl} denotes the residual random error.

A significant difference was established with a p-value < 0.05. Every effort was made to generate p-values to aid in the interpretation of the data. Some departures from the assumptions of normality and equal variances were allowed since the results were always interpreted in the context of variation observed in the conventional varieties.

Composition Methods

Test, control, and reference tubers for the compositional assessment were collected from the same 2012 and 2013 field trial locations listed in Table 7-1. Each combination of year, site, material, and replicate represents one sample of six tubers in the compositional assessment. Samples analyzed at 3, 6, and 9 months of storage consisted of 3 tubers and were taken from all sites in 2012 but not from the 2013 locations.

Samples were obtained by randomly selecting mid-sized tubers at harvest from each site and rep. Tuber samples were powdered in an industrial blender with liquid nitrogen and stored at -70°C until analysis. For acrylamide testing, five pound samples of the potatoes were processed into chips prior to analysis, using standard practices.

Analytical testing was conducted by Covance Laboratories, Inc. in Madison, WI with the exception that acrylamide testing was conducted by Covance Laboratories, Inc. in Greenfield, IN.

Statistical Analysis for Composition

The statistical analysis was performed by Simplot using SAS 9.3. All data were subjected to analysis of variance using the following linear mixed model:

$$Y_{ijkl} = \alpha_i + \beta_j + \gamma_{k(j)} + (\alpha\ \beta)_l + \varepsilon_{ijkl}$$

- α = mean of treatment (fixed)
- β = effect of site (random)
- γ = rep[site] (random)
- ε = residual random error

Where α_i denotes the mean of the i^{th} treatment (fixed effect), β_j denotes the effect of the j^{th} site (random effect), $\gamma_{k(j)}$ denotes the random rep effect (within site), $(\alpha\ \beta)_{ik}$ denotes the interaction between the i^{th} treatment and random k^{th} site effect, and ε_{ijkl} denotes the residual random error.

A significant difference was established with a p-value < 0.05. Every effort was made to generate p-values to aid in the interpretation of the data. Some departures from the assumptions of normality and equal variances were allowed since differences were always interpreted in the context of variation observed in the conventional varieties.

The tolerance intervals were calculated to contain, with 95% confidence, 99% of the values in the population. Tolerance intervals were used for compositional data to represent the natural variability among potatoes. The tolerance interval attempts to predict, with a specified level of confidence, the range in which most values of a population will fall. Conventional potato varieties used in the tolerance interval are shown in Table C-5 and include varieties suitable for fresh use, for frying, for chipping, and the V11 parental control, Snowden.

Table C-5. Number of Data Points for Each Test, Control and Reference Variety

Variety	N Per Attribute	Used in Tolerance Interval?
V11	22	No
Atlantic	8	Yes
Bintje	8	Yes
C0095051-7W	16	Yes
Gala	16	Yes
Golden Sunburst	8	Yes
Nicolet	8	Yes
Norkotah	16	Yes
Purple Majesty	16	Yes
Snowden	21	Yes
TX278	8	Yes
Total N	147	125 in tolerance interval