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Petition for Determination of Nonregulated Status for Innate™ Potatoes with Late Blight Resistance, Low Acrylamide Potential, Reduced Black Spot, and Lowered Reducing Sugars: Russet Burbank Event W8

We submit this petition under 7 CFR 340.6 to request that the Administrator make a determination that the articles should not be regulated under 7 CFR Part 340.

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J.R. Simplot Company

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Petition for Determination of Nonregulated Status

The undersigned submit this petition under 7 CFR 340.6 to request that the Administrator make a determination that the articles should not be regulated under 7 CFR part 340.

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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
ALA	Alanine
A. tumefaciens	*Agrobacterium tumefacians*
ARG	Arginine
ASN	Asparagine
ASN	Southern blot probe used to detect *Asn1* sequence (Chapter 5)
Asn1	Asparagine synthetase-1 gene
ASP	Aspartic acid
Backbone DNA	DNA associated with plasmid/vector backbone
BLAST	Basic Local Alignment Search Tool
Chs	Chalcone synthase gene
cwt/A	Unit of measure equal to 100lbs/ acre
CYS	Cysteine
DNA	Deoxyribonucleic acid
DNA insert	The DNA sequence from pSIM1278 located between the LB and RB intended to be integrated into the potato genome
dsRNA	Double-stranded RNA
EB	Ethidium bromide
EPA	U.S. Environmental Protection Agency
EUP	Experimental Use Permit (application to Environmental Protection Agency)
fASN1	Fragment of the *Asn1* gene
FDA	Food & Drug Administration
G0	First generation greenhouse-grown tuber seed
G1	First generation field-grown tuber seed
G2	Second generation field-grown tuber seed
G3	Third generation field-grown tuber seed
GBS1 or GBS2	Southern blot probe used to detect *Gbss* promoter (3′ and 5′ ends, respectively)
GLN	Glutamine
GLU	Glutamic acid
GLY	Glycine
GM	Genetically modified
gus	β-glucuronidase gene
HIS	Histidine
IPD	Inter-genebank Potato Database
ipt	Isopentyltransferase gene – produces cytokinin hormones associated with plant growth and development
ILE	Isoleucine
INV	Southern blot probe used to detect *VInv* sequence
LB	Left Border (a 25-base pair sequence) similar to *A. tumefaciens* T-DNA border
LEU	Leucine
LYS	Lysine

Abbreviations, Acronyms, and Definitions (Continued)

Abbreviation	Definition
MAFF	Japan Ministry of Agriculture, Forestry, and Fisheries
MET	Methionine
MHLW	Japan Ministry of Health, Labor and Welfare
NCBI	National Center for Biotechnology Information
Non-coding DNA	DNA not coding for translated RNA
ORFs	Open reading frames
pAgp	Promoter of the ADP glucose pyrophosphorylase gene
PCR	Polymerase chain reaction
pGbss	Promoter of the granule-bound starch synthase gene
PHE	Phenylalanine
PhL	Phosphorylase-L gene
P. infestans	*Phytophthora infestans*, pathogen that causes late blight
Ppo5	Polyphenol oxidase-5 gene
PRO	Proline
qPCR	Quantitative / real-time PCR
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
Rpi-vnt1	R-gene that is resistant to *Phytophtora infestans* (*Rpi*) from *Solanum venturii*
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantitative PCR
Sclerotia	Compact mass of hardened mycelium containing food reserves for the pathogen, *Rhizoctonia solani*
SER	Serine
siRNA	Small interfering RNA from RNAi pathway
Somaclonal variation	Genetic and/ or phenotypic variation among clonally propagated plants of a single donor clone; generated by tissue culture and other forms of vegetative propagation
sRNA	All classes of small cellular RNAs
TPS	True potato seed
T-DNA	Transfer DNA from *A. tumefaciens* delineated by left and right border sequences
THR	Threonine
Tomato	Currently known as *Solanum lycopersicum*, previously known as *Lycopersicon esculentum*. May be referred to as *Lycopseriscon esculentum* in older papers and database searches such as BLAST.
TRP	Tryptophan
TYR	Tyrosine

Abbreviations, Acronyms, and Definitions (Continued)

Abbreviation	Definition
VAL	Valine
VNT1	Southern blot probe used to detect *Rpi-vnt1* promoter sequence (Chapter 5)
VNT1	Protein expressed from *Rpi-vnt1* gene

Summary

The J. R. Simplot Company has advanced plant breeding by employing Simplot's proprietary Innate™ branded technologies to transform plants with plant genomic DNA. In the latest innovation in potato breeding, we added late blight resistance, low acrylamide potential, reduced black spot, and lowered reducing sugars into the most popular potato variety, Russet Burbank.

To explain further, we used Innate™ branded technologies to provide resistance to the devastating disease, late blight, caused by the oomycete, *Phytophthora infestans*. Late blight was the cause of the Irish potato famine in the mid-1800s, resulting in mass starvation, disease, and immigration. Late blight is still a serious pest of potatoes and results in significant costs from fungicide applications but also reduces yield because of loss of vitality associated with diseased plants. Reducing fungicide application would save cost but has the additional benefit of releasing less fungicide into the environment.

In addition, we address three critical potato quality issues:

(1) Large amounts of asparagine, a non-essential free amino acid that is rapidly oxidized to form acrylamide upon frying or baking; (2) accumulation of reducing sugars leading to dark spots and sugar ends and (3) susceptibility to enzymatic browning and discoloration.

Enzymatic browning occurs when polyphenol oxidase leaks out from the damaged plastids of bruised potatoes. In the cytoplasm, the enzyme oxidizes phenols, which then rapidly polymerize to produce dark pigments. Browning is also triggered non-enzymatically as a consequence of the partial degradation of starch into glucose and fructose. When heated, these reducing sugars react with amino acids, such as asparagine, through the Maillard reaction to produce a variety of desired compounds contributing to flavor, aroma, and browning, but also acrylamide. By silencing invertase in tubers, the conversion of sucrose to reducing sugars is reduced, potentially allowing for storing potatoes at colder temperatures. Other advantages to lower storage temperatures include reducing the use of chemicals to inhibit sprouting, less disease and moisture loss, all resulting in higher yield of usable potatoes and anticipated economic advantages.

Russet Burbank W8 potatoes provide the potato industry the opportunity to keep the highly desired characteristics of the leading french fry and fresh potato variety with multiple enhancements that could not be added through traditional breeding. Overall improvement in yield and lowered fungicide use with late blight resistance should benefit all members of the food value chain by reducing environmental impacts associated with fungicide use, and potentially reducing acreage devoted to potato production. The combination of low asparagine and reducing sugars results in greater than 70% reduction in acrylamide even after extended cold storage, addressing this potential health risk for consumers and the food industry. Quality improvements related to lower levels of reducing sugars and black spot provide benefits to processors and consumers, and invertase silencing could positively impact quality and yield from potato storage. There is evidence that invertase silencing reduces the incidence of high sugar potatoes and sugar ends, both quality attributes that result in economic loss by causing potatoes to be rejected by french fry and chip processors. Finally, reduced invertase could enable storage of processing potatoes at significantly lower temperatures, decreasing loss from disease and yield losses from higher respiration rates related to typical storage at 46 – 48 ˚F.

Simplot's Innate™ technologies allow researchers to isolate genetic elements from any plant genome, rearrange them, or link them together in desired permutations, and introduce them back into the

genome. More specifically, in these events, the genomic DNA comes from potato or wild potato, a group of related plant species that are sexually-compatible with potato. We incorporate no viral markers, and no plasmid backbone sequences, into the plant genome.

Because our Innate™ technologies effectively accelerate the process of conventional crossing, it allows changes in traditional varieties to occur much faster, while maintaining the desired characteristics of the original parent plant. For potatoes, Innate™ technologies are particularly attractive because potatoes are notorious for having a high degree of heterozygosity, suffering from inbreeding depression, and limited predominantly to clonal propagation. Historically, these inherent factors have significantly hindered and prevented the commercial introduction of quality, sought-after traits into this valuable crop. Traditional breeding techniques result in random genomic rearrangements and trait segregation, and do not allow for the simultaneous addition of multiple desired traits.

To illustrate this point, there currently are no varieties available that produce tubers with late blight resistance, low acrylamide potential, reduced black spot and reducing sugars, while displaying all other traits important to the food industry. Therefore, instead of attempting to develop new varieties, the J.R. Simplot Company used the techniques of modern biotechnology to improve the quality of the existing Russet Burbank variety by transforming with potato genomic DNA that introduces late blight resistance and silences the genes related to expression of black spot, asparagine, and reducing sugars in tubers.

The plasmids pSIM1278 and pSIM1678 were used to transform the Russet Burbank potatoes resulting in selection of W8. These plasmids contain DNA sequence for silencing genes through the mechanism of RNA interference (RNAi) resulting in lower levels of reducing sugars, asparagine, and black spot in potatoes. In addition, the pSIM1678 plasmid contains an expression cassette for the *Rpi-vnt1* gene, which relies on expression of the VNT1 protein for resistance to late blight. See Table 1 for the OECD unique identifier for W8.

Table 1. Innate™ event W8 and the OECD unique identifier

Event number	OECD Unique Identifier
W8	SPS-∅∅∅W8-4

A rationale is presented for the safe use of W8 potatoes. Among the supporting evidence are the phenotypic as well as ecological interactions data collected in a robust field trial program at 11 sites over two crop years. From those studies, a compositional assessment shows the W8 potatoes substantially equivalent to Russet Burbank controls. Open reading frames associated with the DNA inserts, including the late blight resistance gene itself, were screened for allergen and toxin potential, but no safety concerns were identified. The W8 potatoes were tested for glycoalkaloids and found no different from the Russet Burbank controls and well below the accepted safety limit. Similarity of the *Rpi-vnt1* to other gene sequences in related species was assessed and greater than 90% homology discovered between this late blight resistance gene and the Tomato Mosaic Virus resistance gene (ToMV Tm-2) identified in tomatoes, which has been widely bred into tomato varieties in the fresh market and is consumed by humans.

Among the risk considerations is the presence of the VNT1 protein, and although effective at controlling late blight, levels in potato tubers are below our detection limit of 30 ppb. Also, nucleic acids such as those added through plasmids pSIM1278 and pSIM1678 have long been considered safe under the FFDCA by EPA and FDA. An in depth review is included on the topic of the safety of using RNA

interference for gene silencing. Numerous physiological barriers exist to uptake of RNA, confirming that ingested RNA is rapidly metabolized in the gut where it is converted to nutrients, and therefore unlikely to adversely affect human health. Regulatory actions supporting safe use of the Coat Protein Gene of Plum Pox Virus and Potato Leaf Roll Virus Resistance Gene provide additional evidence of the safety of W8.

Accordingly, based on the results of the studies presented here, there is no reason to believe that the VNT1 protein will have any impact to human health through allergenicity or toxicity, or any environmental impacts to non-target mammals, birds, fish, or insects due to the low expression and lack of toxic effects of the protein. There is negligible risk of environmental contamination and no persistence in the environment because of the low expression of the VNT1 protein in potato tubers. The prevalence of similar resistance genes throughout edible crops suggests that extremely low levels of similar proteins are widespread in nature and unlikely to pose risk to human health, non-targets or the environment.

Extensive analyses demonstrated the achievement of the desired traits, including effective resistance to late blight, reduced black spot, lower asparagine, and lower levels of reducing sugar. Ultimately, the combination of lower asparagine and reducing sugars led to greater than 70% reductions in acrylamide levels in cooked potatoes and, importantly, we showed that the transformed tubers were otherwise substantially equivalent to untransformed potatoes.

The propagation of commercial potato varieties through cloning of seed potatoes mitigates concerns about increased weediness or plant pest potential such as seed dispersal, survival outside of cultivation, or outcrossing. The potatoes that were transformed, the Russet Burbank variety, produce few flowers and are male sterile. Other factors limiting outcrossing include the tendency for most fertile varieties to be self-pollinated, an inability to attract honey bees because they lack nectar, and limitation of the pollen transfer range to about 20 meters. In addition, true seeds would be unlikely to grow into mature potatoes since 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. In addition, potatoes are not known to escape from commercial fields or show weediness potential. 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 modifications described in this petition were intended to enhance disease resistance and quality, not agronomic characteristics, of potatoes. Planting, cultivation, management and harvesting techniques were not affected by the incorporated traits, with the exception that late blight resistant potatoes should require less fungicide. The Innate™ branded W8 variety is likely to be planted in areas that are already growing potatoes, and would not result in a significant expansion of planted acres or a change in the areas where potatoes would otherwise be grown. The modifications described in this petition are highly unlikely to increase the weediness or invasiveness of potato because the incorporated traits (late blight resistance, reduced free-asparagine, black spot tolerance, and lowered reducing sugars) would not influence the fundamental biological characteristics or ecological competitiveness. Field trials over multiple years with W8 did not provide any evidence for altered growth characteristics such as accelerated tuber sprouting, increased plant vigor, increased tuber set, or delayed senescence. The Potato Late Blight Resistance Gene (*Rpi-vnt1*) expresses a resistance protein, VNT1, which reacts with a pathogen-specific effector protein to halt the spread of infection. This resistance should partially replace fungicide treatments but would not enhance the potatoes

survivability beyond what would be normally expected for potatoes. Thus, modified potatoes are unlikely to display enhanced weediness. The mechanism of action for resistance proteins like that coded by *Rpi-vnt1* is highly specific and extremely unlikely to impact insects and other non-target organisms, weed or disease susceptibility, endangered species or biodiversity.

We therefore seek nonregulated status for Russet Burbank event W8 based on the weight of evidence demonstrating safety when compared to untransformed potatoes. The technologies presented in this petition and the resultant transformed plants and their tuber products satisfy the requirements for nonregulated status. The transformed plants and subsequent traits described in this petition should not be considered plant pests. In conclusion, plants displaying the incorporated traits are as safe to grow and as safe to eat as untransformed potatoes based on all of the data contained and referenced in this submission. The J.R. Simplot Company requests a determination from APHIS that event W8 described in this petition, and any progeny derived from this event, are granted nonregulated status and would no longer be considered regulated articles under 7 CFR Part 340.

The genetic services and research and development methods and associated genetic tools, disclosed in this petition, such as expression vectors, and the transformed plants and plant cells, and methods of transformation, have been trademark-branded by Simplot as Innate™.

1.0 Rationale for Potatoes with Late Blight Resistance, Low Acrylamide, Reduced Black Spot, and Lowered Reducing Sugars

The Russet Burbank potatoes were transformed using Innate™ technologies in order to address the need of the potato industry and consumers for potatoes with late blight resistance, improve quality by reducing expression of the enzyme responsible for black spot and to reduce asparagine and reducing sugars, and thus reduce acrylamide through lowering the concentration of the reactants, asparagine and reducing sugars. With Innate™ branded technologies, we transformed potatoes using desirable traits which were simultaneously incorporated into the most popular potato processing variety, Russet Burbank. Such multiple trait addition would not be possible to achieve through traditional breeding because potato is tetraploid, highly heterozygous and sensitive to inbreeding depression. The resulting Innate™ branded potatoes contain highly sought after traits, none of which would contribute to weediness or other undesirable agronomic or environmental characteristics as verified by phenotypic and molecular characterization.

1.1 Basis for Determination of Nonregulated Status

Under the authority of the Plant Protection Act and Part 340 of title 7 of the Code of Federal Regulation (7 CFT Part 340), USDA-APHIS regulates, among other things, the introduction of organisms and products altered or produced through genetic engineering that are plant pests or are believed to be plant pests. The event described in this petition was transformed with DNA that does not alter the pest characteristics of the Russet Burbank potatoes. Instead, the addition of the Potato Late Blight Resistance Gene (also known as *Rpi-vnt1*) would potentially reduce the need for pesticides and promote better health of the potato plants. The W8 event was confirmed to be free of *Agrobacterium* and free of backbone DNA. In addition, the scientific evidence presented here shows that the inserted genetic material results in a new potato event that is as safe as the untransformed potatoes.

Field evaluations demonstrated that the event displayed similar agronomic characteristics compared to the untransformed controls, except that the event is much more resistant to late blight. This submission reviews the biosafety implications of all minor differences observed. In addition to the desired agronomic difference of late blight resistance; the phenotypic changes in W8 potatoes include reduced levels of the free amino acid, asparagine, and reduced levels of reducing sugars in tubers. As predicted, we confirmed that changes in asparagine and reducing sugar levels were associated with a reduced potential to form acrylamide upon frying. The Russet Burbank event W8 also contains a silencing cassette to reduce the PPO enzyme in tubers, resulting in a reduced incidence of black spot.

W8 contains expression cassettes that influence reducing sugars by multiple mechanisms. Included in the first transformation using pSIM1278, we introduced a silencing cassette for the promoters of the starch associated gene (*R1*) and the phosphorylase-L gene (*PhL*) inserted between promoters that are predominantly active in tubers. The result of this silencing strategy was lowered levels of the reducing sugars glucose and fructose at harvest or when analyzed at one month after harvest (Collinge and Clark 2013). Another gene silencing cassette, for the acid invertase gene (Ye et al. 2010), was introduced into event W8 using the transformation vector pSIM1678. A reduction in invertase through gene silencing reduces the conversion of sucrose to reducing sugars during cold storage, inhibits formation of sugar related defects such as sugar ends in french fries, and results in even lower levels of acrylamide upon frying.

The event W8 produced by transformation of the Russet Burbank variety with the specified transfer DNA is well characterized and safe as determined by composition, agronomic, and phenotypic evaluations, when compared to the untransformed controls.

1.2 Rationale and Benefits of Developing Potatoes with Late Blight Resistance

A resistance gene, referred to as *Rpi-vnt1,* to the most important potato pathogen, *Phytophthora infestans*, was introduced into the Russet Burbank potato variety using *Agrobacterium*-mediated transformation. The *Rpi-vnt1* gene confers resistance to late blight, a major pest of potatoes in North America and throughout the world. Late blight was the cause of the Irish potato famine in the mid-1800s, resulting in mass starvation, disease, and immigration; and remains a serious plant disease today.

Late Blight Potato Breeding History

Resistance to late blight (*Phytophthora infestans*) occurs in many tuber-bearing wild Solanum species that belong to the highly diverse section Petota Dumort. Early potato breeding programs from the mid-twentieth century used *Solanum demissum*, a common species in central Mexico and a great source of resistance against late blight. In total, 11 *S. demissum* resistance (R) genes designated R1-R11 are distinguished in a potato differential set made by Black and Mastenbroek (Black et al. 1953; Malcolmson and Black 1966). R1, R3, and R10, and to a lesser extent R2 and R4, have been widely used for introgression in European breeding programs (Colon 1994) to help control late blight. The introduction of new cultivars containing these R genes was initially successful, but rapidly evolving populations of *P. infestans* reduced their efficacy (Fry 2008; McDonald and Linde 2002; Pink and Puddephat 1999; Wastie 1991). However, durability in the field of a particular R gene is variable (Leach et al. 2001), and additional novel resistance genes against *P. infestans* (Rpi-genes) are being discovered from other wild Solanum species. To date, 68 Rpi-genes, from wild Solanum species, have been characterized (Rodewald and Trognitz 2013) and about 20 of them have been cloned. In addition, germplasm from those wild Solanum species has been integrated into numerous cultivars through breeding, for example but not limited to: *S. bulbocastanum* (Park et al. 2005; Ramanna and Hermsen 1971), S*. stoloniferum* (Hutten and van Berloo 2001, referred to as sto or CPC 2093), *S. microdontum* (Tan et al. 2008) and *S. phureja* (Sliwka et al. 2010; 2013).

***Rpi-vnt1* History and Origin**

Recently, the first Rpi-gene (*Rpi-vnt1*) from a South America wild Solanum species, *S. venturii*, was cloned (Pel et al. 2009; Foster et al. 2009). Three functional alleles of the gene were identified, *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*. The gene was first located on chromosome 9 (same locus as Tm-2(2) from *S. lycopersicum* which confers resistance against Tomato mosaic virus (ToMV)) of the potato wild species *S. venturii*. The gene was identified by a classical genetic and physical mapping approach including nucleotide binding site profiling and bulked segregant analysis, as well as a Tm-2(2) based allele mining strategy (Pel et al. 2009; Foster et al. 2009). Transgenic potato and tomato plants carrying *Rpi-vnt1.1* were shown to be resistant to *P. infestans*. Of 11 *P. infestans* isolates tested, only isolate EC1 from Ecuador was able to overcome *Rpi-vnt1.1* and cause disease on the inoculated plants (Pel 2010). However, *Rpi-vnt1.1* remains of major importance since it brings resistance against the most dominant late blight isolate in Europe, Blue 13, by responding to the presence of the cognate effector protein AVR-VNT1 (Cooke et al. 2012). Alleles of *Rpi-vnt1* (*Rpi-vnt1.1, Rpi-vnt1.2 and Rpi-vnt1.3*) which differed by only a few nucleotides were found in other late blight resistant accessions of *S. venturii*. The late blight resistance gene *Rpi-phu1* from *S. phureja* (Sliwka et al. 2006) has been shown to be identical to *Rpi-vnt1.1* (Pel 2010; Sliwka et al. 2013), suggesting either that this strong resistance gene has been maintained since a common ancestor, due to selection pressure for blight resistance, or that genetic

exchange between *S. venturii* and *S. phureja* has occurred at some time. In addition, a collection containing 200 wild Solanum species with 5 genotypes each was screened. *Rpi-vnt1* alleles were found in *S. venturii*, as expected, *S. mochiquense* and *S. weberbaueri* (Pel 2010; Vleeshouwers et al. 2011; SOLRgene database). Moreover, *Rpi-phu1* (also called *Rpi-vnt1*) was introgressed into *S. tuberosum* (tetraploid potato varieties) from an interspecific cross between *S. phureja* and *S. stenotomum* (known as pinta boca in Peru and Bolivia). Both of these species are edible and research programs are underway in Europe to introgress *Rpi-phu1*/*Rpi-vnt1* (identical genes) in cultivated potatoes (Sliwka et al. 2010; 2013).

Mode of action

Expression of *Rpi-vnt1* in wild and cultivated potato confers broad-spectrum resistance to late blight caused by *P. infestans*. Resistant potato parts include leaves, stems and tubers. This resistance is based on the recognition of a pathogen-specific protein (effector protein) by a host-specific protein (resistance or R protein). Upon infection, AVR-VNT1, an effector protein produced by *P. infestans*, is recognized by the R-protein VNT1 expressed in potato (Pel 2010). R-protein mediated immunity, known as Effector Triggered Immunity (ETI), results in a form of programmed cell death called a hypersensitive response (Morel and Dangl 1997). Consequently, VNT1 activates a signal transduction pathway that leads to localized plant cell death or hypersensitive response. Death is restricted to a few plant cells and limits the growth and spread of *P. infestans* throughout the rest of the plant.

Benefits of Potatoes with Late Blight Resistance

As one of the most important potato pathogens, resistance to late blight could have significant benefits with respect to reduced use of pesticides, but also could effectively improve yield compared with diseased potatoes. A reduction in pesticide use could reduce the cost of production through less chemical, fuel, and labor costs. Also, the result could be less chemical residue in food, land, and waste water. Even with pesticide application, some disease tends to persist in potato fields so effective resistance could result in healthier plants and better yield. Late blight affects foliage and tubers. There's evidence to suggest that tuber damage is far more likely to occur if the plants are infected than by merely picking up the disease agent through soil contact. Tuber infection with late blight results in lesions, rot, and ultimately economic loss in storage.

1.3 Rationale and Benefits of Developing Potatoes with Low Acrylamide Potential

The Swedish National Food Administration and Stockholm University announced in April 2002, a relationship between the formation of certain blood adducts and the consumption of foods later found to contain acrylamide (NTP 2012). Other researchers also studied the mechanism for acrylamide formation in food, and the J.R. Simplot Company had also earlier predicted that acrylamide would form as a result of the well-known but complex Maillard browning reaction. Cooking or heat treating of foods that are rich in reducing sugars and amino acids may result in browning, commonly referred to as the Maillard reaction. This reaction occurs between sugars and amino acids, affecting changes in the color, flavor, functional properties and nutritional value of food (Obrien and Morrissey 1989). More specifically, we found acrylamide formed primarily when the amino acid asparagine along with reducing sugars were heated at temperatures above 120°C, as would occur during frying and baking. The biochemical basis of acrylamide formation was later published by Stadler et al. (2002).

Various governments responded to the news about finding acrylamide in food by providing guidance and surveys. Through one such survey, the FDA determined that potato products contribute 35% of the acrylamide exposure through diet in the US. The scientific community has since gathered additional

information, including epidemiology and toxicology studies. Along with studies to mitigate the levels of acrylamide in food, the scientific community has also been working to understand the health implications of acrylamide consumption (NTP 2012).

Based on a recent 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. These include variety development and selection, focusing on varieties that are more resistant to cold-induced sweetening, and lower levels of reducing sugars or asparagine. Many of the methods in FDA's Guidance document are consistent with those reported in the Acrylamide Toolbox published by Food Drink Europe (2011).

The introduction of Innate™ potatoes with low acrylamide potential would provide potatoes that are largely indistinguishable from existing varieties. The reduction in asparagine and sugars using Innate™ technologies and the resulting reduction in acrylamide upon heating will address food industry needs with respect to the FDA's Draft Guidance (2013). As such, this biotechnology approach to lowering acrylamide was mentioned in the guidance document as a promising method to develop potato varieties with potentially reduced acrylamide. After deregulation, these Innate™ branded potatoes could be adopted readily by the food industry following completion of the FDA consultation process (57 Federal Register 22984, May 29, 1992).

In addition, litigation in the state of California as a result of Proposition 65 (OEHHA 2011a; OEHHA 2011b) resulted in legal settlements with restaurant chains and the retail french fry and potato chip manufacturers regarding perceived risks from acrylamide. Some settlements required signs warning consumers, but in addition, some manufacturers of retail french fries and potato chips would be required to reduce acrylamide in their products or add warning labels. As of December 1, 2011, some potato chip manufacturers began including a statement on packaging warning consumers of the presence of acrylamide and its relationship to browning in potatoes. Potato processors affected by these rulings in California could be motivated to adopt the low acrylamide, Innate™ potato products.

Following the discovery of acrylamide in foods and in response to governmental concerns, many additives were studied and some have shown effectiveness at reducing acrylamide. Some of the more promising options include citric acid, enzymes such as asparaginase, and amino acids that substitute for asparagine in the Maillard reaction and therefore form products other than acrylamide when heated (FDA 2013; Food Drink Europe 2011).

Another approach to reducing acrylamide could be based on either traditional breeding or biotechnology to reduce the precursors for acrylamide. The use of biotechnology allows for specific deactivation of genes involved in the formation of asparagine and reducing sugars with tissue specificity. In a crop like potatoes, which is highly heterozygous and suffers from inbreeding depression, the speed to develop such changes could take decades through traditional breeding. In contrast, biotechnology allows for specific changes in traditional varieties much faster, while maintaining the desired characteristics of the original parent.

The W8 event contains expression cassettes that could lower levels of reducing sugars by multiple mechanisms. Through the transformation with pSIM1278, we introduced a silencing cassette for the

promoters of the starch associated gene (*R1*) and the phosphorylase-L gene (*PhL*) inserted between promoters that are predominantly active in tubers. Together, these traits function by slowing the conversion of starch to reducing sugars (glucose and fructose). The result of this silencing strategy was lowered levels of the reducing sugars glucose and fructose at harvest or when analyzed at one month after harvest (Collinge and Clark 2013). Another gene silencing cassette, for the invertase gene (Ye et al. 2010), was introduced into event W8 using the transformation vector pSIM1678. A reduction in invertase from gene silencing should reduce the conversion of sucrose to reducing sugars during cold storage, inhibit formation of sugar related defects such as sugar ends in french fries, and result in even lower levels of acrylamide upon frying. Overall benefits of silencing *R1, PhL, and VInv* include improved quality, especially relating to color control, and thus contributing to the desired golden brown colors required by most french fry or chip customers. Also, the reducing sugars react with amino acids, such as asparagine, to produce Maillard products including acrylamide.

The Russet Burbank W8 produces tubers with greatly reduced potential to form acrylamide, thus addressing a potentially critical food safety issue for the potato industry, particularly in light of recent toxicology studies (NTP 2012).

Many methods have been tested and research is ongoing to reduce acrylamide through process changes, reduction in dextrose, and additives such as asparaginase, citrate, and competing amino acids. The required capital expense to implement process changes throughout the potato industry would cost millions of dollars. In addition to the expense, these process changes have significant drawbacks including potentially negative flavors associated with additives such as asparaginase or citrate. Typically, fry manufacturers add dextrose during processing of french fries to develop the desired golden brown color, but dextrose also increases the formation of acrylamide through the Maillard reaction. Significant reductions in acrylamide occur by merely omitting dextrose from the process; however, the signature golden brown colors must then be developed some other way (such as though the addition of colors like annatto). The use of alternate colors, results in an absence of the typical flavors that develop through those browning reactions. Another challenge with the use of additives to reduce reactants like asparagine will be the principle of moisture migration that occurs during frozen storage resulting in a return of asparagine to the surface and increased acrylamide compared with the initial testing at the time of processing. In contrast, the Innate™ potatoes will significantly reduce acrylamide without the use of new additives or process changes. The resulting foods will have the same appealing color, flavors, and aromas as they currently have today.

1.4 Rationale and Benefits of Developing Potatoes with Reduced Black Spot

The blackening that occurs after potatoes are bruised affects quality and recovery in processing french fries and chips. Potatoes that have been damaged and show black spot must be trimmed or could be rejected before processing, resulting in quality challenges or economic loss or both. In many instances potato growers have contracts that provide incentives for delivering “bruise free” potatoes. A significant reduction in black spot could result in higher profit for the farmer. Also, these black spots are considered defects in potato chip or french fry processing, causing economic loss from trimming or culling potatoes with black spot. Potatoes may develop black spots from pressure bruising resulting from the weight of potatoes in deep stacks during storage. The weight of the potatoes causes damage to the potato tissue resulting in dark colors that must be removed through trimming. Another possible advantage of silencing black spot could be the enabling of new markets for “freshly cut” potatoes, without pre-cooking or using sulfites or other preservatives to maintain color and flavor.

1.5 Rationale and Benefits of Developing Potatoes with Reduced Levels of Reducing Sugars

In breeding programs for processing potatoes, one of the most desired characteristics is low levels of the reducing sugars glucose and fructose. High levels of reducing sugars lead to undesirable dark colors and bitter flavors after frying of fries and chips. High sugar levels can result from any stress to the plants during growing or harvesting such as drought or heat stress (Bethke et al 2009), but develop rapidly with low temperature storage (Driskill et al. 2007). Typical storage temperatures for potatoes for the frozen fry market are 46 - 48˚F (Driskill et al. 2007). This temperature is the optimum for maintaining sugar levels while minimizing the chance for disease. If potatoes could be stored at a lower temperature without adversely affecting sugar level, the result would be less damage from disease and higher yield because of lower respiration rates resulting in a greater net yield of potatoes.

Multiple pathways exist for carbohydrate metabolism in potatoes and the DNA inserted into W8 targets several of those paths through gene silencing. The vector pSIM1278 contains silencing cassettes for promoters of the water dikinase *R1* gene and starch phosphorylase *Phl* gene. The *R1* gene functions by phosphorylating the C6 position on glucosyl residues in starch (Ritte et al. 2006). Silencing of this gene could lead to an accumulation of starch in plants and a reduction in cold sweetening as shown by Lorberth et al. (1998). The phosphorylase *Phl* gene is also thought to be responsible for degradation of starch to sugar during cold storage (Sonnewald et al. 1995). Kamrani et al. (2011) showed a reduction in sugar accumulation in potatoes with silencing of the starch phosphorylase L gene. The vector pSIM1678 silences the vacuolar acid invertase gene *(VInv)*, responsible for catalyzing the reaction converting sucrose into glucose and fructose.

With introduction of pSIM1278 into Russet Burbank potatoes (Collinge and Clark 2013), we found silencing of the promoters for *R1* and *Phl* to result in slight reductions in reducing sugars at the time of harvest or after 1 month of storage. In the event W8, we have silenced the *VInv* gene through introduction of a gene silencing cassette contained in the pSIM1678 DNA insert. Silencing of *VInv* will result in lower levels of reducing sugar throughout the storage period. The *VInv* silencing may also allow for lower temperature storage which will reduce yield loss from respiration and disease. There is also evidence that *VInv* silencing reduces the incidence of high sugar potatoes and sugar ends, both quality attributes that result in economic loss by causing potatoes to be rejected by processors, resulting in lower prices as they are only suitable for making dehydrated potatoes.

An early discovery of high sucrose levels in wild tomatoes led researchers to determine that some tomatoes contained a gene that silenced the production of acid invertase (Klann et al. 2006). Researchers assayed the tomatoes for several common enzymes and found that the cause of high levels of sucrose was associated with lack of acid invertase activity.

More specific work with silencing of vacuolar acid invertase in potatoes has demonstrated the efficacy of that approach for lowering levels of reducing sugars in cold stored potatoes (Ye et al. 2010; Bhaskar et al. 2010). Both research groups reported effective reductions in reducing sugars during cold storage with silencing of vacuolar acid invertase. In addition, with less reducing sugar, significantly lower levels of acrylamide were found after frying.

Benefits of Potatoes with Reduced Levels of Reducing Sugars

At the time of harvest, most potatoes have low levels of the reducing sugars, fructose and glucose. For optimum quality, potatoes used for processing into french fries and chips, should be low in reducing sugars to give the processor ultimate control in creating the desired finished product color. When

potatoes are low in reducing sugar, colors can be readily developed by adding reducing sugars, such as glucose before frying. If potatoes contain too much reducing sugar, they can become excessively brown during cooking, losing their ideal flavor and appearance. Many potatoes must be stored for as long as 3 to 12 months before processing, and the ideal storage potato maintains a low level of reducing sugar throughout storage. A consistent focus of breeders for processing potatoes would be to have low reducing sugars at the time of harvest that remain low throughout their storage life (Driskill et al. 2007). High levels of reducing sugars can result in an overall excessive browning during processing or may result in dark ends or mottled appearance. With excessively high sugar levels, potatoes may become unusable for fries or chips and must be sold at a lower price into the dehydrated potato sector. Management of reducing sugars presents significant challenges and potential financial loss because processors reject loads with reducing sugar content above 2%, which is approximately 20% of potatoes produced.

High levels of reducing sugars affect the overall potato color, but sugars are often concentrated into excessively dark areas either throughout the potato or at the end of a potato strip. If levels of sugar ends are extremely high, the potatoes cannot be used for premium french fries, and may result in loss of economic value to the processor. For the Russet Burbank event W8 presented here, there should be significant economic and quality benefits from lower levels of reducing sugars. In addition, less reducing sugar will result in lower levels of acrylamide after cooking, a significant advantage considering the health concerns associated with acrylamide (NTP 2012; FDA 2013).

1.6 Conclusions: Rationale for Potatoes with Late Blight Resistance, Low Acrylamide, Reduced Black Spot, and Lowered Reducing Sugars

In summary, there is an important need to introduce late blight resistance into the Russet Burbank potato variety, while simultaneously lowering reducing sugars, black spot, and the potential to form acrylamide in fried and baked potatoes. Considering that the most popular North American potato variety, the Russet Burbank, is sterile, it was not feasible to make such changes using traditional breeding. Therefore, we accomplished this goal by applying Innate™ technologies. The Russet Burbank represents 20.3% of seed acres in the US (NPC 2013), and is widely used in french fry and dehydrated processed potatoes, and also remains the standard of excellence for table stock. We now seek nonregulated status for Russet Burbank event W8 based on the weight of evidence demonstrating the safety of the Russet Burbank W8 potatoes. We have transformed plants with genomic DNA from potato and wild potato where the integrated genetic material and the transformed potatoes are as safe as the Russet Burbank controls.

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2.0 The Biology of Potato

This section is based, in part, on the Organization for Economic Co-Operation and Development's (OECD) series on harmonization of regulatory oversight in biotechnology no. 8, Consensus document on the biology of Solanum tuberosum, 1997.

This section describes potato biology and how it contributes to the effective management of the Innate™ potatoes. All commercial potato varieties must be propagated through cloning, effectively mitigating many concerns about increased plant pest potential such as seed dispersal, survival outside of cultivation, or outcrossing.

2.1 History of Potato

The cultivated potato traces its origin to Andean and Chilean landraces developed by pre-Colombian cultivators. These landraces exhibit morphological and genetic diversity, and are distributed from western Venezuela to northern Argentina, and in southern Chile (Spooner et al. 2005). Introduced to Europe by Spain in 1536, the potato was subsequently conveyed by European mariners to territories and ports throughout the world. Once established in Europe, the potato soon became an important food staple and field crop. But lack of genetic diversity, due to the fact that very few varieties were initially introduced, left the crop vulnerable to disease. In 1845, a plant disease known as late blight, caused by the fungus-like oomycete pathogen *P. infestans*, spread rapidly through the poorer communities of western Ireland, resulting in the crop failures that led to the Great Irish Famine. Late blight remains a serious disease of potatoes today.

Potato remains an essential crop in Europe, where per capita production is still the highest in the world, but the crop also made its way back over the Atlantic Ocean to North America. The industry gained greater importance particularly in western states after the horticulturist Luther Burbank developed the Russet Burbank potato in 1872. In 2012, the United States harvested 46.7 billion lbs of potatoes, enough to make it the world's fourth biggest producer (NPC 2013). Potatoes in the United States are grown in many states, although about half of the crop comes from Idaho and Washington. The top ten potato producing states of Idaho, Washington, Wisconsin, Oregon, Colorado, North Dakota, Minnesota, California, Michigan, and Maine account for almost 88% of the United States potato crop (NPC 2013). Most potatoes are harvested in July through October. Americans eat, on average, approximately 50 kg of potatoes per person per year (NPC 2013). The most rapid expansion in production and consumption of potatoes has occurred in southern and eastern Asia. China is now the world's largest potato-producing country, and nearly a third of the world's potatoes are harvested in China and India.

2.2 Use of the Potato as Food and Feed in the USA

Most harvested potatoes are used for food but some are grown for seed for planting. According to the National Potato Council, the commercial uses include 36% as frozen, 26% sold fresh, 15% chips and shoestrings (julienne cut crispy snacks), 11% dehydrated, 6% seed potatoes, 4% other frozen products, and 1% canned (NPC 2013).

Raw potato waste products (peels, out of specification raw potatoes, or other non-processed raw potato products) and processed discards (french fry, hash brown, etc.) are routinely incorporated into feed rations at livestock feedlot operations including those owned by the J.R. Simplot Company. Approximately 15,000 tons of processed potato waste and 60,000 tons of raw potato waste are

integrated into livestock feed at the J.R. Simplot Company's Grandview, ID feedlot on an annual basis. The typical feed ration used for cattle finishing at J.R. Simplot Company feedlots includes 8-14% potato waste. One of the major benefits of using the potato waste is that it is not discarded into landfills, but used in a sustainable manner as part of a long-term approach to reducing waste and integrating sustainability with respect to the Earth's valuable natural resources.

2.3 Taxonomy of the Genus *Solanum*

The Solanaceae family contains several well-known cultivated crops such as tomato (*S. lycopersicum* also referred to as *Lycopersicon esculentum*), eggplant (*S. melogena*), tobacco (*Nicotiana tabacum*), pepper (*Capsicum annuum*) and potato (*S. tuberosum*). Within the genus *Solanum*, over a thousand species have been recognized. Potatoes will not hybridize with non-tuber bearing species (tomato, eggplant, etc.) including weeds commonly found in and around commercial potato fields (Love 1994).

The genus *Solanum* is divided into several subsections, of which the subsection *potatoe* contains all tuber-bearing potatoes. The subsection *potatoe* is divided into series, of which *tuberosa* is relevant to this document. Within the series *tuberosa* approximately 54 species of wild and cultivated potatoes are found. One of these is *S. tuberosum*.

S. tuberosum is divided into two subspecies: *tuberosum* and *andigena*. The subspecies *tuberosum* is the cultivated potato widely in use as a crop plant in, for example, North America and Europe. The subspecies *andigena* is also a cultivated species, but cultivation is restricted to Central and South America (Hanneman 1994).

2.4 Genetics of Potato

The basic chromosome number in the genus *Solanum* is twelve. *S. tuberosum* subsp. *tuberosum* can be diploids (2n=2x=24) or tetraploids (2n=4x=48). The diploids have a limited range in parts of South America, while the tetraploids are the most commonly cultivated all over the world. How tetraploidy originated in potato is unclear. The cultivated *S. tuberosum* subsp. *tuberosum* can be either an autotetraploid (doubling of the chromosomes of a diploid species) or an allotetraploid (doubling of the chromosomes of a diploid hybrid between two related species).

While nearly all diploid species are self-incompatible, the cultivated tetraploid *S. tuberosum* subsp. *tuberosum* is capable of self-pollination (selfing). Plaisted (1980) has shown that under field conditions selfing is most likely for tetraploid *S. tuberosum,* with 80-100 percent of the seeds formed due to selfing. Conner and Dale (1996) collected outcrossing data from several field experiments with genetically modified potatoes, performed in New Zealand, the United Kingdom and Sweden. In each study, the outcrossing rate was zero when receiving plants were separated by more than 20 meters from the genetically modified ones. Although many Solanum species are fertile, it appears that a large number of the tetraploid cultivated *S. tuberosum* subsp. *tuberosum* cultivars have reduced fertility. The Russet Burbank variety is sterile and has no outcrossing potential, and the same is true with respect to the W8 Russet Burbank.

2.5 Potato Growth and Life Cycle

Potatoes grown in both commercial and organic farming operations go through the following five general growth stages of development (Dwelle 2003):

1) Sprouts develop from eyes on seed tubers, grow upwards, and emerge from the soil. Roots initiate development at the base of the emerging sprouts.
2) Vegetative growth begins. Leaves and branch stems develop from aboveground nodes. Roots and stolons develop at belowground nodes.
3) Tubers begin to form at stolon tips, but do not enlarge. Flowering is initiated at the end of this stage.
4) Tuber cells expand with the accumulation of water, nutrients, and carbohydrates.
5) Vines turn yellow and lose leaves, tuber growth slows and vines die. Tuber maturation is completed as tuber skins set.

In greater detail, the potato life cycle begins with certified seed potatoes, which are cut into pieces containing at least one eye and are typically planted in the field from February through May. Plants will establish by forming vegetative tissues, including roots, leaves, stems, and stolons. Tuber formation begins approximately 30-60 days after planting. Tubers are derived from lateral underground buds that develop at the base of the main stem, when kept underground. These develop into stolons due to diagravitropical growth. When conditions are favorable for tuber initiation, the elongation of the stolon stops, and cells located in the pith and the cortex of the apical region of the stolon first enlarge and then divide longitudinally. The combination of these processes results in the swelling of the subapical part of the stolon. During enlargement, tubers become the largest nutrient sink of the potato plant storing large amounts of carbohydrates (mainly starch) and also significant amounts of protein. Furthermore, potato tubers decrease their general metabolic activity and as such behave as typical storage sinks.

Potato tubers of the Russet Burbank variety are harvested from 140 to 150 days after planting (PAA 2013), which may vary with production area and marketing conditions. Typical potatoes are about 20-24% dry matter, of which approximately 60-80% consists of starch. After potato vines die back, the tuber skins thicken and harden which provides greater protection to tubers during harvest and blocks entry of pathogens. Although dormancy is defined as the absence of visible growth, dormant tuber meristems are metabolically active. In general, rates of many cellular processes such as respiration, transcription, and translation are suppressed during dormancy and non-dividing, dormant tuber meristems are arrested in the G-1 phase (a period prior to the synthesis of DNA in mitosis). Activation of cell metabolism during early spring triggers the development of sprouts from the eyes of the primary tuber.

2.6 Seed Propagation

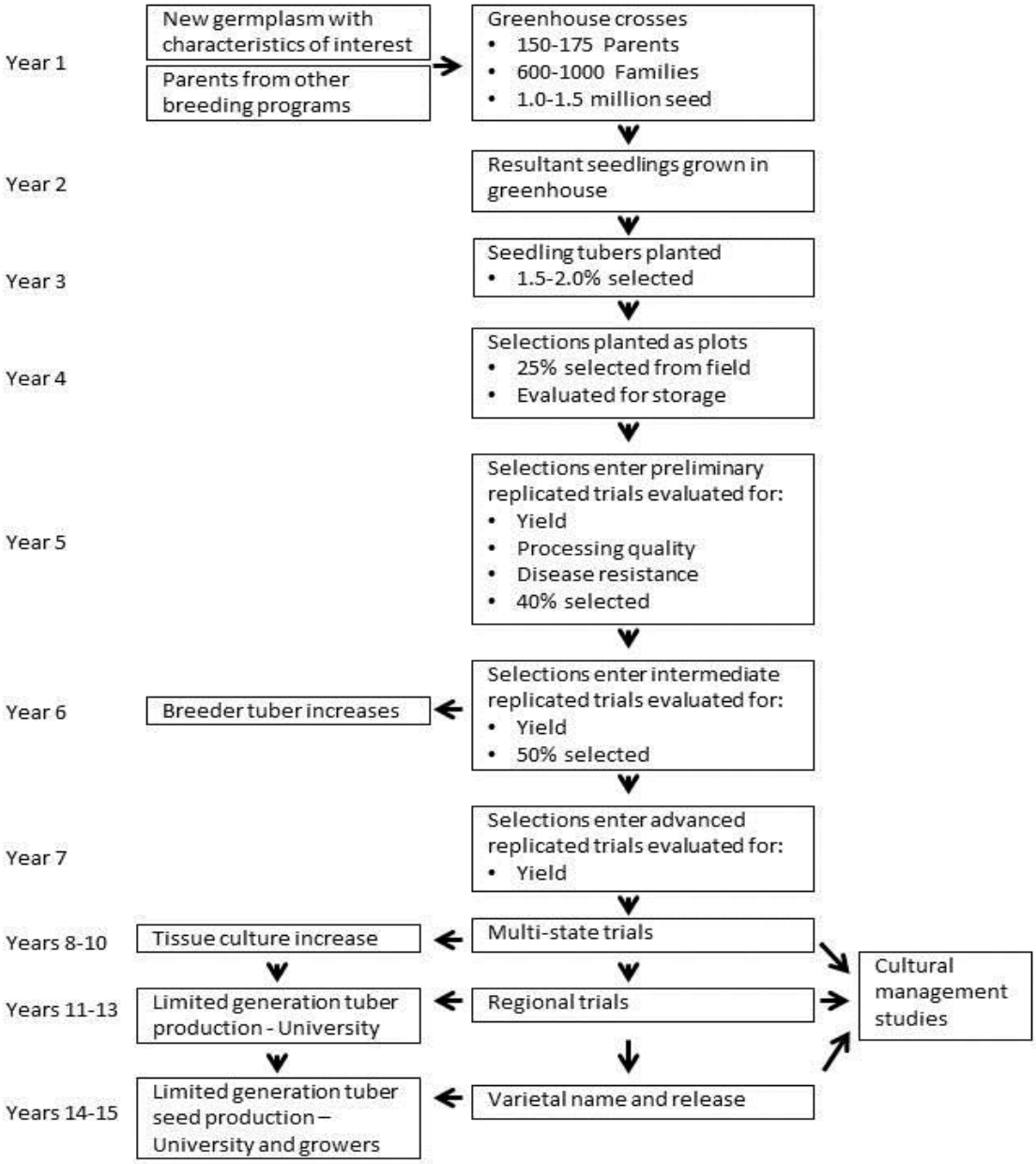
Potato is clonally propagated, which means that tubers rather than seeds are used for planting. A single potato plant produces approximately 12 tubers, therefore it takes multiple years to propagate a new potato variety and generate sufficient amounts of tuber seed needed for commercialization. A typical seed bulk-up program would progress as follows. In the initial year, propagation in tissue culture would produce about 100 plants, and then these plants are grown in a greenhouse to produce thousands of mini-tubers, also called “nuclear seed”. In the second year, mini-tubers are planted at field sites with the lowest possible incidence of diseases and pests, producing generation-1 (G1) seed. Those G1 tubers are grown in the third year to increase the amount of tubers, producing G2 seed. In the fourth, fifth, and

sixth years, bulk-up continues with commercial seed often representing G3, G4, and G5 tubers respectively.

2.7 Variety Development

Potato varieties take many years to develop (see Figure 2-1). The decision to establish a new variety is based on many factors such as need in the market place, potential consumer acceptance, and pest tolerance or resistance. Potato varieties do not have a high frequency of introduction and discontinuation compared to some other crops such as field corn or soybeans. Since potatoes are clonally propagated, there is a reduced risk of varietal dilution due to cross pollination.

Figure 2-1. Potato Variety Development Schematic



2.8 Recipient Potato Varieties

The potato variety chosen for modification, Russet Burbank, represents significant value to the potato industry and a relatively large percentage of the overall acreage. Used for fries and table stock, in 2012 it comprised 20.3% of seed acreage in the US (NPC 2013).

2.9 Typical Agronomic Practices

Examples of typical agronomic practices for a successful potato crop are described in Table 2-1.

Table 2-1. Example of Agronomic Inputs for Russet Varieties

	Russet Varieties
Planting Date	April 1 to May 10
Planting Rate	15,000 - 18,000 seed pc or 17 – 23 cwt/A
Row Spacing	34-36" between rows
Seed Spacing	10-12" within row
Fertilizer	For 600 cwt/A yields and optimum soil test levels: 250 lb N – 100 lb P_2O_5 – 330 lb K_2O per acre
Yield/Plant	2-4 lb
Yield/Acre	400-700 cwt/A
Harvest Date	September 1 to October 15

2.10 Pollination and Outcrossing

Potato is clonally propagated, which means that tubers rather than seeds are used for planting. Therefore, pollination to produce seed is not a factor in major commercial potato production. For example, if Innate™ potatoes were planted in close proximity to organic potatoes, any cross pollination would not impact the organic tubers.

Some of the most important potato varieties, including Russet Burbank, produce few flowers and are male sterile. Many cultivars flower less than wild material, and flowers that do form often drop after pollination resulting in no berry formation. Flowering has no impact on tuber development.

Approximately 80% of seed produced by fertile varieties is derived from self-pollination (Plaisted 1980). Cross-pollination is typically mediated by certain insects including bumblebees (*Bombus* spp.) that typically do not travel much more than 3 kilometers, rather than wind pollination (OECD 1997). Additionally, other bee species such as honey bees (*Apis mellifera*) are not pollinators of potatoes since potato flowers do not contain ample nectar (OECD 1997). Field evaluations in New Zealand, the United Kingdom and Sweden demonstrated through genetic testing that outcrossing rates were zero when receiving plants were separated by more than 20 meters from genetically modified plants (Conner and Dale 1996). Additionally, potato pollen grains are extremely temperature sensitive. Pallais et al. (1988) showed that pollen viability decreased by 30 to 70% when exposed to temperatures of 30°C for up to 30 minutes.

2.11 Wild potatoes in the U.S.

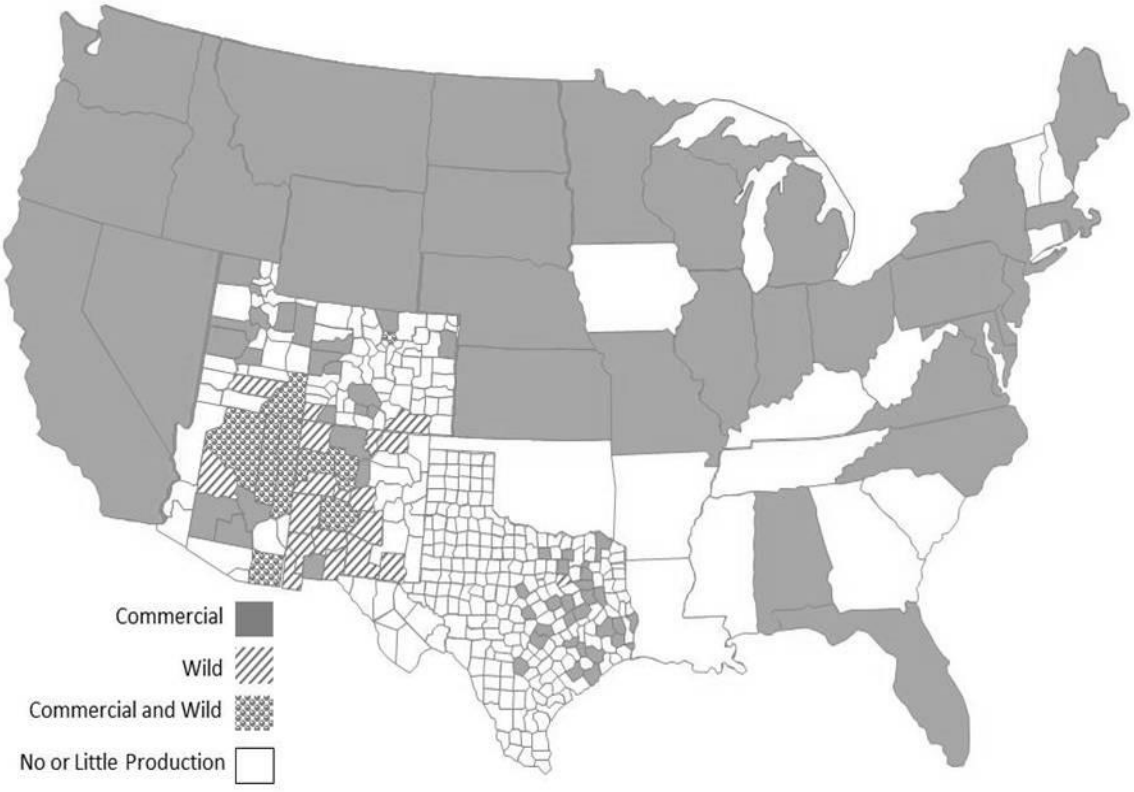
This discussion is based on data in the U.S. Potato Genebank and on a monogram published by the American Society of Plant Taxonomists entitled Wild Potatoes (*Solanum* section *Petota; Solanaceae*) of North and Central America (Spooner et al. 2004). The USDA maintains the US Potato Genebank and participates in the Association of Potato Inter-genebank Collaborators (APIC), which has produced a global inventory of wild potato genetic resources available on the Internet, the Inter-genebank Potato Database (IPD 2011), which includes the U.S. Potato Genebank records. Many of the IPD records from the U.S. were obtained by John Bamberg and associates (including Spooner) during a decade of collecting wild potatoes in the Southwest U.S. (Bamberg et al. 2003).

The only two wild potato species that grow within the borders of the USA, and for which specimens exist in gene banks, include the tetraploid species *S. fendleri* (recently reclassified as *S. stoloniferum*; however, some sources, including the IPD, still use the *S. fendleri* designation) and the diploid species *S. jamesii* (Bamberg et al. 2003; IPD 2011; Bamberg and del Rio 2011a; Bamberg and del Rio 2011b; Spooner et al. 2004). Love (1994) reported that a third species, *S. pinnatisectum*, is also a native species in the USA. However, Spooner et al. (2004) determined that what was previously thought to be *S. pinnatisectum* was in fact *S. jamesii.* Through more than 10 years of field work and assessments of existing records, Bamberg et al. (2003) and Spooner et al. (2004) established the presence of only these two species, *S. fendleri* and *S. jamesii,* in the U.S. These researchers also attempted to verify previously recorded locations, and through this process, updated the maps of current known locations of these species, providing latitude and longitude locations for each documented population (Bamberg et al. 2003) and distribution maps (Spooner et al. 2004). These species mostly reside in dry forests, scrub desert, and sandy areas at altitudes of 5,000 to 10,000 feet, well isolated from most commercial production areas (Bamberg and del Rio 2011a).

While there is some overlap between the acreage used for commercial production and occurrence of wild species on a county level, the majority of the potato production in the United States is not in wild potato zones (Figure 2-2). However, there is a possibility that a few wild potato plants may be growing near potato fields (Love 1994). Spooner et al. (2004) describe *S. jamesii* habitat in the U.S. as among boulders on hillsides, sandy alluvial stream bottoms, in gravel along trails or roadways, rich organic soil of alluvial valleys, sandy fallow fields, grasslands, juniper-pinyon scrub deserts, oak thicket, coniferous and deciduous forests at elevations between 4,500 to 9,400 feet. They describe *S. fendleri* habitat similarly, and at elevations between 4700 to 11,200 feet. The risk of hybridization between commercial varieties and wild species is low because of the lack of geographical overlap and the fact that potatoes are grown from tuber seed. Furthermore, studies discussed in Conner and Dale (1996) indicated that a separation of 20 meters will be sufficient to prevent outcrossing between wild and commercial potatoes.

Love (1994) evaluated the risk associated with growing transgenic potatoes in Canada and the USA and concluded that, given the number and potency of barriers to hybridization and more specifically to introgression and stabilization, the only sound conclusion is that gene introgression into wild *Solanum* species will not occur under natural conditions in these geographies. Therefore, according to Love (1994), potato gene movement from commercial fields to wild potato species would not occur.

Figure 2-2. Potato Production in the United States
(County Information is for states with wild potato populations (Hijmans and Spooner 2001))



Based on conversations with Bamberg, Love (1994) reported that no one has ever reported finding hybrids between native and cultivated potatoes in the U.S., although gene transfer has been accomplished using special laboratory techniques (Love 1994). Love concluded that, based on the barriers that exist (including geographic isolation as described previously), endosperm imbalances, and multiple ploidy levels, natural hybridization is highly unlikely, and gene introgression (cross hybridization over multiple generations) is impossible or at least highly improbable (Love 1994). More recently, the US EPA has concluded that, based on its review of the scientific literature, successful gene introgression between native and cultivated potatoes in the U.S. is virtually excluded due to constraints of geographical isolation and other barriers to natural hybridization. These barriers include incompatible (unequal) endosperm balance numbers that lead to endosperm failure and embryo abortion, multiple ploidy levels and incompatible mechanisms that do not express reciprocal genes to allow fertilization to proceed. No natural hybrids have been observed between these species and cultivated potatoes in the U.S. (US EPA 2011). Based upon these biological barriers to hybridization, it is unlikely that inter-species pollination would occur with the Innate™ potatoes that are the subject of this petition.

2.12 Weediness

Standard growing practices for potatoes make it highly unlikely that potatoes would persist in a field from one crop cycle to the next. Where potatoes are a rotation crop, as is often the case, other crops such as alfalfa, corn or wheat would be grown following potatoes. In that situation, any potatoes left in

the field would be eliminated by tilling, field preparations with herbicides, and harsh winters. In the rare event that potatoes survived mixed in with another crop, they would have to face the same challenges during the next year.

Potatoes are typically grown on a 3 year minimum rotation to minimize soil-borne disease buildup such as white mold, pink rot, *Pythium* leak, *Verticillium* wilt, and powdery scab (Hopkins et al. 2003). Potatoes left to grow in the field are not desired as they can harbor disease, have no protection with fungicide, and could compete with the desired rotation crop the following year. For these reasons, it is standard practice for potato growers to monitor and control such growth, either with herbicide or tillage. Soil is disturbed greatly during potato growth and harvest operations, therefore requiring tillage prior to planting the rotation crop. Typically a broad spectrum pre-emergence herbicide is applied in the rotation crop to control a wide variety of weeds. Potato growers rarely leave the ground fallow following harvest to maximize economic return. If potatoes grew inadvertently in the field left fallow, they would not be protected with insecticide or fungicide and would be susceptible to insects such as Colorado potato beetle and diseases such as early blight, further decreasing the chance of survival and reproduction.

Outside of cultivation, potato seedlings from tubers have difficulty establishing themselves as they cannot compete effectively with other plants (Love 1994). Potato is not known as a colonizer of unmanaged ecosystems. In climax vegetation, potatoes are not able to compete with other species such as grasses, trees and shrubs (CFIA 1996), virtually eliminating any chance for them to become feral. Furthermore, potatoes generally do not cause toxic effects to other plants or animals including non-target organisms.

Although almost all growers use tubers for planting, there is a small market for “true potato seed” (TPS). TPS is seed that is produced as a result of pollination. The seed is produced inside fruits that resemble small tomatoes on the potato vine. The major disadvantage of TPS is that it segregates for numerous traits because potato is highly heterozygous and each seed produced is potentially a new variety. When TPS is used for planting, harvested tubers will be variable in quality. TPS plants take longer to establish themselves and set tubers, resulting in lower yield compared to plants grown from tuber seed. Some growers in tropical areas use TPS because it is easy to ship and free of pathogens (Dwelle 2003). Love (1994) reported that botanical seed (TPS, not tubers or “seed potatoes”) can survive and germinate for periods of time in excess of seven years however, it is unlikely that plants from TPS would persist for the reasons stated above.

2.13 Characterization of the Recipient Potato Cultivar

Russet Burbank. Luther Burbank developed this variety in the early 1870s. Plants are vigorous and continue vine growth throughout the season. Stems are thick, prominently angled and finely mottled. Leaflets are long to medium in width and light to medium green in color. The blossoms are few, white and not fertile. The cultivar is tolerant to common scab but is susceptible to *Fusarium* and *Verticillium* wilts, leafroll and net necrosis, potato virus Y, and late blight. Plants require conditions of high and uniform soil moisture and controlled nitrogen fertility to produce tubers free from knobs, pointed ends and dumbbells. Jelly-end and sugar-end develop in tubers when plants are subjected to stress. The tubers produced are large brown-skinned and white-fleshed, display good long-term storage characteristics, and represent the standard for excellent baking and processing quality. The variety is sterile and widely grown in the Northwest and Midwest, especially for the production of french fries.

2.14 Conclusions: The Biology of Potato

The propagation of commercial potato varieties through cloning mitigates concerns about increased plant pest potential such as seed dispersal, survival outside of cultivation, or outcrossing. Potatoes are rarely grown from true potato seed because the offspring would show such extreme diversity they would not be useful as commercial potatoes. In addition, potatoes are not known to escape from commercial fields or show weediness potential. Wild potato varieties are rare in the United States and geographically isolated from commercial production areas, further reducing concerns about cross-pollination with wild species.

An understanding of the biology of potatoes shows the extremely difficult challenges associated with simultaneously incorporating multiple traits through traditional breeding. Thus, potatoes benefit greatly by the use of Innate™ technologies which provide a superior alternative to breeding, through the addition of non-coding DNA, and result in new potato varieties that are not plant pests.

2.15 References

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3.0 Development of W8 Russet Burbank: Description of Marker-Free DNA Transformation

3.1 Introduction

Simplot's Innate™ technologies comprise many aspects of plant biology all working together to produce traits of interest in transformed plants. Specifically, the potatoes were modified to introduce late blight resistance while decreasing acrylamide potential, reducing sugars, and black spot in the tuber. The expression cassettes, including promoters and their silencing or expression cassettes, used to confer these traits are all derived from the genomes of potatoes or sexually compatible species. The trait specific genes were combined with backbone elements to create the vectors for *Agrobacterium*-mediated transformation resulting in potatoes that specifically incorporate the expression cassette and not backbone elements. We chose the most prominent variety for table stock and french fries, Russet Burbank, as the target for transformation.

The W8 event was developed by first transforming Russet Burbank with the plasmid pSIM1278 to reduce expression of asparagine synthetase (ASN1), polyphenol oxidase (PPO5), and reducing sugars. In a second transformation, another construct, pSIM1678, was introduced which includes the late blight resistance gene and an invertase silencing cassette. These constructs are described in detail in Chapter 4: Donor Genes and Regulatory Sequences.

3.2 Description of the Transformation System

The C58-derived *Agrobacterium* strain AGL1 was developed by precisely deleting the transfer DNA of the hyper-virulent plasmid, pTiBo542 (Lazo et al. 1991). Transformed plants were grown on media containing the antibiotic, timentin, which prevents survival of *Agrobacterium*, and thus selects for plants free of *Agrobacterium.* Following selection, plants are both antibiotic and *Agrobacterium* free, with the potato-derived expression cassettes inserted into the plant's genome.

3.3 Transformation Method

Stock plants were maintained in magenta boxes with 40 ml half-strength M516 (Phytotechnology) medium containing 3% sucrose and 2 g/l gelzan (propagation medium). Potato internode segments of four to six mm were cut from four-week old plants, infected with the *Agrobacterium* AGL1 strain carrying pSIM1278, and transferred to tissue culture media containing 3% sucrose and 2 g/l gelzan (co-cultivation medium). Infected explants were transferred, after two days, to M404 (Phytotechnology) medium containing 3% sucrose, 2 g/l gelzan, 300 mg/l timentin and 1.2 ml plant protection medium (Phytotechnology) to eliminate *Agrobacterium* (hormone-free medium). Evidence that the plants were *Agrobacterium*-free was obtained by incubating stem and/or leaf fragments of transformed events on nutrient broth-yeast extract (NBY medium) for 2 weeks at 28^{O}C (repeated twice) with no outgrowth. In accordance with 7 CFR Part 340, transformed plants were transported and planted in the field only when free of live *Agrobacterium*. Details of the methods are described elsewhere (Richael et al. 2008).

Although *Agrobacterium* is effective in cleaving at the Right Border (RB) site, it often fails to fully release the DNA insert from its plasmid vector by also cutting at the Left Border (LB) site (Gelvin 2003). Consequently, some infected plant cells received the DNA insert itself as well as additional plasmid backbone sequences containing the backbone marker gene, isopentenyltyransferase (ipt), for a plant hormone cytokinin, which commonly regulates growth and development processes in plants. Overexpression results in stunted phenotypes, abnormal leaves, or the inability to root due to the

cytokinin overproduction, which were used to select against plants containing backbone DNA (Richael et al. 2008). Every two weeks, the infected explants were transferred to fresh medium lacking any synthetic hormones and incubated in a Percival growth chamber under a 16-hr photoperiod at 24^{o}C where they started to form shoots. Many shoots expressed the *ipt* gene and displayed the cytokinin-overproduction phenotype; these shoots were discarded and not considered for further analyses. PCR genotyping demonstrated that about 0.3 to 1.5% of the remaining shoots contained at least part of the DNA insert while lacking the *ipt* gene.

Following development and isolation of events containing an insert associated with the pSIM1278 vector, those plants were transformed using the methods described for a second plasmid, pSIM1678, to produce stacked events containing two independent inserts.

The following methods were used to establish that backbone portions of the plasmids pSIM1278 and pSIM1678 were not present in events developed for commercial purposes: 1) If plants had phenotypes associated with the negative selectable isopentenyl isomerase (*ipt*) marker gene in the plasmid backbone, they were discarded; 2) Absence of the backbone DNA was confirmed with Southern blot hybridization; 3) PCR was used to confirm no fragments of the backbone DNA were present. Evidence showing that event W8 does not contain backbone DNA is provided in Chapter 5: Genetic Characterization**.**

3.4 References

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4.0 Donor Genes and Regulatory Sequences

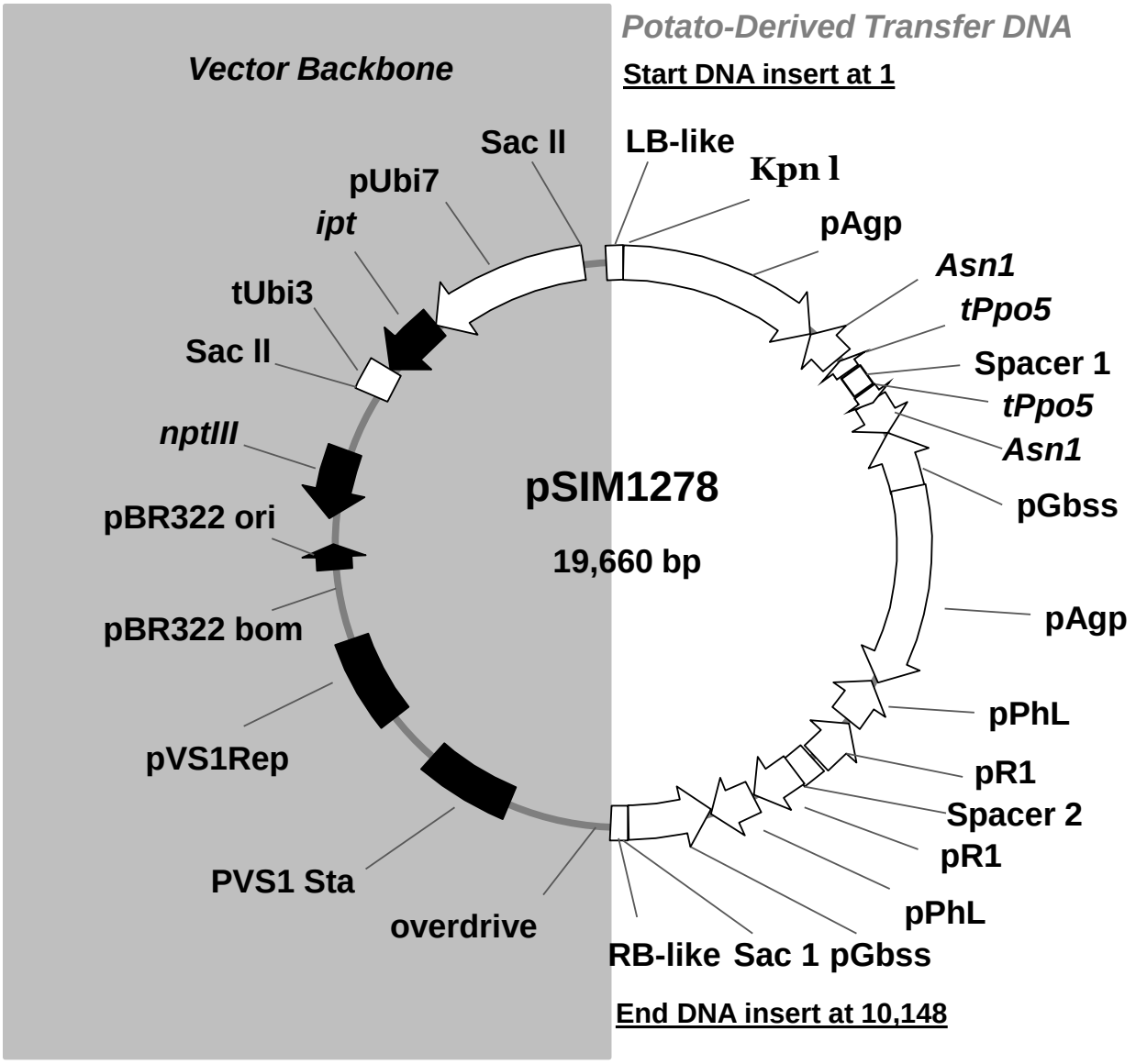
4.1 Plasmids used for Transformation

W8 was developed by first transforming Russet Burbank with plasmid, pSIM1278, (Figure 4-1) followed by stacking with a second plasmid, pSIM1678 (Figure 4-2). The pSIM1278 insert contains cassettes to reduce expression of asparagine synthetase (ASN1), polyphenol oxidase (PPO5), and reducing sugars by silencing the *R1* and *PhL* genes, whereas the pSIM1678 construct provides the late blight resistance gene (*Rpi-vnt1*) and a vacuolar invertase (*VInv*) silencing cassette. The plasmids, pSIM1278 and pSIM1678, are binary transformation vectors that consist of two parts: plasmid backbone and the DNA insert.

The plasmids were developed using the same parental plasmid and thus share identical backbone sequences (Table 4-1), containing well-characterized bacterial origins of replication from plasmids pVS1 and pBR322, and the *nptIII* 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).

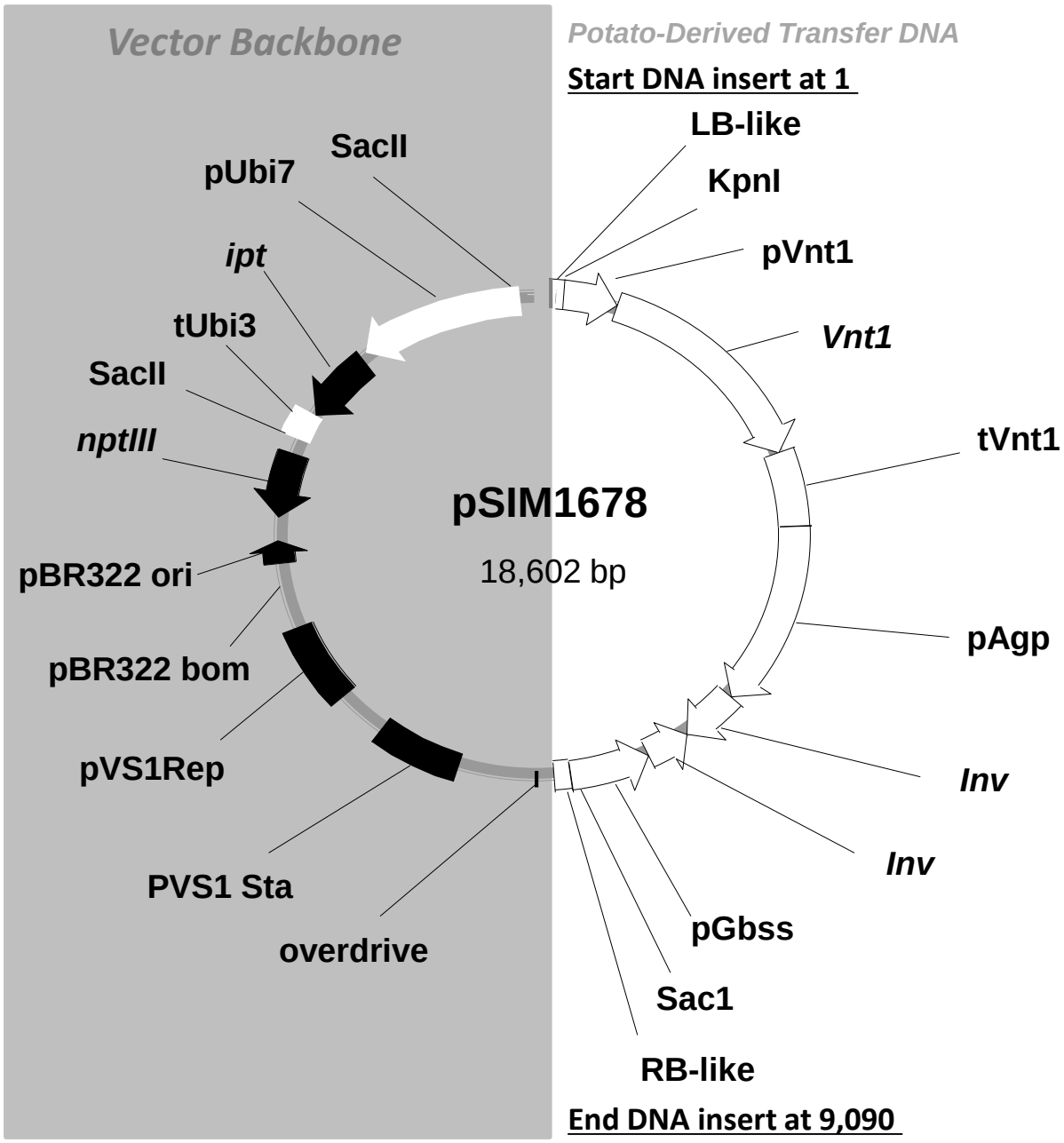
Maps of the transformation plasmids, pSIM1278 and pSIM1268, are provided in Figures 4-1 and 4-2 with descriptions of the genetic elements for the DNA inserts provided in Tables 4-2 and 4-3. Briefly, the pSIM1278 insert consists of two independent inverted repeats and their associated spacer elements flanked by opposing plant promoter elements, pGbss and pAgp. One inverted repeat consists of sequence from the potato *Asn-1* gene adjacent to sequence from the potato gene, *Ppo5*. The other inverted repeat consists of adjacent sequences derived from the promoters of two potato genes, *PhL* and *R1*. The pSIM1678 insert consists of the *Rpi-vnt1* expression cassette and a silencing cassette for the plant vacuolar invertase gene, *VInv*. The *Rpi-vnt1* gene cassette consists of the VNT1 protein coding region regulated by its native promoter and terminator sequences, whereas the silencing cassette consists of an inverted repeat of sequence from the potato *VInv* gene flanked by opposing plant promoters, pGbss and pAgp.

Figure 4-1. pSIM1278 Vector



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-like sequences and potato genomic DNA which are intended for stable integration into the plant genome.

Figure 4-2. pSIM1678 Vector



The vector backbone region, indicated in grey, starts at position 9,091-bp and ends at 18,602-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, including flanking Border sequences, is on the right from 1-bp to 9,090-bp, which is a total of 9090-bp. The DNA insert consists of T-DNA border-like sequences and potato genomic DNA which are intended for stable integration into the plant genome.

Table 4-1. Table 4-1 Genetic elements of the pSIM1278 and pSIM1678 backbone[1]

Genetic Element	*Origin*	Accession Number	Position (pSIM1278) [1]	Function
SacII restriction site	*S. tuberosum*	AJ272136.1	19,411-19,416	Restriction site used to connect Ubi7 promoter with LB flanking sequence.
Polyubiquitin promoter (**Ubi7**) including the coding sequence for a 76- amino-acid potato ubiquitin monomer (**UBQmon**)	*S. tuberosum* var. Ranger Russet	U26831.1	17,671-19,410	Promoter to drive expression of the *ipt* backbone marker gene
Isopentenyl transferase (***ipt***) gene	*Agrobacterium tumefaciens*	NC_002377.1	16,936-17,658	Condensation of AMP and isopentenylpyrophosphate to form isopentenyl-AMP, a cytokinin in the plant. Results in abnormal growth phenotypes in plant (Smigocki and Owens 1988)
Terminator of the ubiquitin-3 gene (**tUbi3**)	*S. tuberosum*	GP755544.1	16,230-16,584	Terminator for *ipt* gene transcription (Garbarino and Belknap 1994)
Neomycin phosphotransferase III (***nptIII***) gene	*E. coli*	FJ362602.1	15,240-16,034	Aminoglycoside phosphotransferase (Courvalin et al. 1977)
Origin of replication for pBR322 (**pBR322 ori**)	*E. coli*	J01784.1	14,669-14,949	Bacterial origin of replication [2]
(**pBR322 bom**)	*E. coli*	J01749.1	14,269-14,529	pBR322 region for replication in *E. coli* [2]
pVS1 replicon (**pVS1Rep**)	*Pseudomonas fluorescens* plasmid pVS1	AJ537514.1 (4,501-5,501)	12,859-13,859	pVS1 region for replication in *Agrobacterium* [2]
pVS1 partitioning protein StaA (**PVS1 Sta**)	*Pseudomonas fluorescens* plasmid pVS1	AJ537514.1 (6,095-7,095)	11,266-12,266	pVS1 stability [2]
Overdrive	*Agrobacterium tumefaciens*	K00549.1 (103-132)	10,155-10,184	Enhances cleavage at the Right Border site [2]

[1] Numbering system based on pSIM1278, but backbone sequences identical for pSIM1678.
[2] http://www.cambia.org/daisy/cambia/585.html - (General structure map of pCAMBIA vectors)

Table 4-2. Genetic elements of the DNA Insert of pSIM1278, from Left Border site to Right Border

Genetic Element	*Origin*	Accession Number	Position (pSIM1278)	Intended Function
1. Left Border (LB) site[1]	Synthetic	AY566555[2] (bases 1-25)	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.	AY566555[2] (bases1-187)	1 – 187	Supports secondary cleavage at LB
3. KpnI restriction site	*S. tuberosum*	AF393847.1	188 –193	Site for connection of DNA insert with LB flanking sequence.
4. Promoter for the ADP glucose pyrophosphorylase gene (pAgp), 1st copy	*S. tuberosum* var. Ranger Russet	HM363752	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	HM363759	2,454-2,858	Generates with (10) double stranded RNA that triggers the degradation of Asn1 transcripts to impair asparagine formation (Chawla et al. 2012[3])
6. 3'-untranslated sequence of the polyphenol oxidase-5 gene (Ppo5) (1st copy, in antisense orientation)	*S. verrucosum*	HM363754	2,859-3,002	Generates with (9) double stranded RNA that triggers the degradation of Ppo5 transcripts to block black spot development
7. XbaI restriction site	*S. tuberosum*	DQ478950.1	3,003-3,008	Site for connection of the first Ppo5 copy to spacer-1.
8. Spacer-1	*S. tuberosum* var. Ranger Russet	HM363753	3,009-3,166	Sequence between the 1st inverted repeats
9. 3'-untranslated sequence of the polyphenol oxidase-5 gene (Ppo5) (2nd copy, in sense orientation)	*S. verrucosum*	HM363754	3,167-3,310	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	HM363759	3,311-3,715	Generates with (5) double stranded RNA that triggers the degradation of Asn1 transcripts to impair asparagine formation (Chawla et al. 2012[3])
11. EcoRI restriction site	*S. tuberosum* var. Ranger Russet	X73477	3,716-3,721	Site for connection of the second Asn1 copy to Gbss promoter.
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	HM363755	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. Spe1 / KpnI restriction sites	*S. tuberosum* var. Ranger Russet	X95996 / AF393847.1	4,408-4,423	Polylinker site for connection of Gbss promoter to the second Agp promoter.
14. pAgp, 2nd copy	*S. tuberosum* var. Ranger Russet	HM363752	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	HM363758	6,684-7,192	Generates with (20) double stranded RNA that triggers the degradation of PhL transcripts to limit the formation of reducing sugars through starch degradation
16. Fragment of promoter for the potato R1 gene (pR1) (1st copy, in antisense orientation)	*S. tuberosum* var. Ranger Russet	HM363757	7,193-7,724	Generates with (19) double stranded RNA that triggers the degradation of R1 transcripts to limit the formation of reducing sugars through starch degradation
17. Pst1 restriction site	*S. tuberosum* var. Ranger Russet	DQ478950.1	7,725-7,730	Site for connection of the first R1 promoter fragment to the spacer2
18. Spacer-2	*S. tuberosum* var. Ranger Russet	HM363756	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	HM363757	7,989-8,520	Generates with (16) double stranded RNA that triggers the degradation of R1 transcripts to limit the formation of reducing sugars through starch degradation

Table 4-2 (Continued). Genetic elements of the DNA Insert of pSIM1278, from Left Border site to Right Border

Genetic Element	*Origin*	Accession Number	Position (pSIM1278)	Intended Function
20. Fragment of promoter for the potato phosphorylase-L (pPhL) gene (2nd copy, in sense orientation)	*S. tuberosum* var. Ranger Russet	HM363758	8,521-9,029	Generates with (15) 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	HM363755	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. SacI restriction site	*S. tuberosum*	AF143202	9,954 – 9,962	Site for connection of DNA insert with RB flanking sequence.
23. Right Border region sequence including RB	*S. tuberosum* var. Ranger Russet	AY566555[2] (bases 231-416)	9,963 – 10,148	Supports primary cleavage at RB-Like site
24. Right Border (RB) sequence[1]	Synthetic	AY566555[2] (bases 392-416)	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]GenBank Accession AY566555 was revised to clarify the sources of DNA for the Border regions.
[3]ASN1 described as genetic elements 5 and 10 is referred to as StAst1 in Chawla et al. 2012.

Table 4-3 Genetic elements of the DNA Insert of pSIM1678, from Left Border site to Right Border

Genetic Element	*Origin*	Accession Number	Position (pSIM1678)	Intended Function
1. Left Border (LB) site[1]	Synthetic	AY566555[2] (bases 1-25)	1 – 25	Site for secondary cleavage to release single-stranded DNA insert from pSIM1678
2. Left Border region sequence including LB	*S. tuberosum* var. Ranger Russet.	AY566555[2] (bases 1-187)	1 - 187	Supports secondary cleavage at LB
3. KpnI restriction site	*S. tuberosum*	AF393847.1	188 - 193	Site for connection of DNA insert with LB flanking sequence.
4. Native promoter for the late blight resistance gene (*Rpi-vnt1*)	*S.* venturii	FJ423044.1	194 -902	Drives expression of late blight resistance gene *vnt1*
5. Late blight resistance gene (*Rpi-vnt1*)	*S.* venturii	FJ423044.1	903 -3,578	Solanum venturii late blight resistance protein gene
6. Native terminator for the *Rpi-vnt1* gene	*S.* venturii	FJ423044.1	3,579 -4,503	Ends transcription of late blight resistance gene *vnt1*
7. Apa1	*S. tuberosum*t	HM363755	4,504 -4,509	Site for connection of *vnt1* terminator with Agp promoter
8. Promoter for the ADP glucose pyrophosphorylase gene (pAgp)	*S. tuberosum* var. Ranger Russet	HM363752	4,510 - 6,770	One of the two convergent promoters that drives expression of an inverted repeat containing fragments of acid invertase gene.
9. BamH1	*S. tuberosum* var. Ranger Russet	DQ206630	6,771 - 6,776	Site for connection of Agp promoter with invertase
10. Fragment of the acid invertase (sense orientation)	*S. tuberosum* var. Ranger Russet	DQ478950.1	6,777 - 7,455	Generates with (12) double stranded RNA that triggers the degradation of invertase transcripts
11. EcoRI	*S. tuberosum* var. Ranger Russet	X73477	7,456 - 7,461	Site for connection of an invertase fragment (sense) with an invertase fragment (anti-sense)
12. Fragment of the acid invertase (anti-sense orientation)	*S. tuberosum* var. Ranger Russet	DQ478950.1	7,462 - 7,965	Generates with (10) double stranded RNA that triggers the degradation of invertase transcripts
13. Spe1	*S. tuberosum* var. Ranger Russet	X95996	7,966 - 7,971	Site for connection of an invertase fragment (anti-sense) with GBSS promoter
14. Promoter for the granule-bound starch synthase (pGbss) gene (convergent orientation relative to the pAgp)	*S. tuberosum* var. Ranger Russet	HM363755	7,972 - 8,895	One of the two convergent promoters that drives expression of an inverted repeat containing fragments of invertase gene, especially in tubers
15. SacI restriction site	*S. tuberosum*	AF143202	8,896 – 8,901	Site for connection of DNA insert with RB flanking sequence.
16. Right Border region sequence including RB	*S. tuberosum* var. Ranger Russet	AY566555[2] (bases 231-416)	8,902 – 9,090	Supports primary cleavage at RB-Like site
17. Right Border (RB) sequence[1]	Synthetic	AY566555[2] (bases 392-416)	9,066 – 9,090	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]GenBank Accession AY566555 was revised to clarify the sources of DNA for the Border regions.

4.2 References

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5.0 Genetic Characterization of Russet Burbank Event W8

5.1 Introduction

The Russet Burbank W8 event contains inserts derived from two separate transformations with different plasmids. The first insert, plasmid pSIM1278, contains two cassettes consisting of inverted repeats designed to silence up to four potato genes, *Asn1*, *Ppo5*, *R1*, and *PhL*, in tubers. Similarly, the second plasmid, pSIM1678, contains a cassette consisting of an inverted repeat to silence the *VInv* gene in tubers, while also containing a copy of the *Rpi-vnt1* gene under its native potato promoter. The following studies were carried out as part of the characterization and biosafety assessment of the Russet Burbank W8 event.

In the following section, we provide a detailed characterization of the genetic inserts associated with transformation of the Russet Burbank variety to produce event W8. The characterization includes a combination of Southern blot analyses, polymerase chain reactions (PCR), and DNA sequencing to assess the structure, stability, and flanking regions of each insert. After determining the DNA insert and flanking region structures, bioinformatic methods were used to evaluate putative open reading frames to show that modifications to the genome did not generate new or modify existing open reading frames to produce potential allergens or toxins (see Chapter 9: Safety of the W8 Potato for the detailed analysis). The methods used in the following studies are described in Appendix A: Genetic, Molecular, and Biochemical Methods.

5.2 Copy number and structure of the DNA Inserts

An ideal transformation event consists of the plasmid insert being introduced into a single locus within the plant genome including intact copies of all expression cassettes. In some cases, the structure of the insert within the plant genome matches the structure from the original plasmid. However, in other cases, recombination events can lead to changes in the structure and/or duplications of cassettes within the original plasmid. Although one result is not better than the other, the presence of rearrangements and duplications can make structural determination more challenging.

Our characterization of copy number and structure of the Russet Burbank W8 Event (W8) included: (1) Southern blot verification that pSIM1278 and pSIM1678 each integrated into a single genomic locus, (2) Southern blot determination that the structure of the pSIM1678 insert was similar to the original construct, whereas the pSIM1278 insert was significantly more complex, and (3) structural determination of both inserts using numerous Southern blot analyses combined with PCR and sequencing analysis.

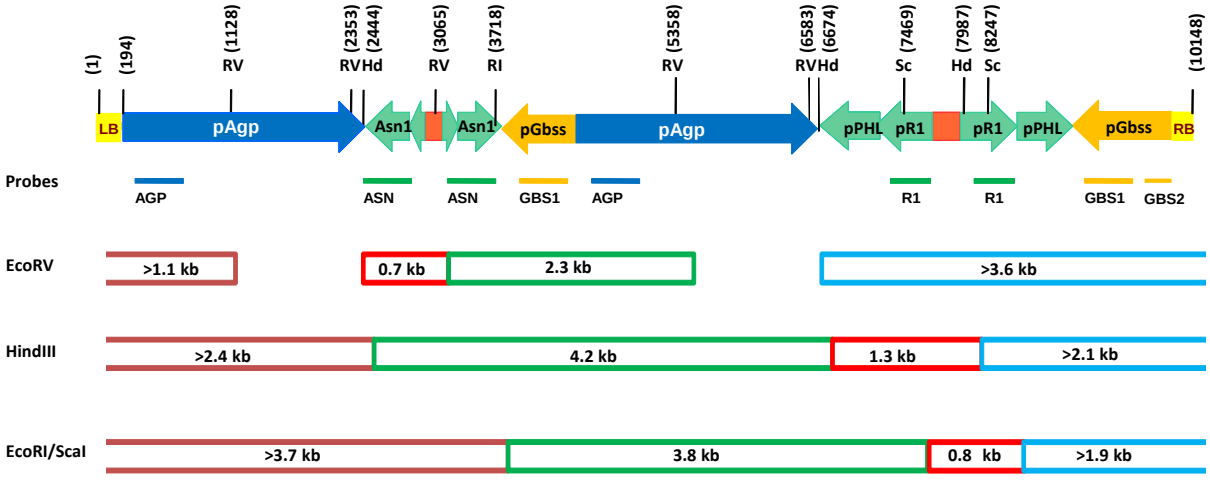
The Southern blot analyses presented in this section use a set of probes that specifically hybridize to elements contained within our inserts, but also recognize endogenous sequence within the plant genome. The AGP probe hybridizes to the 5′ end of the *Agp* promoter, which exists at two locations in the original pSIM1278 construct. The ASN probe hybridizes to both sides of the Asn1/Ppo5 silencing cassette, whereas the R1 probe hybridizes to both sides of the pR1/pPHL silencing cassette. The *Gbss* promoter associated with the Asn1/Ppo5 silencing cassette is a truncated form of the *Gbss* promoter associated with the pR1/pPHL silencing cassette. Whereas the GBS1 probe detects both promoters, the GBS2 probe can distinguish between these two promoters as it specifically detects the longer one associated with the pR1/pPHL silencing cassette.

As shown in Figure 5-1A, the pSIM1278 insert consists of an Asn1/Ppo5 silencing cassette flanked by converging *Agp* and *Gbss* promoters and a second pPHL/pR1 silencing cassette flanked by the same set of converging promoters. Probes that hybridize to the pAgp, Asn1, pGbss, pR1 elements were used to analyze the copy number and structure using Southern blot analysis following digestion with restriction enzymes. A number of informative restriction enzyme sites are highlighted, including EcoRV, HindIII, EcoRI, and ScaI, with a map showing the expected fragment sizes provided below the insert map for convenience.

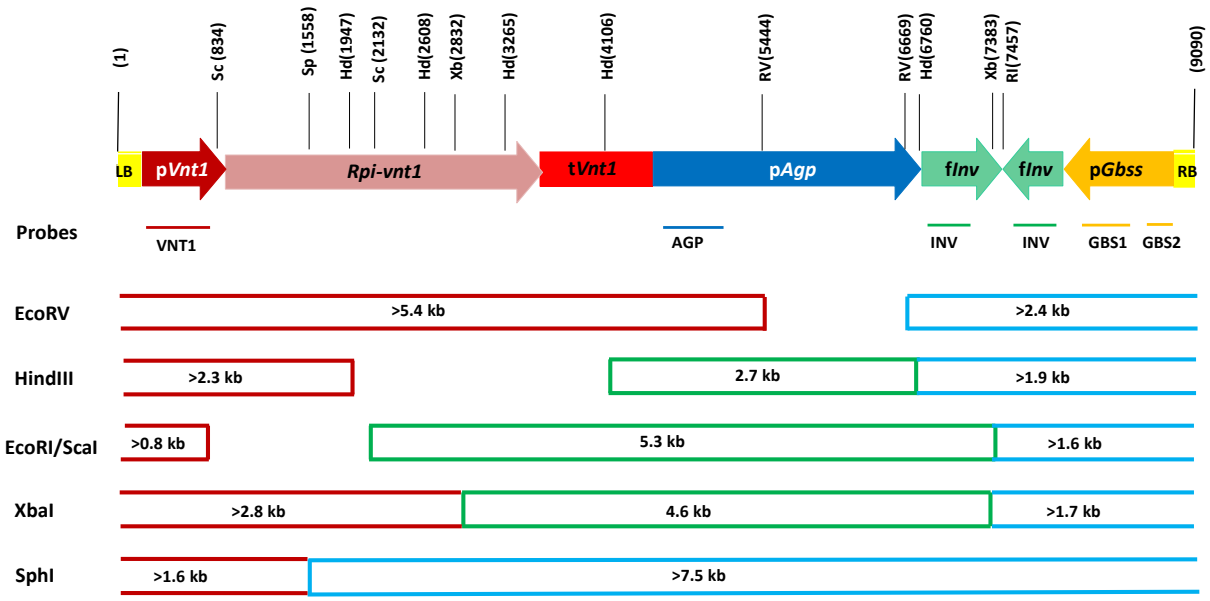
Similarly, Figure 5-1B shows the structure of the original pSIM1678 insert along with Southern probes and a map of commonly used restriction digests. Note there are two promoter elements, pAgp and the longer pGbss, in common between the pSIM1278 and pSIM1678 inserts. Therefore, the AGP, GBS1, and GBS2 probes will detect bands from both inserts on all Southern blots. However, the VNT1 and INV probes are specific to the *Rpi-vnt1* promoter and *VInv* silencing cassette of the pSIM1678 insert, respectively.

Figure 5-1. Plasmid constructs and Southern Probes

A. pSIM1278



B. pSIM1678



Restriction enzyme abbreviations: RV=EcoRV, RI=EcoRI, Sc=ScaI, Hd=HindIII, Xb=XbaI, Sp=SphI
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.

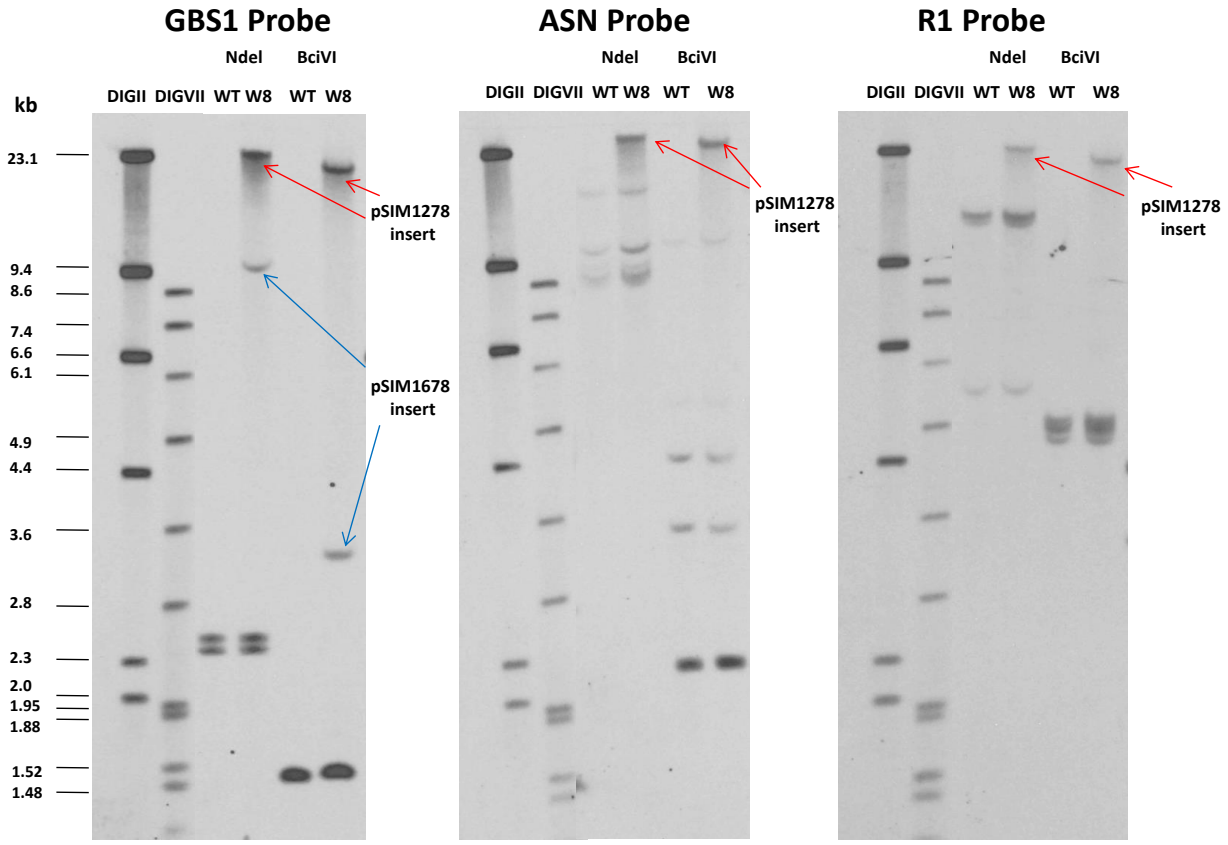
5.2.1 Characterization of insertion number for pSIM1278 and pSIM1678

Southern blots were performed using genomic DNA isolated from Russet Burbank W8 Event and Russet Burbank controls (WT) to identify the number of insertion events associated with each plasmid. These analyses were performed using restriction enzymes that frequently cut within the potato genome, but do not cut within our inserts, resulting in individual intact loci on Southern blots.

Single insertion site associated with pSIM1278

To assess the number of insertion events for the pSIM1278 plasmid, we digested genomic DNA with the restriction enzymes, BciVI and NdeI, which do not cut within the insert itself. Since the restriction enzyme recognition site is common within the potato genome, it is unlikely that more than one insertion event will contain the same size following digestion and that two insertion events will fall within the same fragment.

Figure 5-2. pSIM1278 DNA inserted at a single site in the genome



Red arrows indicate bands associated with pSIM1278 DNA and blue arrows indicate bands associated with pSIM1678 DNA.

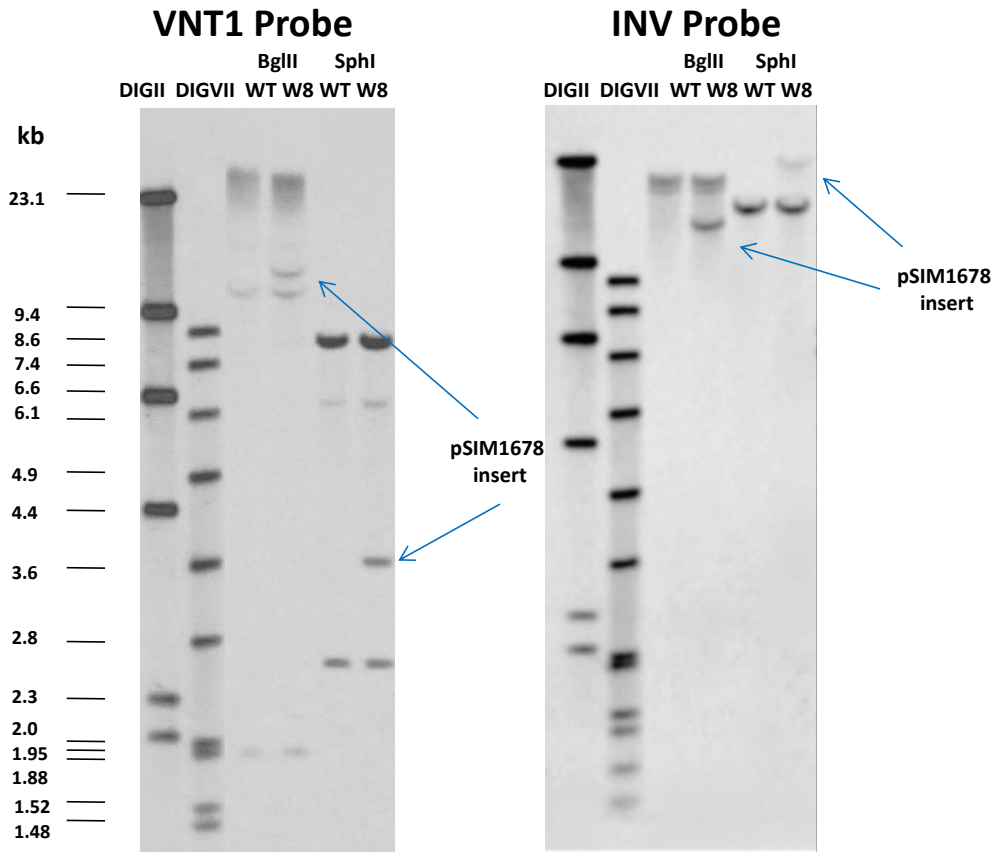
DNA digests were separated on agarose gels (TAE) using extended electrophoresis to ensure good separation and resolution of high-molecular weight bands. Southern blots were hybridized with the GBS1, ASN, and R1 probes depicted in Figure 5-1 to verify that a single band is detected with each probe (Figure 5-2). In addition to the two endogenous bands common to both samples, two higher molecular weight species were identified in the NdeI digested samples. The lower band corresponds to the

pSIM1678 insert, whereas the larger band contains the pSIM1278 insert. A similar digestion pattern was observed following digestions with BciVI, a single pSIM1278 band and a smaller pSIM1678 band. The identity of the smaller bands was consistent with known NdeI and BciVI restriction sites within the pSIM1678 construct and was confirmed by INV and VNT1 probes specific to this insert. The identity of the high molecular weight pSIM1278 bands was confirmed by Southern blots probed with ASN and R1, which are specific to that insert (Figure 5-2, gels on right). Collectively, these data show the pSIM1278 insert was integrated at a single locus within the Russet Burbank genome.

Single insertion site associated with pSIM1678

To assess the number of integration sites for the pSIM1678 insert, genomic DNA was digested with the restriction enzymes, BglII and SphI. The BglII restriction enzyme does not cut within the pSIM1678 DNA insert, and thus should generate a single band containing the entire construct. As shown in Figure 5-3, a single band of the same size was detected when BglII digested DNA was probed with either the VNT1 or INV probe. As shown in Figure 5-1(B), the regions detected by these probes are located on opposite ends of the insert and are not found in pSIM1278. To corroborate these findings, we also digested with the restriction enzyme, SphI, which cleaves the pSIM1678 insert within the *Rpi-vnt1* gene (Figure 5-1b), which resulted in a single band when probed with either VNT1 or INV, as expected. Since the SphI restriction site is located between the VNT1 and INV probe binding sites, this digest was expected to yield the two different sized species that was observed (Figure 5-3).

Figure 5-3. pSIM1678 DNA inserted at a single site in the genome



Blue arrows indicate bands associated with pSIM1678 DNA.

Summary of Integration Analysis

Our Southern blot analyses indicated a single insertion site associated with each plasmid transformation. As discussed in Section 5-3, the flanking sequence for the pSIM1278 insert indicated the presence of BciVI restriction sites within 1kb of the insert junctions. Given the close proximity of these restriction sites to our inserted DNA, a much smaller band was expected in our Southern blots (Figure 5-2) if the insertion represented the structure shown in Figure 5-1. Since the observed band was roughly 2-fold larger than expected, it suggested the structure of the insert was more complex than the original construct of pSIM1278 (Figure 5-1). A similar analysis could not be performed for pSIM1678 as there were no SphI or BglII restriction sites identified in the flanking sequence, but as described later the structure of this insert was very similar to the input sequence.

5.2.2 Structure of the pSIM1278 DNA insert

We used Southern blot analyses to test our hypothesis that the pSIM1278 construct was more complex than a simple, single copy integration of our plasmid DNA insert. Table 5-1 presents a summary of a set of Southern blots highlighting differences between the expected and observed band numbers, sizes, and intensities. The expected values are based upon integration of a single copy of the pSIM1278 DNA insert described in Figure 5-1A.

Table 5-1. Predicted and observed bands based on Southern blots for pSIM1278

Enzyme	Probe	Expected sizes (kb)	Observed sizes (kb)*	References
EcoRV	AGP	>1.1, 2.3	10, **2.3**	Figure 5-6
	ASN	0.7, 2.3	**0.7**, **2.3**	Figure 5-8
	GBS1	2.3, >3.6	**2.3**, 14.0	Figure 5-11
	R1	>3.6	14.0	Figure 5-13
HindIII	AGP	>2.4, 4.2	**4.2**	Figure 5-6
	ASN	4.2	**4.2**	Figure 5-8
	GBS1	4.2, >2.1	**4.2**, 6.0	Figure 5-11
	R1	1.3, >2.1	1.3, 4.3, 6.0	Figure 5-13
EcoRI/ScaI	AGP	>3.7, 3.8	3.8, **4.2**	Figure 5-6
	ASN	>3.7	**4.2**	Figure 5-8
	GBS1	3.8, >1.9	3.7, 3.8, **4.2**, 9.0	Figure 5-11
	R1	>3.7, 3.8, 0.8	**0.8**, **3.8**, 9.0	Figure 5-13

*Does not include endogenous or pSIM1678 bands.
- Sizes marked by bold print indicate bands had higher than normal intensity, suggesting >1 copy.

The presence of unexpected bands and signal intensity confirmed our expectation that the actual insert consisted of a complex structure. As we were unable to reconcile the Southern data presented in Table 5-1 with a tandem or inverted duplication of the pSIM1278 insert DNA, a number of additional Southern blots were performed in combination with PCR and sequencing studies to elucidate the structure of the insert at this locus. A detailed analysis of these studies is upcoming, but a summary of the final structure is provided in Figure 5-4 (top) and is broken into sections (Left, Middle, and Right) to facilitate discussion as shown in Figure 5-4a and 5-4b. Briefly, the Middle section consists of a full-length copy of the original pSIM1278 insert with a deletion of the T-DNA left border. The Middle section is flanked on the left (Left Section) by a tandem repeat of the Asn1/Ppo5 silencing cassette, both of which are in their original orientation, but also include the T-DNA left border deletion. The Right Section consists of an inverted repeat containing the pR1/pPHL silencing cassette flanked by converging *Gbss* promoters, where the right edge of the inverted repeat is juxtaposed to a tandem copy of the longer GBSS promoter separated by right border and a small amount of pPHL sequence (Figure 5-4).

Although the structure associated with pSIM1278 is more complicated than anticipated, the duplicated silencing cassettes are still under the control of the tissue-specific promoters and the current structure does not negatively impact safety or trait efficacy of the product (See Chapter 9: Safety of Russet Burbank W8 Potato and Chapter 10: Trait Efficacy). The data supporting the proposed structure will be presented next.

Southern blot presentation notes
Each Southern blot has been presented with a table that indicates the estimated sizes of the observed bands and whether they are interpreted as either endogenous original bands (OB), internal bands (IB) or

junction bands (JB). The endogenous original bands represent sequences found in the Russet Burbank control and are not related to the DNA inserts. The internal bands are those bands that reside completely within the junctions of the inserted DNA and thus have predicted sizes. The junction bands correspond to digestion products that contain sequence from the DNA insert as well as the plant genome. These bands have unique sizes that are not always predictable due to uncertainty in the plant genome sequence. Nonetheless, the minimal size of these bands can be predicted based upon the distance between the location of the restriction site within the DNA insert and the junction between the DNA insert and plant genome sequence. The junction bands are valuable for confirming the number of integration sites in the genome as there should never be more than two junction bands for a single integration event.

We have observed two phenomena that make it challenging to accurately determine the size of digestion products from potato genomic DNA. First, we have observed a tendency for bands above ~3kb to migrate slightly faster than similarly-sized molecular weight markers in the same gel. The anomalous migration appears to be related to residual polysaccharides in the genomic DNA samples as molecular weight markers also run faster when they are mixed with potato DNA samples. That said, the markers were not generally spiked with genomic DNA as the presence of genomic DNA has a tendency to make the ladder difficult to interpret due to hybridization of probes to endogenous DNA. Instead, we validated the size of IBs, such as the numerous 4.2 kb IBs and the 7.0 kb IB, by comparing their migration with digested plasmid DNA that had been mixed with Russet Burbank genomic DNA. Under these conditions, the plasmid and genomic bands co-migrated, although their migration was consistently faster than the unspiked molecular weight markers. In addition, we confirmed the identity of selected, faster migrating species by PCR and sequencing as described in Appendix A: Genetic, Molecular, and Biochemical Methods.

Additionally, many of the higher molecular weight bands in these samples have a tendency to produce smiles during electrophoresis. Size measurements taken from the top of these bands have proven to be the most reliable and reproducible so all sizes reported here used this technique (see line marking top of band in following example). 

Figure 5-4. Overall structure of pSIM1278 DNA Insert with digestion and hybridization patterns

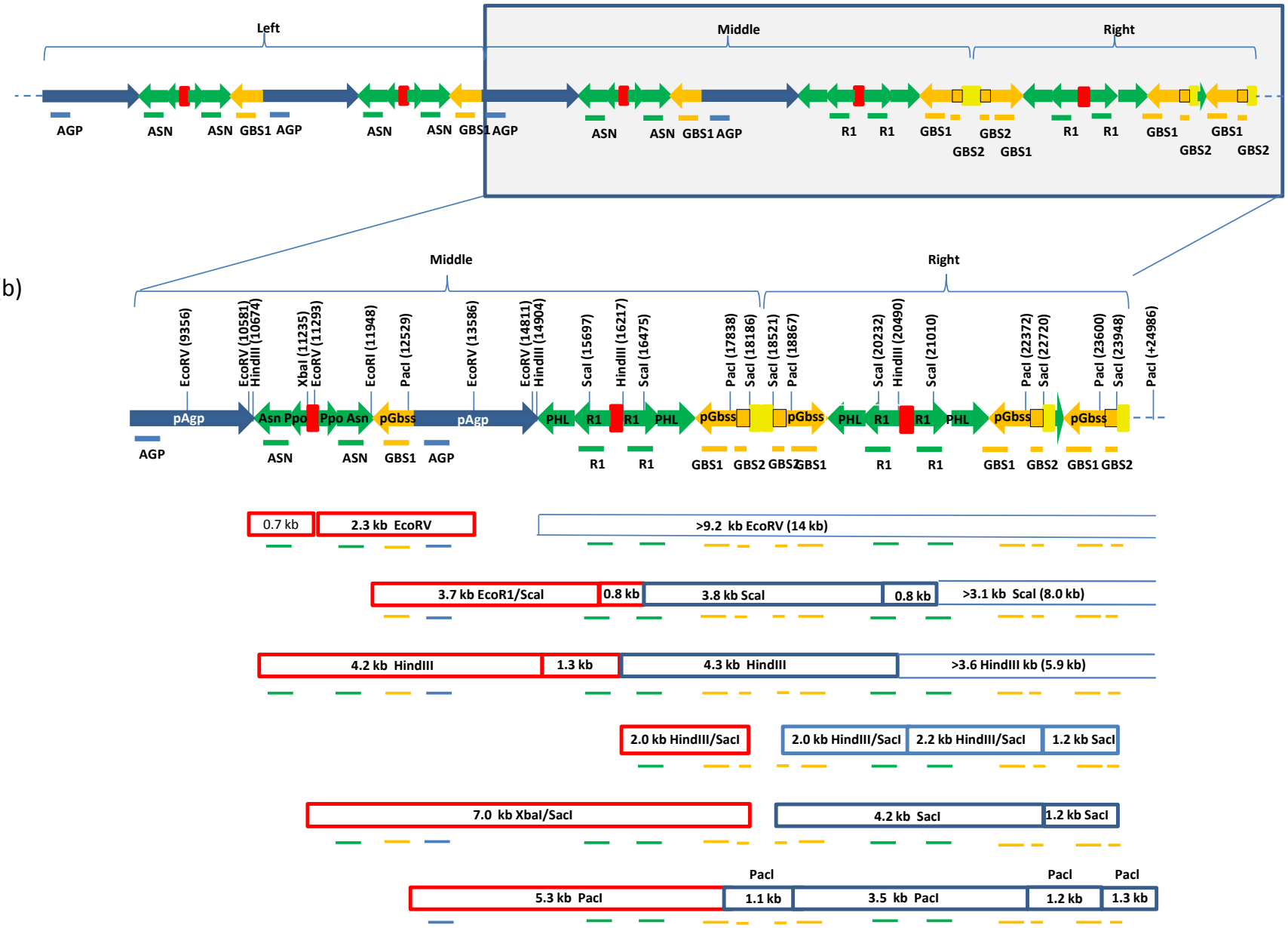

Left
Middle
Right
AGP
ASN
ASN GBS1 AGP
ASN
ASN GBS1 AGP
ASN
ASN GBS1 AGP
R1 R1
GBS1
GBS2
GBS2 GBS1
R1 R1
GBS1
GBS2
GBS1
GBS2
(b)
Middle
Right
EcoRV (9356)
EcoRV (10581)
HindIII (10674)
XbaI (11235)
EcoRV (11293)
EcoRI (11948)
PacI (12529)
EcoRV (13586)
EcoRV (14811)
HindIII (14904)
ScaI (15697)
HindIII (16217)
ScaI (16475)
PacI (17838)
SacI (18186)
SacI (18521)
PacI (18867)
ScaI (20232)
HindIII (20490)
ScaI (21010)
PacI (22372)
SacI (22720)
PacI (23600)
SacI (23948)
PacI (+24986)
pAgp
Asn Ppo
Ppo Asn
pGbss
pAgp
PHL
R1
R1
PHL
pGbss
pGbss
PHL
R1
R1
PHL
pGbss
pGbss
AGP
ASN
ASN
GBS1
AGP
R1
R1
GBS1
GBS2
GBS2
GBS1
R1
R1
GBS1
GBS2
GBS1
GBS2
0.7 kb
2.3 kb EcoRV
>9.2 kb EcoRV (14 kb)
3.7 kb EcoR1/ScaI
0.8 kb
3.8 kb ScaI
0.8 kb
>3.1 kb ScaI (8.0 kb)
4.2 kb HindIII
1.3 kb
4.3 kb HindIII
>3.6 HindIII kb (5.9 kb)
2.0 kb HindIII/SacI
2.0 kb HindIII/SacI
2.2 kb HindIII/SacI
1.2 kb SacI
7.0 kb XbaI/SacI
4.2 kb SacI
1.2 kb SacI
PacI
PacI
PacI
5.3 kb PacI
1.1 kb
3.5 kb PacI
1.2 kb
1.3 kb

Figure 5-4 legend. The upper figure in (a) and (b) represents the entire pSIM1278 insert containing the left, middle, and right sections. Underneath is an enlarged image of (a) the left and middle sections or (b) the middle and right sections, 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 provided in this petition. Red boxes denote internal bands (IB) associated with the original pSIM1278 DNA construct. Blue closed boxes indicate internal bands or 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 structure discussion

Our molecular studies led to the elucidation of the model for the pSIM1278 insert presented in Figure 5-4. As mentioned earlier, we have separated the structure into three sections (Left, Middle, and Right) and will describe the molecular data supporting each of those sections independently for simplicity and clarity. Although not all bands will be discussed, all of the digests and probes depicted in Figure 5-4 are represented in the Southern blots shown in Figures 5-6 through 5-15. In addition, the size of each digestion product shown in Figure 5-4 can be cross-referenced against the tables adjacent to the appropriate Southern blot.

Structure of the Left Section and Junctions

Based upon sequence information (Section 5.3 Flanking Regions of Inserted Sequences) we determined that the left side of the insert consists of a slightly truncated (35 bp) *Agp* promoter fused to Russet Burbank DNA, which contained useful restriction sites as described in Figure 5-4. These restriction sites predicted precise junction band sizes for the ScaI/EcoRI (4.1 kb) and XbaI (3.3 kb) digests, which were observed in gels hybridized with AGP and ASN probes (Figures 5-6 and 5-7 for AGP; Figures 5-8 and 5-9 for ASN). The expected sizes of the junction bands associated with the other three digests were not known; however, after extending the flanking sequence using the reference genome available from Michigan State University's repository, a HindIII site was predicted to exist ~2 kb upstream of the insert and result in a 4.2 kb junction band, which is what was observed (Figure 5-6).

Initial evidence that the left end of the insert consisted of a duplicated Asn1/Ppo5 silencing cassette came from the number and intensity of 4.2 kb bands in our Southern blots when W8 DNA was digested with the enzymes, HindIII, XbaI, PacI, and EcoRI/ScaI, and probed with AGP, ASN, and GBS1, which all hybridize to sequence within the 4.2 kb Asn1/Ppo5 silencing cassette. The 4.2 kb bands present in the EcoRI, HindIII, and XbaI digests were all consistent with tandem duplications of the silencing cassette and spanned the border between the two sections (Figure 5-4a). This arrangement and duplication was further corroborated by higher intensity 2.3 and 0.7 kb bands on EcoRV blots hybridized with the same set of probes (Figures 5-6, 5-8, and 5-11). The second copy of the 2.3 kb band covers the border between the left and middle sections to further establish the tandem arrangement. Lastly, the 4.2 kb band observed when the PacI digests were probed with the AGP, ASN and GBS1 probes (Figures 5-7, 5-9 and 5-12) strongly supports the tandem arrangement of two Asn1/Ppo5 silencing cassettes. Not only does it cross the junction between the left and middle sections, but it also supports the existence of a *Gbss* promoter at the junction between the two sections. The *Gbss* promoter is the only element in the pSIM1278 insert that contains a PacI restriction site, which is necessary to generate this 4.2 kb band containing ASN, AGP, and GBS1 hybridization sites (consider PacI digestion pattern in Figure 5-4).

The intensity of the 4.2, 2.3, and 0.7 kb bands described above appeared greater than expected for a structure including a simple tandem duplication of the Asn1/Ppo5 silencing cassette. We performed a

set of digests to determine the size of the left side of the insert and determine whether there were two or three copies of the Asn1/Ppo5 cassette on that side. The restriction enzymes, ScaI and BsrGI, both cut the insert exclusively in or adjacent to R1, leaving the entire left side of the insert intact. A known restriction site was identified in the flanking region for ScaI and another was predicted for MfeI by the reference genome at the insertion site. Thus, these two digests were capable of determining the size of the insert to the left of the internal R1 cassette. Southern blots probed with an ASN probe identified a single band, as expected. The sizes of the MfeI/BsrGI and ScaI digests were 17.6 and 16.3 kb, respectively (Figure 5-10). These sizes were both 4.2 kb larger than expected for an insert containing a single Asn1/Ppo5 cassette in the left section. This additional cassette does not alter the digestion patterns of any of our other digests due to the repetitive nature of the 4.2 kb duplication, but the third cassette identified by these digests does account for the higher than expected intensity of the bands corresponding to this region. Thus, these data support the tandem duplication of the Asn1/Ppo5 cassette represented in the left section of the model (Figure 5-4). We did not uncover any sequence differences among the pGbss-pAgp junctions within these repeats using PCR and Sanger sequencing.

Structure of the Middle Section: The middle section of the structure represents a complete pSIM1278 DNA insert except for a deletion of the T-DNA left border, originally adjacent to the *Agp* promoter (compare middle section with Figure 5-1). All of the expected internal bands shown as red boxes in Figure 5-4 were observed on the appropriate Southern blot (Figures 5-6 through 5-9 and Figures 5-11 through 5-15). These digests confirm the existence of a full copy of the pSIM1278 DNA insert in the middle section of our structure.

The EcoRV digests produced the high intensity 2.3 kb band already mentioned that exists in the left and middle sections and is detected by AGP, ASN, and GBS1 probes (Figures 5-6, 5-8, and 5-11). The 4.2 kb internal band generated by the HindIII digest was detected by the AGP, ASN and GBS1 probes, but appears as a high intensity band due to the presence of other 4.2 kb bands in those same digests as described in Figure 5-4. Similarly, the 0.8 kb and 1.3 kb internal bands generated by digestion with ScaI and HindIII, respectively, were detected by the R1 probe (Figure 5-13). Additionally, the 5.3 kb and 3.7 kb internal bands resulting from PacI and EcoRI/ScaI digests were detected by AGP, R1, and GBS1 probes. Lastly, the 7.0 kb internal band generated by a combined XbaI/Sac1 digest was detected by R1 probe (Figure 5-14).

Right Section and Junction: The right section of the structure is essentially an inverted repeat of the pPhl/pR1 silencing cassette adjacent to a *pGbss*-RB tandem repeat (Figure 5-4, right). The *pGbss*-RB tandem repeat was initially determined by identification and sequencing of the right junction (Section 5.3). In addition to the repeat and the right flanking sequence, these data revealed a small intervening region of sequence derived from the *Phl* promoter as shown by the small green arrow at the right side of Figure 5-4b. The flanking sequence also identified a nearby PacI restriction site as indicated in the structure.

The SacI and SacI/XbaI digests probed with the R1 probe were central to the elucidation of a second copy of the pPhl/R1 silencing cassette (Figure 5-14). The XbaI enzyme only cuts pSIM1278 DNA within the Asn1/Ppo5 inverted repeat, which results in a single, large right junction band (~18kb) when probed with R1. However, when XbaI is combined with SacI, we observe a 4.2 kb band, in addition to the 7.0 kb internal band, indicating the presence of a second copy of R1. This conclusion was corroborated by the presence of two appropriately sized R1 bands in the PacI digests (Figure 5-14). From the same set of gels, the 5.3 kb band corresponded to the internal band described in the previous section, whereas the 3.5 kb band corresponds to the second copy. Lastly, the numerous small, but distinct internal bands

ranging from 1.0 to 2.2 kb shown in Figure 5-4b were detected on the GBS1 and GBS2 blots (Figures 5-12 and 5-15).

Summary of pSIM1278 structure

The structural data presented support a single integration event for pSIM1278 where the structure contains multiple copies of the two silencing cassettes as depicted in Figure 5-4. The flanking sequences were identified and mapped to chromosome 2 as described in Section 5.3.

5.2.3 Structure of the pSIM1678 DNA insert

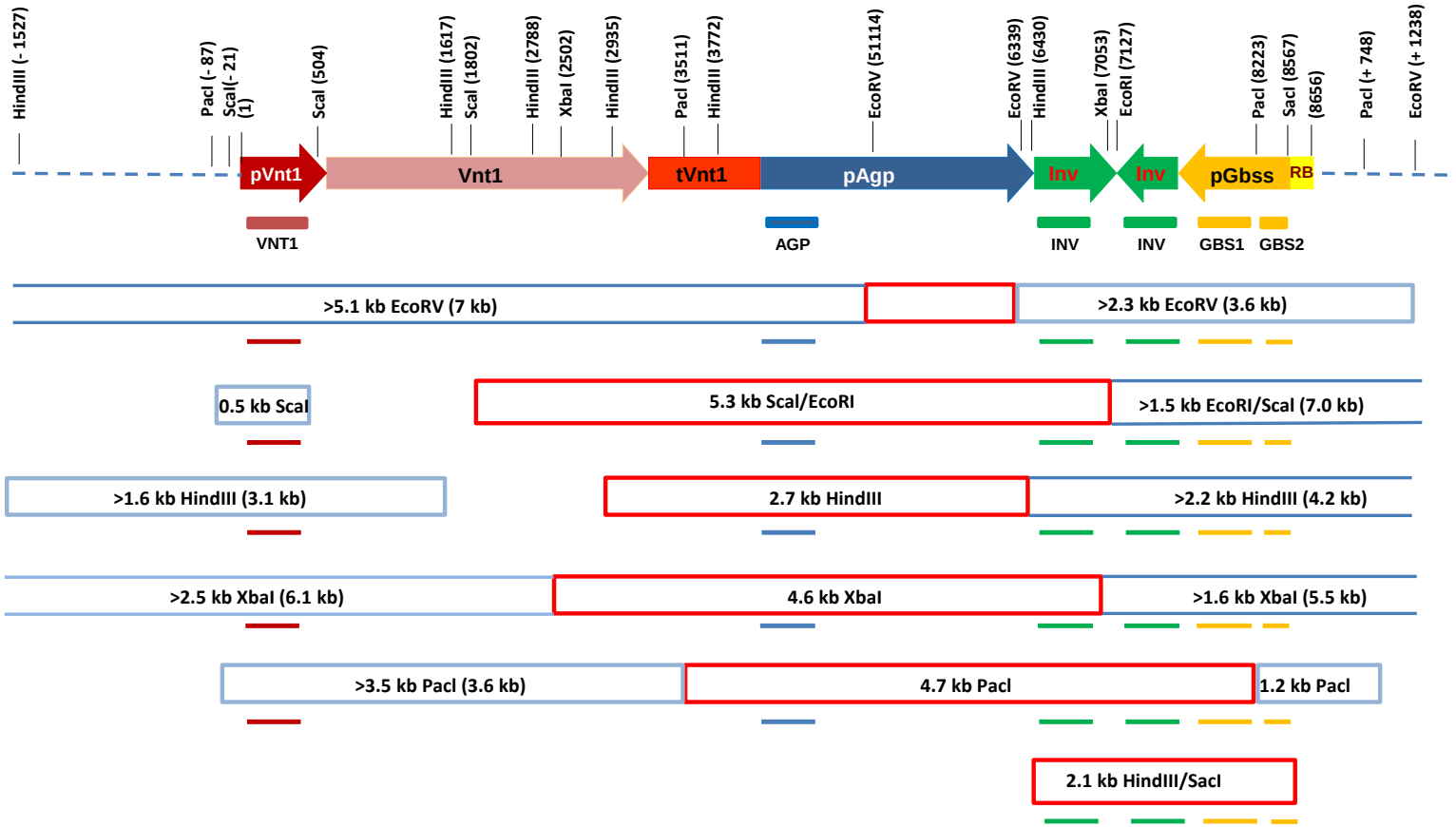
As established earlier, the pSIM1678 DNA insert integrated at a single location in the Russet Burbank genome. Identification of the flanking regions (Section 5.3) revealed a number of nearby restriction sites that were valuable in our Southern blot analyses and are depicted in Figure 5-5. Many of the same Southern blots already discussed for pSIM1278 were instrumental in elucidating the structure of the insert derived from the pSIM1678 plasmid as both DNA inserts contain *Agp* and *Gbss* promoter elements. The data did not suggest the inserted structure deviated from the original pSIM1678 DNA insertion except for a deletion of the T-DNA left border and a small piece (137 bp) of the *Rpi-vnt1* promoter (Figure 5-5).

The structure of the left junction, including the promoter truncation, was confirmed by the presence of the 0.5 kb ScaI and 3.1 kb HindIII (Figure 5-16) bands and the 3.6 kb PacI band (Figure 5-17) detected using the VNT1 probe. All three junction bands involved restriction sites predicted by the left flanking sequence. As expected, only a single junction band was identified for each of the digests probed by VNT1. The internal bands depicted in Figure 5-5 by red boxes were all identified by the appropriate probes suggesting integrity of the internal region (Figures 5-6, 5-7, 5-18, and 5-19).

Consistent with a single integration event, we observed a single right junction band for each of the digests described in Figure 5-5, including 1.2 kb PacI band (GBS2 probe, Figure 5-15) and a 3.6 kb EcoRV band (GBS1 and INV probes; Figures 5-11 and 5-19) bands as predicted by the flanking sequence at the right junction. The 2.1 kb internal HindIII/SacI band detected by the GBS2 probe confirmed the integrity of the *VInv* inverted repeat and adjacent *Gbss* promoter (Figure 5-15).

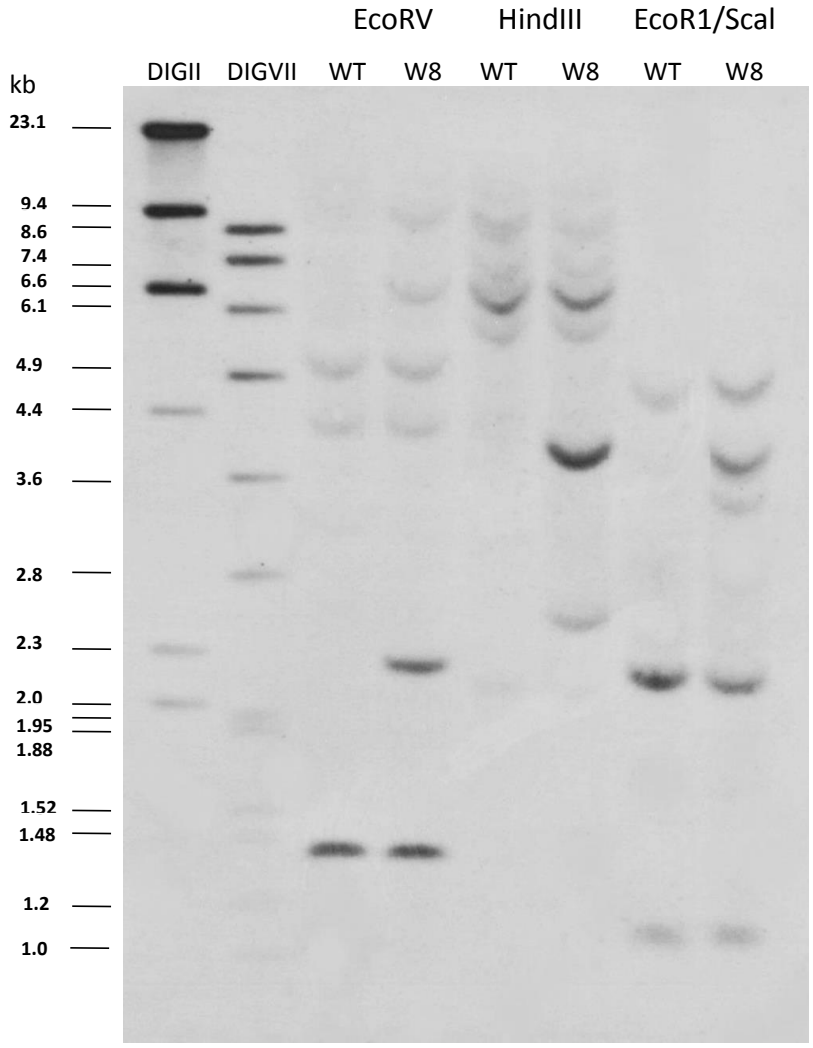
Collectively, our data are consistent with a single copy and insertion of the pSIM1678 DNA insert with a deletion of the left border and a small region of adjoining promoter sequence.

Figure 5-5. Structure of pSIM1678 DNA Insert Digestion and Hybridization Pattern



The upper figure represents the structure of the insert associated with the pSIM1678 plasmid, 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 provided in this petition. Red boxes denote internal bands (IB) associated with the original pSIM1678 DNA construct. Blue closed boxes indicate internal bands or 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.

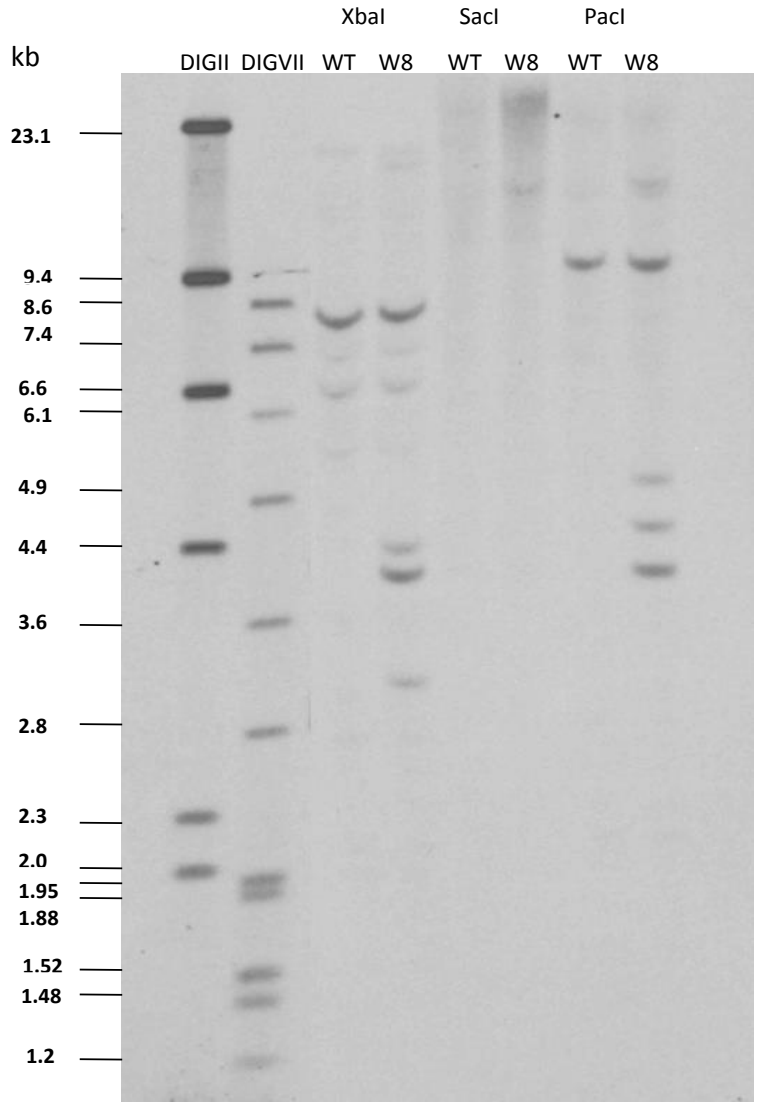
Figure 5-6. Russet Burbank DNA Hybridization with the AGP Probe



EcoRV	HindIII	EcoRI / Scal
JB 10	OB 9	
JB 7	OB 7.5 OB 7.0 OB 6.0	IB 5.3
OB 5.3		OB 5.1
OB 4.4	IB 4.2 IB 4.2 IB 4.2 JB 4.2	IB 4.2 IB 4.2 JB 4.2 IB 3.8
IB 2.3 IB 2.3 IB 2.3	IB 2.7	
		OB 2.2
OB 1.4		
		OB 1.1

Genomic DNA of Russet Burbank control (WT) and event W8 were 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

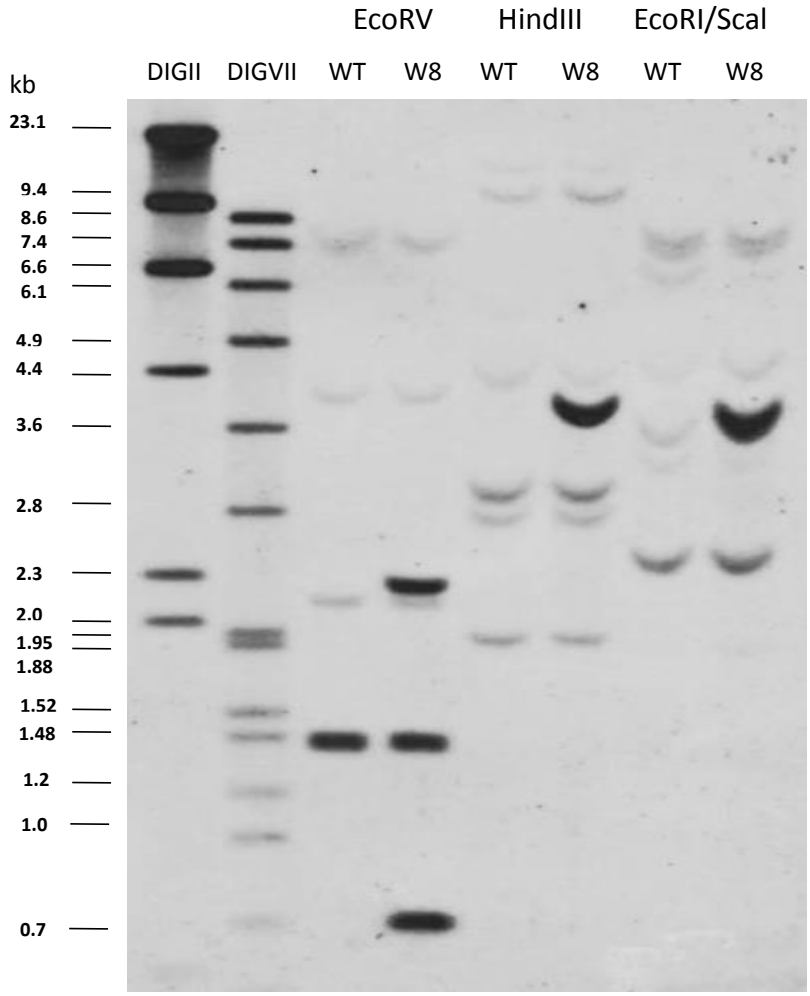
Figure 5-7. Russet Burbank DNA Hybridization with the AGP Probe



XbaI	SacI	PacI
	JB 25	OB 23
OB 20		
JB 18		JB 15
	JB 18	OB 14
		OB 12
OB 8.2		
OB 7.4		
OB 6.7		
		IB 5.3
IB 4.6		IB 4.7
IB 4.2		IB 4.2
		IB 4.2
JB 3.3		

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with XbaI, Sac1, and PacI 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

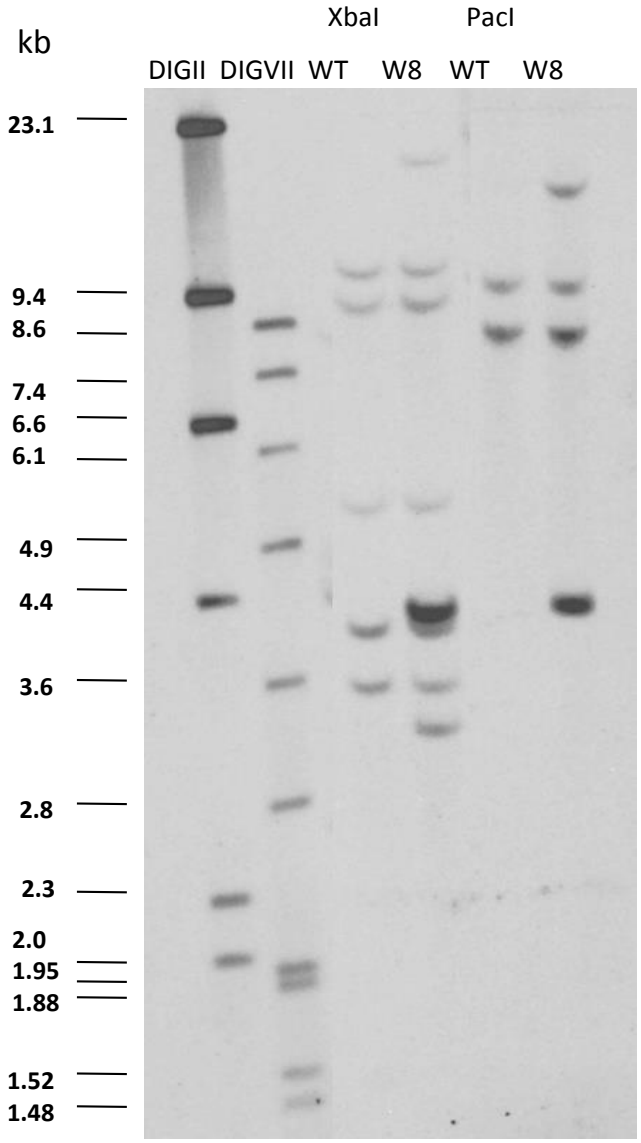
Figure 5-8. Russet Burbank DNA Hybridization with the ASN Probe



EcoRV	HindIII	EcoRI / ScaI
	OB 10	
OB 8		OB 8
		OB 7.5
		OB 6.5
	OB 4.5	OB 4.8
OB 4.3	IB 4.2	OB 4.2
	IB 4.2	IB 4.2
	IB 4.2	IB 4.2
	OB 3.1	JB 4.2
	OB 2.8	OB 3.5
IB 2.3		
IB 2.3		OB 2.5
IB 2.3		
OB 2.2	OB 1.9	
OB 1.4		
IB 0.7		
IB 0.7		
IB 0.7		

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the 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 three groups based on the structure of the DNA insert: original endogenous bands (**OBs,** in green), internal bands (**IBs**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. All molecular weights are presented in kilobases.

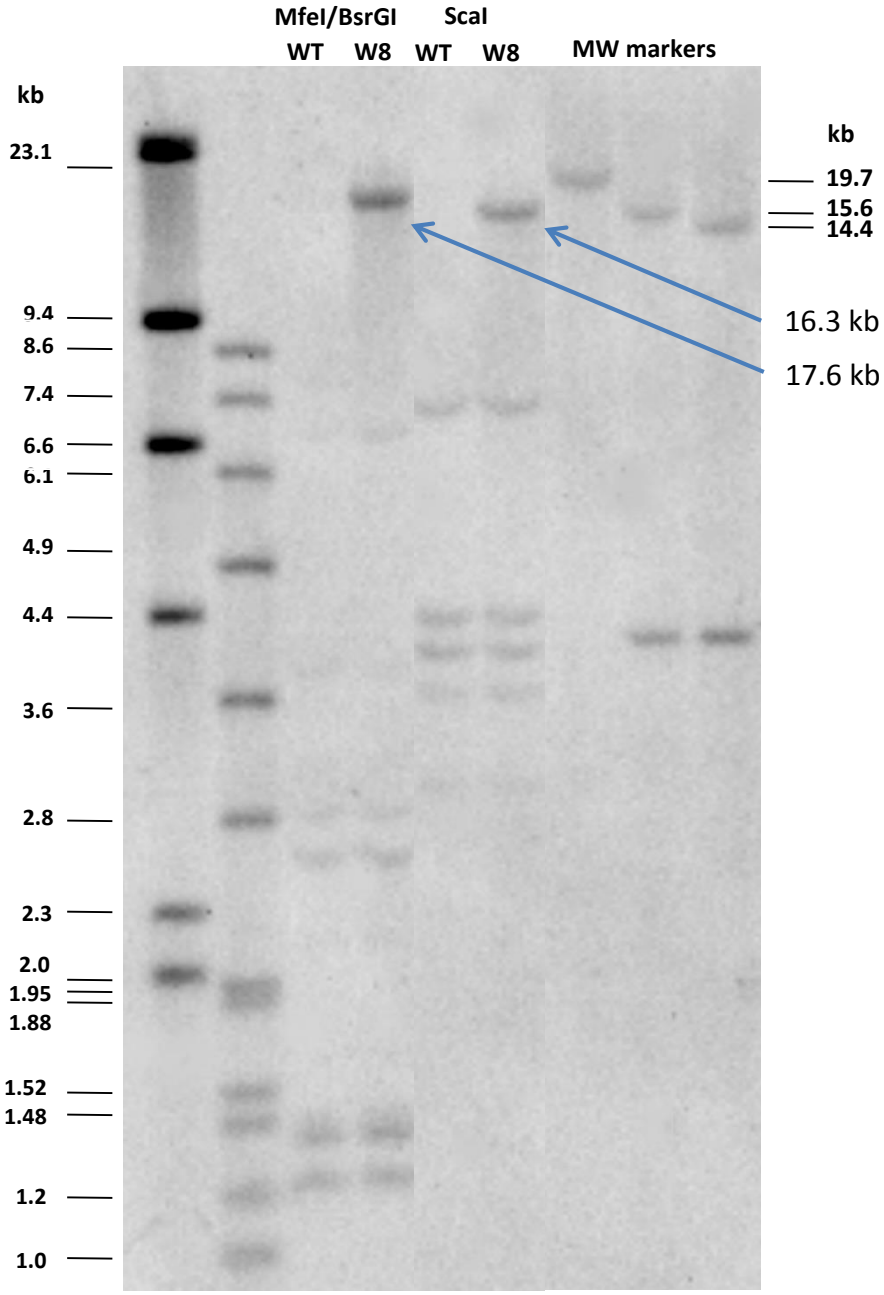
Figure 5-9. Russet Burbank DNA Hybridization with the ASN Probe



XbaI	PacI
JB 18	JB 15
OB 12	
OB 9.0	OB 11
	OB 8.0
OB 5.3	
IB 4.2	IB 4.2
IB 4.2	IB 4.2
OB 4.0	
OB 3.6	
JB 3.3	

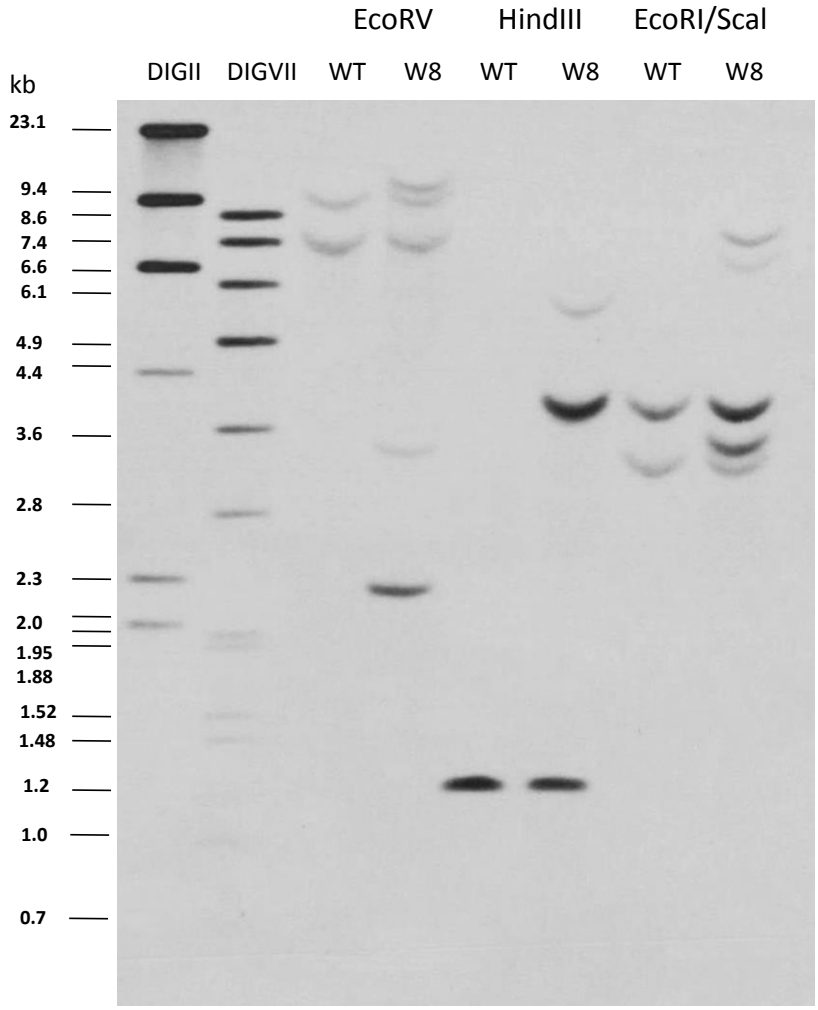
Genomic DNA of Russet Burbank control (WT) and event W8 were digested with XbaI and PacI and hybridized with the ASN probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. The estimated sizes of bands are summarized in the table and classified into three groups based on the structure of the DNA insert: original endogenous bands (**OBs,** in green), internal bands (**IBs**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. All molecular weights are presented in kilobases.

Figure 5-10. Russet Burbank DNA Hybridization with the ASN Probe



Genomic DNA of Russet Burbank control (WT) and event W8 were digested with MfeI/BsrGI and ScaI and hybridized with the ASN probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image on the left. pSIM1278 digests were used as additional size markers and are shown at the right with their sizes indicated in kb. The sizes of the two digests are indicated with the observed sizes 4.2kb greater than expected for a single Asn1/Ppo5 cassette in the left section. All molecular weights are presented in kilobases.

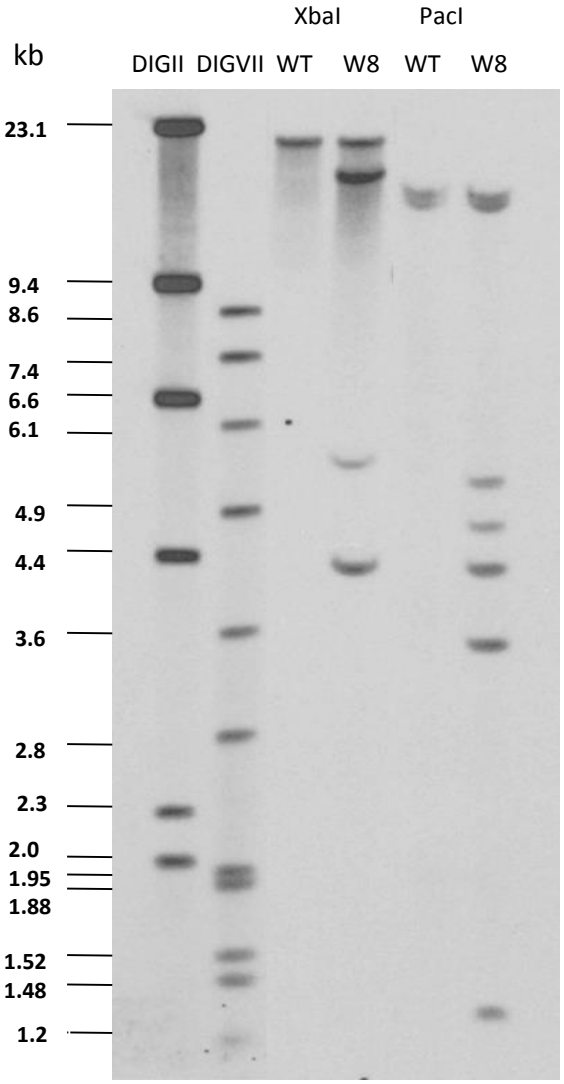
Figure 5-11. Russet Burbank DNA Hybridization with the GBS1 Probe



EcoRV	HindIII	EcoRI / ScaI
JB 14		
OB 9.5		JB 9.0
OB 8.0		JB 7.0
	JB 6.0	
	IB 4.3	
	IB 4.2	OB 4.2
	IB 4.2	IB 4.2
JB 3.6	IB 4.2	IB 4.2
	JB 4.2	IB 3.8
		IB 3.7
		OB 3.5
IB 2.3		
IB 2.3		
IB 2.3		
	OB 1.3	

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the GBS1 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

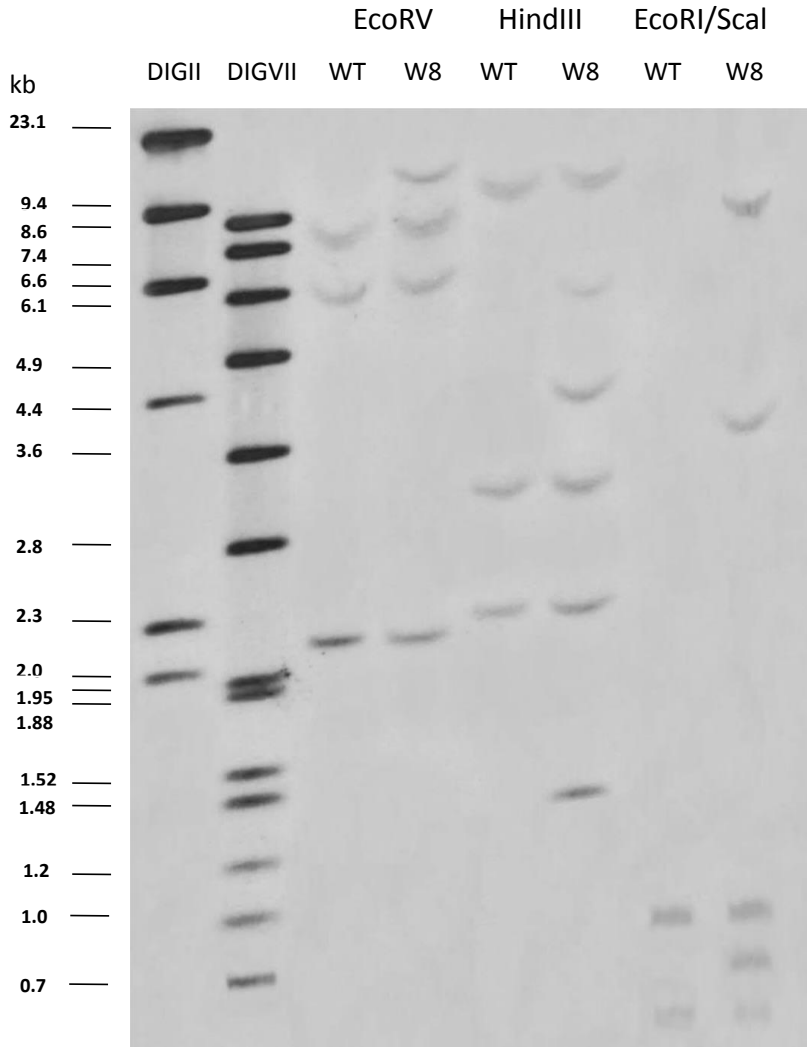
Figure 5-12. Russet Burbank DNA Hybridization with the GBS1 Probe



XbaI	PacI
OB 20	
JB 18	
	OB 16
	OB 15
	JB 15
JB 5.5	
	IB 5.3
IB 4.2	IB 4.7
IB 4.2	IB 4.2
	IB 4.2
	IB 3.5
	IB 1.3

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with XbaI and PacI and hybridized with the GBS1 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

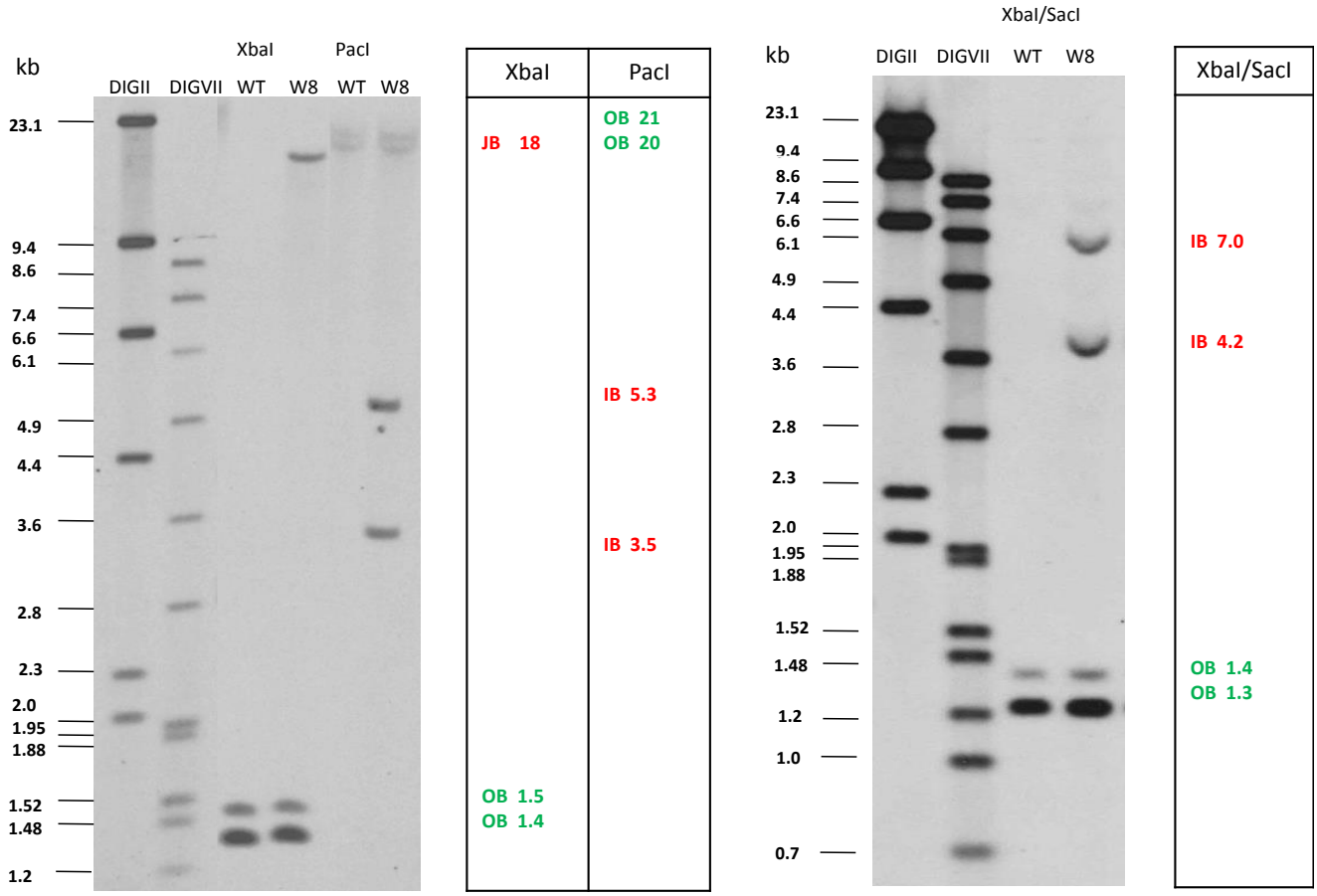
Figure 5-13. Russet Burbank DNA Hybridization with the R1 Probe



EcoRV	HindIII	EcoRI / ScaI
JB 14	OB 10	JB 9.0
OB 8	JB 6.0	IB 3.8
OB 6.3	IB 4.3	IB 3.8
OB 2.2	OB 3.2	OB 1.0
	OB 2.3	IB 0.8
	IB 1.3	IB 0.8
		OB 0.5

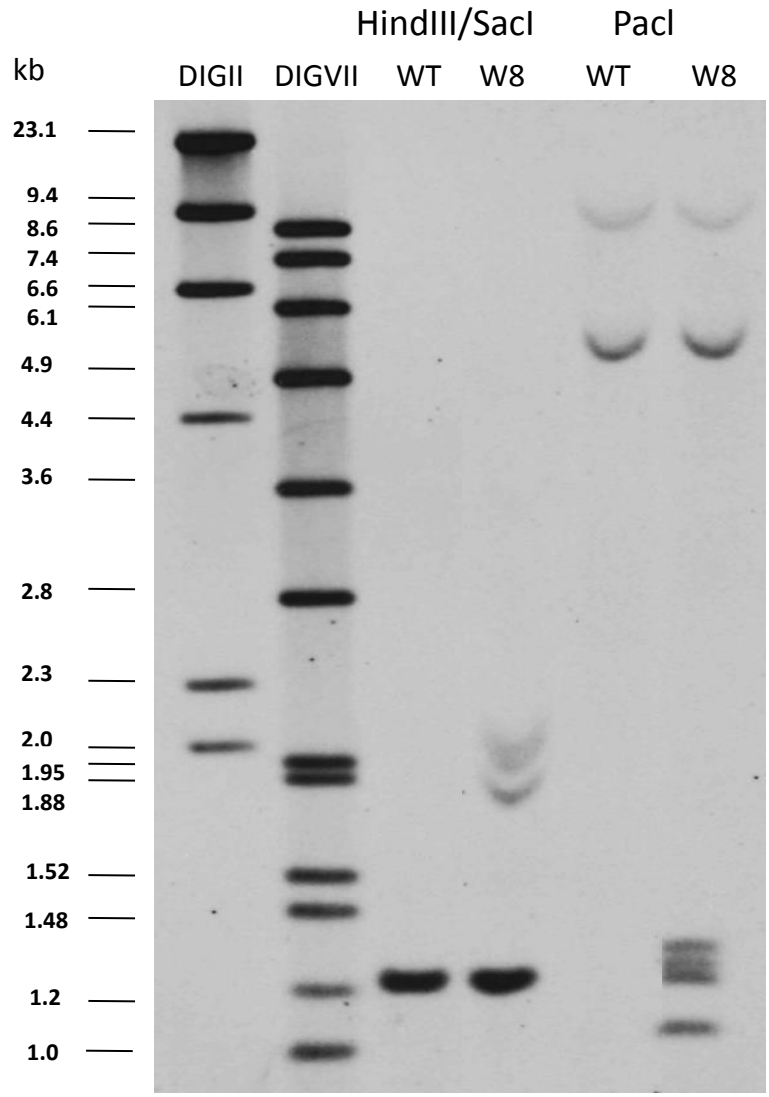
Genomic DNA of Russet Burbank control (WT) and event W8 were digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the R1 probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. The estimated sizes of bands are summarized in the table and classified into three groups based on the structure of the DNA insert: original endogenous bands (**OBs,** in green), internal bands (**IBs**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. All molecular weights are presented in kilobases.

Figure 5-14. Russet Burbank DNA Hybridization with the R1 Probe



Genomic DNA of Russet Burbank control (WT) and event W8 were digested with XbaI, PacI, and XbaI/Sac1 and hybridized with the 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), internal bands (**IBs**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. All molecular weights are presented in kilobases.

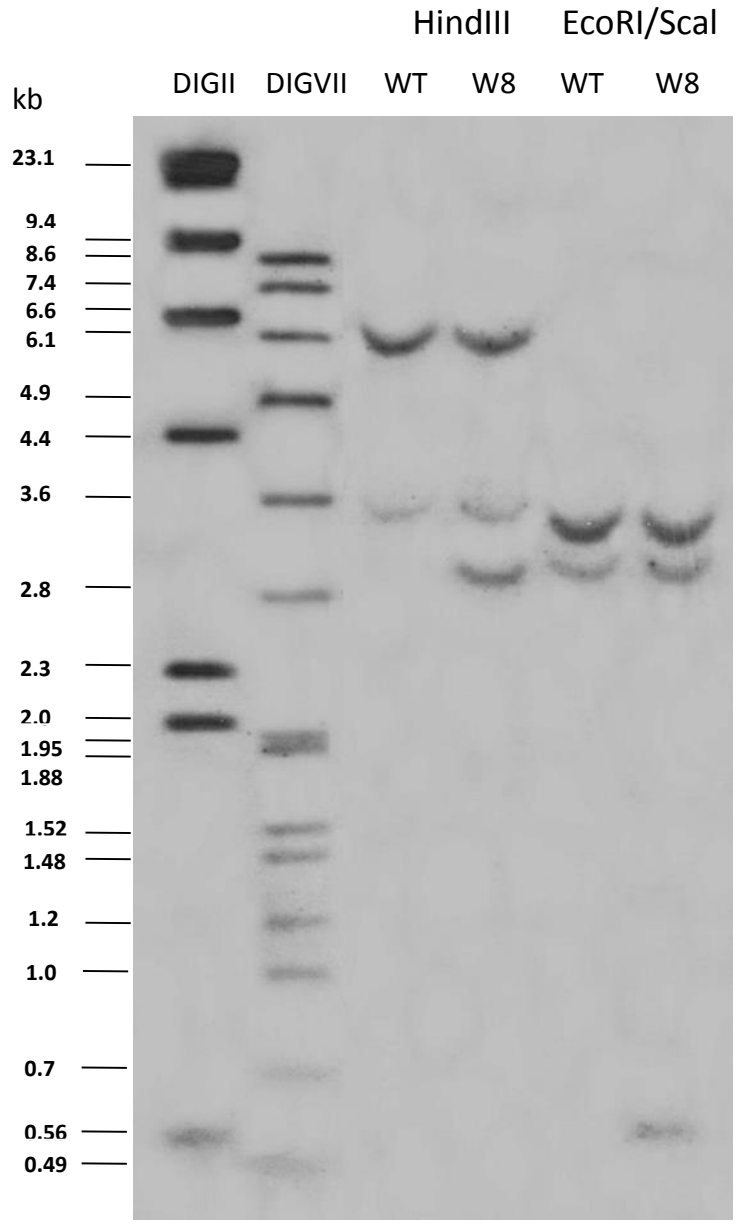
Figure 5-15. Russet Burbank DNA Hybridization with the GBS2 Probe



HindIII/SacI	PacI
	OB 10
	OB 5.5
IB 2.2 kb	
IB 2.1 kb	
IB 2.0 kb	
IB 2.0 kb	
IB 1.2 kb	JB 1.3 kb
OB 1.2 kb	IB 1.2 kb
	JB 1.2 kb
	IB 1.1 kb

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with HindIII/Sac1 and PacI and hybridized with the GBS2 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1278 insert are in red. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

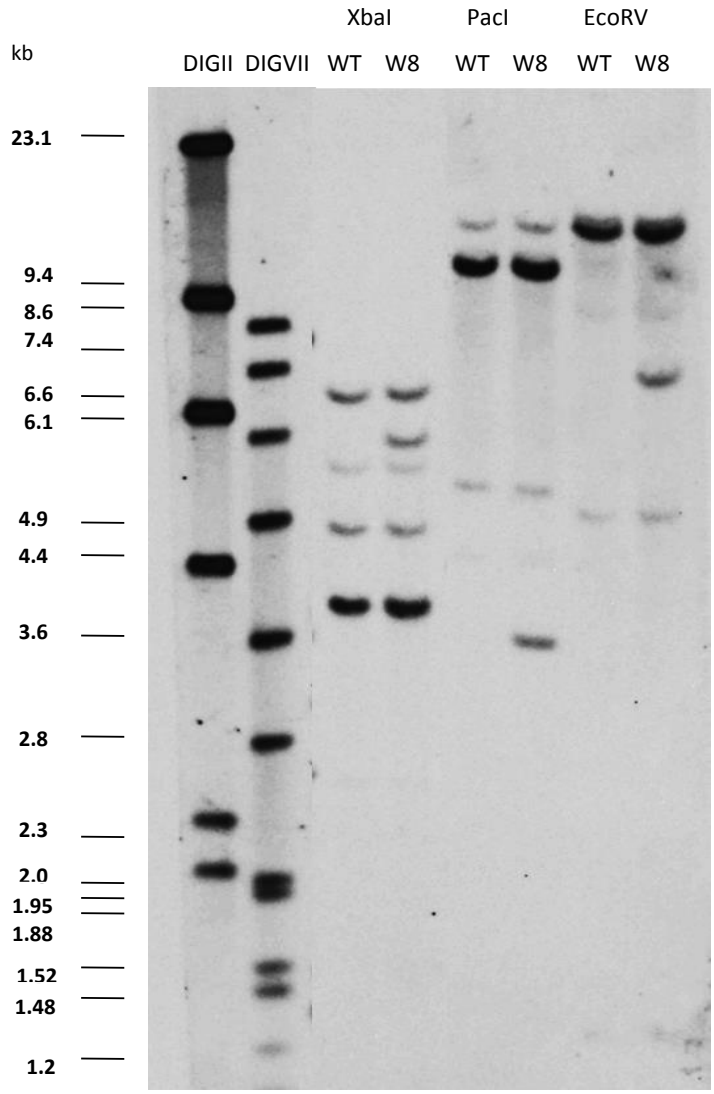
Figure 5-16. Russet Burbank DNA Hybridization with the VNT1 Probe



HindIII	EcoRI / ScaI
OB 6.6	
0B 3.6	OB 3.5
JB 3.1	OB 3.2
	JB 0.5

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with HindIII and EcoRI/ScaI and hybridized with the VNT 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

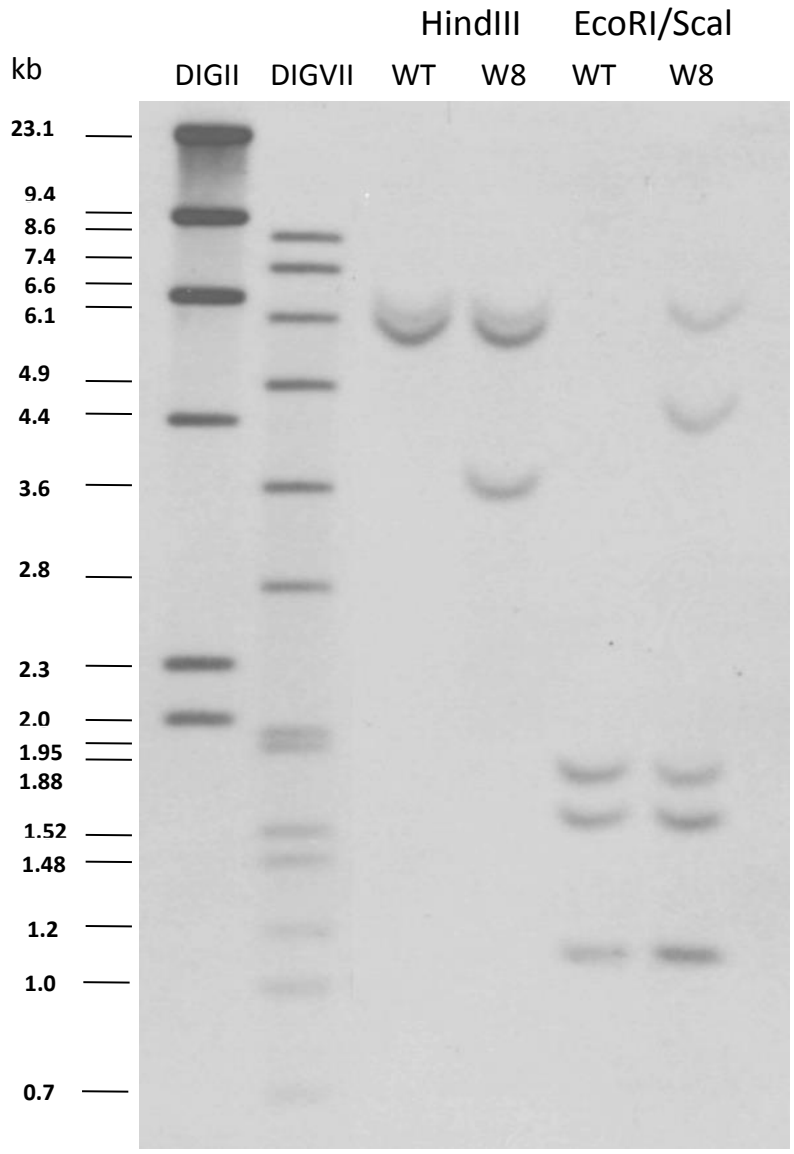
Figure 5-17. Russet Burbank DNA Hybridization with the VNT1 Probe



XbaI	PacI	EcoRV
	OB 15 OB 13	OB 15
OB 6.7		JB 7
JB 6.1 OB 5.5	OB 5.2	OB 4.9
OB 4.8		
OB 4.0	JB 3.6	

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with HindIII/Sac1 and PacI and hybridized with the VNT 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

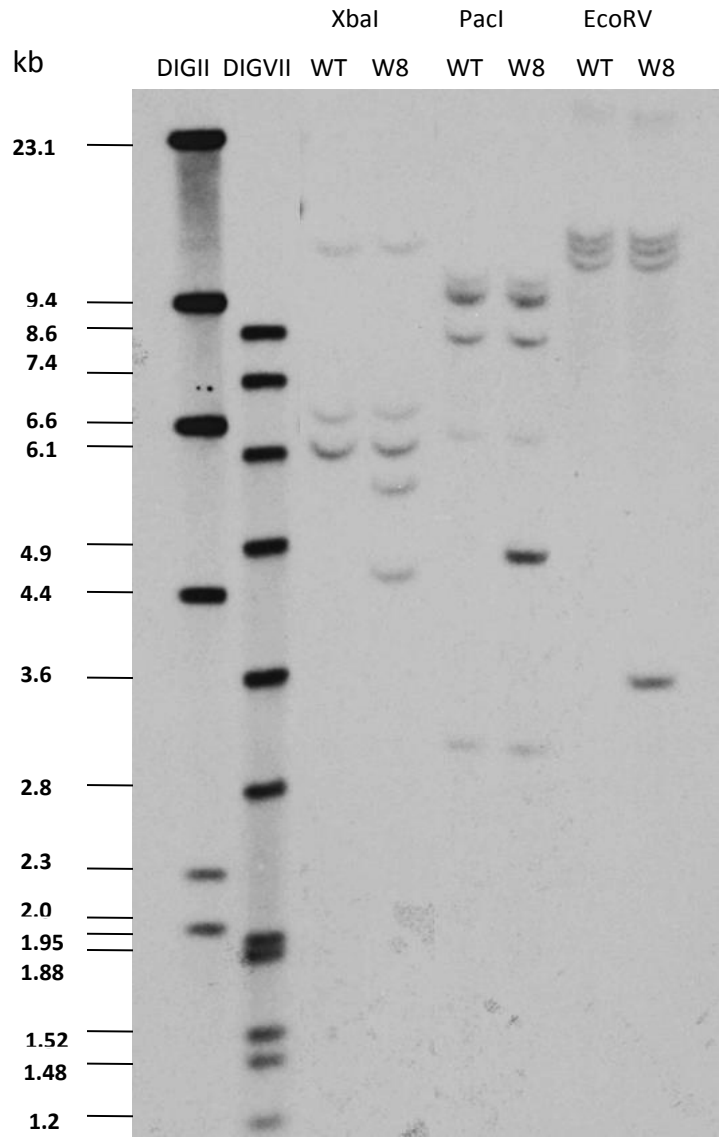
Figure 5-18. Russet Burbank DNA Hybridization with the INV Probe



HindIII	EcoRI / ScaI
OB 6.6 OB 6.1	JB 7.0
	IB 5.3
JB 4.2	
	OB 1.8 OB 1.6
	OB 1.1

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with HindIII and EcoRI/ScaI and hybridized with the INV 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

Figure 5-19. Russet Burbank DNA Hybridization with the INV Probe



XbaI	PacI	EcoRV
		OB 14
OB 13	OB 11	OB 13
	OB 10	OB 12
	OB 8.5	
OB 6.7		
OB 6.2	OB 6.3	
JB 5.5		
	IB 4.7	
IB 4.6		
		JB 3.6
	OB 3.1	

Genomic DNA of Russet Burbank control (WT) and event W8 were digested with XbaI, PacI and EcoRV and hybridized with the INV 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**) and the junction bands (**JBs)**. The DNA fragments of pSIM1678 are in blue. All molecular weights are presented in kilobases.

5.2.4 Conclusions: Copy number and structure of the DNA Inserts

Genetic and structural characterization of the inserts associated with transformation of Russet Burbank by pSIM1278 and pSIM1678 to produce event W8 showed that both transformations resulted in a single integration site for each plasmid. The structure of the DNA derived from transformation of pSIM1278 was complex (Figure 5-4) relative to the structure of the original insert. The inserted DNA appears to have undergone rearrangement during transformation resulting in a structure consisting of a tandem repeat of the Asn1/Ppo5 silencing cassette, followed by a nearly complete pSIM1278 construct, and an inverted repeat containing a duplication of the pR1/pPhl silencing cassette and a tandem duplication of the *Gbss* promoter with intervening Phl sequence (Figure 5-4).

Although this structure is more complicated than anticipated, the duplicated silencing cassettes are intact and remain under the control of the tissue-specific promoters. The structure does not negatively impact safety or trait efficacy of the product (See Chapter 9: Safety of Russet Burbank W8 Potato and Chapter 10: Trait Efficacy).

W8 also contains a single copy of the DNA from pSIM1678 that resides at a single locus of integration (Figure 5-5). The DNA insert of pSIM1678 contains a nearly intact DNA insert with a 330-bp deletion, which removes the entire T-DNA left border and 137-bp of the *Rpi-vnt1* promoter. This small deletion in the promoter does not affect the gene's ability to confer late blight resistance (Chapter 10: Trait Efficacy). Also, RNA expression associated with the *Rpi-vnt1* gene has been demonstrated using RT-PCR, which is included in Chapter 6, Section 6.3: *Rpi-vnt1* Gene Expression.

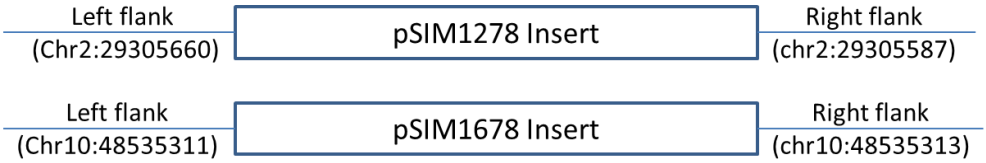
5.3 Flanking Regions of Inserted Sequences

As described in the previous section, each construct was integrated at a single locus in the Russet Burbank genome. We identified the flanking sequence for each construct using standard molecular approaches (see Appendix A: Genetic, Molecular, and Biochemical Methods for details) and aligned them to the reference genome sequence using the genomic resources available through Michigan State University's Plant Biology Group.

The reference genome is based upon sequencing a unique homozygous, doubled monoploid, form of the potato developed using tissue culture techniques (Potato Genome Sequencing Consortium, 2011) and was later integrated with sequences from a heterozygous diploid line. Together, these efforts have provided a reference genome that is valuable for investigating the evolution and genome organization of potatoes, but is still lacking for detailed analysis of individual loci in commercial cultivars, which are mostly tetraploid. Although this reference genome can be valuable, many differences between it and Russet Burbank have been identified through the course of our work, and reliance upon it should be undertaken with caution.

Based upon the reference genome assembly, the pSIM1278 construct appears to have been inserted on chromosome 2, whereas the pSIM1678 construct was mapped to a location on chromosome 10 (Figure 5-20).

Figure 5-20. Predicted Chromosomal Insertion Sites for each Insert



Among the evidence supporting safety relative to the insertion site are the following: (1) most insertions do not disrupt gene expression because DNA integrates at random when no selection is used during transformation (Kim *et al.* 2007) and only approximately 5.5% of the potato genome is occupied by genes (Zhu *et al.* 2008); (2) insertions that do disrupt native potato gene functions are almost always recessive (Hagio *et al.* 2002; Chiou *et al.* 2006; Daxinger *et al.* 2008), which means that disruption of a single copy of the native gene would be unlikely to result in an observable phenotype; and (3) if an unusual and undesirable phenotype occurred, that event would be selected only if equivalent or superior to the untransformed potato variety. As discussed in Chapter 9: Safety of the W8 Potato, there is no evidence suggesting these insertions have disrupted any native Russet Burbank genes or introduce any safety concerns related to generation of allergens or toxins.

5.4 Absence of Plasmid Backbone Sequence

The W8 event was developed by transforming Russet Burbank with two constructs in series, pSIM1278 followed by pSIM1678. Only the potato-derived transfer DNA sequence located between the left and right border sequences is designed to be inserted into the potato genome, but not the plasmid backbone sequences (Chapter 4: Donor Genes, Figures 4-1 and 4-2; grey highlighting). As the backbone sequence of the two plasmids is identical, molecular evidence showing the absence of backbone sequence simultaneously confirms its absence from both transformations.

The following methods were used to establish that backbone portions of the plasmid were not present in events developed for commercial purposes: 1) If plants had phenotypes associated with the negative selectable isopentenyl isomerase (*ipt*) marker gene in the plasmid backbone, they were discarded; 2) Absence of the backbone DNA was confirmed with Southern blot hybridization; 3) PCR was used to confirm no fragments of the backbone DNA were present. Our results indicate that the W8 event does not contain backbone sequence as described below.

5.4.1 Selection of Backbone-free Plants

Agrobacterium-mediated transformation often results in transfer of plasmid backbone DNA, in addition to the intended DNA positioned between the left and right borders (LB and RB) of the plant-derived DNA insert. The frequency of transfer of backbone DNA has been estimated at 75% for tobacco (Kononov *et al.* 1997) and 47% - 67% for *Arabidopsis* (Oltmanns *et al.* 2010). To reduce the number of transformants that must be characterized molecularly, we employed a phenotypic screen for plants that contain the Agrobacterium *ipt* gene, which is present in the plasmid 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. The Russet Burbank W8 event 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. Additional details regarding the transformation process and selection method can be found in Chapter 3: Transformation Method.

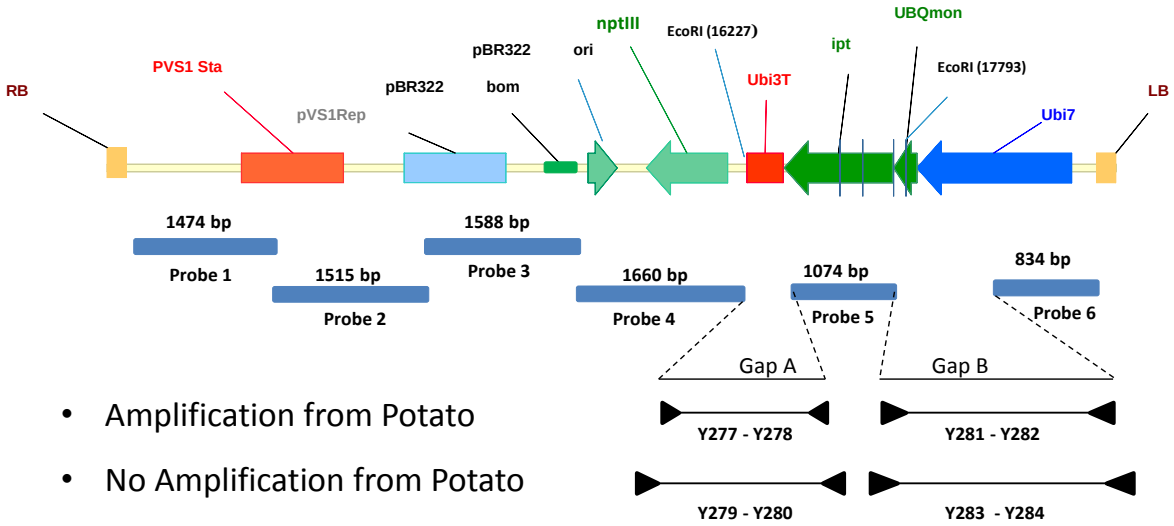
5.4.2 Molecular Evidence for Backbone-free Plants

A detailed molecular characterization of W8 genomic DNA was undertaken to show the absence of backbone DNA using a combination of Southern blot and PCR-based analyses.

Absence of backbone DNA in W8 using Southern blotting methods
A series of Southern probes were designed to span the length of the plasmid backbone for detection of any backbone DNA residing in the genome of the transformed potatoes (Figure 5-21). There were two gaps, Gap A and Gap B, not detected by the probe set, but were instead analyzed by PCR. Since the absence of DNA is based upon a lack of detection or amplification, a positive control (T130) transformant was developed, which contains an integrated copy of the entire plasmid backbone.

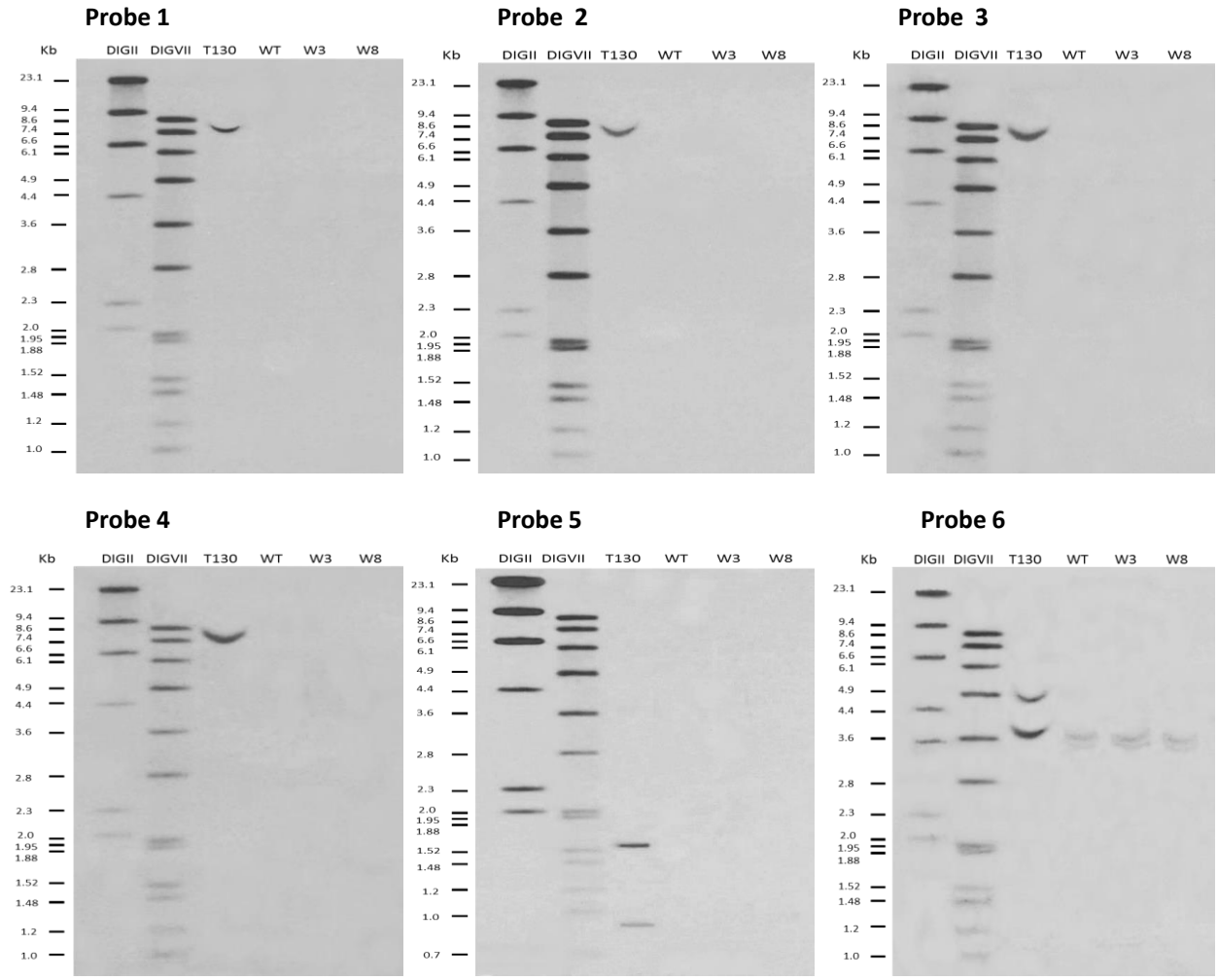
Genomic DNA isolated from W8, T130, and Russet Burbank controls (WT) were digested with EcoRI and probed with each of the six probes. As shown in Figure 5-22, bands were limited to the positive control (T130) samples in all Southern blots, except for probe 6, which contained a pair of bands common between the Russet Burbank control and W8. Backbone would only be confirmed in an event if there were unique bands associated with the positive controls and not found in the untransformed controls. Thus, these bands are not associated with backbone DNA, and all Southern blots are consistent with a lack of backbone DNA in the W8 genome.

Figure 5-21. Probes for the backbone of pSIM1278 and pSIM1678



[1]Numbering system based on pSIM1278 (Chapter 4: Donor Genes, Table 4-1 and Figure 4-1), but backbone sequences are identical to pSIM1678. The backbone DNA is spanned by the probes shown as blue rectangles with two small gaps labeled as Gap A and Gap B detected by PCR using the indicated primer sets. The gapped regions exist in the potato genome and can be amplified using the internal primer sets, while the flanking set of primers is unique to the backbone DNA.

Figure 5-22. Analysis of Plasmid Backbone DNA using Southern Blotting

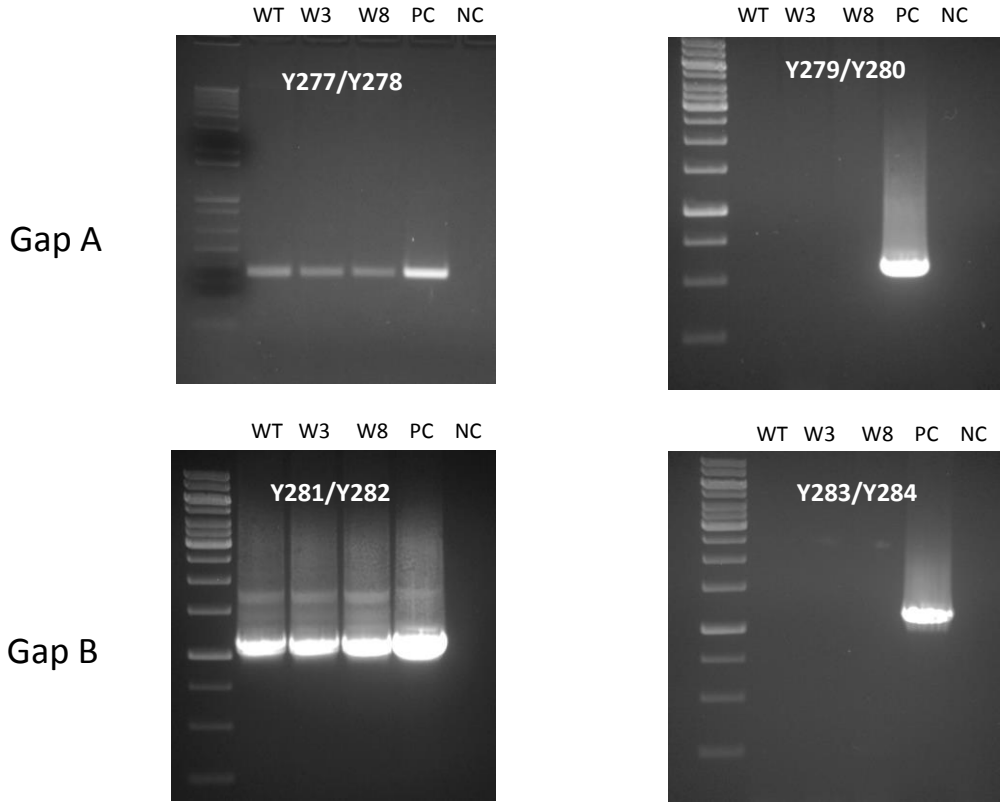


Genomic DNA was digested with EcoRI and analyzed by Southern blot using probes 1-6. WT = Russet Burbank control, W3 = additional event that was not submitted, W8 = Event W8, T130 = positive control containing backbone DNA. Lanes 1 and 2 are molecular weight markers (DIG II, and DIGVII, respectively) with sizes indicated next to gel.

Absence of backbone DNA in W8 using PCR-based methods

The two gapped regions not covered by Southern probes were independently characterized using a set of PCR assays. First, PCR was performed using a set of primers that hybridized fully within the Gap A (Y277-Y278) and Gap B (Y281-Y282) regions (Figure 5-21). Each of these primer sets successfully amplified sequence corresponding to the gapped regions (Figure 5-23, left panel of gels), but the sequence corresponded to the Russet Burbank genome, not to the plasmid backbone. Sequence comparisons showed that the sequence of the W8 and Russet Burbank PCR products were identical, but nucleotide polymorphisms distinguished these sequences from the backbone DNA and T130. Further evidence for the absence of these gapped regions in W8 was obtained by a second set of PCR using primers specific to the flanking regions, just outside of the gaps (Y279-Y280, Gap A; Y283-Y284, Gap B; Figure 5-21). Unlike the internal gap-specific primers, these primers do not amplify Russet Burbank sequence (WT) as shown in Figure 5-23 (right panel of gels), but do amplify the gapped regions from the plasmid backbone as shown in the positive control (PC) sample. Importantly, these primers did not detect backbone in the W8 samples.

Figure 5-23. PCR Amplification of Gapped Regions in Plasmid Backbone



Ethidium bromide stained agarose gels for the PCR reactions using the primer sets indicated. PC = positive control, Lane 1 = 100-bp DNA marker (Invitrogen), WT = Control Russet Burbank, W3 = Additional event that was not submitted, W8 = Submitted Event, PC = Positive control, NC = Negative control.

Another PCR assay was used to verify the absence of sequence containing the junction between backbone and the DNA insert from either pSIM1278 or pSIM1678. As diagrammed in Figure 5-24, each junction was tested using two sets of primers. Since the plasmids have identical right junctions, its presence was tested using a shared set of PCR primers (JY725-JY726, JY915-JY749). The left junction was tested using two sets of primers that were specific to either pSIM1278 or pSIM1678. All of the PCR reactions failed to identify junction regions in either W8 or WT samples, whereas in each case positive controls amplified as expected (Figure 5-25). Thus, the backbone DNA adjacent to the left and right border regions was not introduced into W8 for either transformation. These findings are further supported by the lack of backbone DNA within the flanking sequences for each insert (Section 5.3).

Figure 5-24. PCR Primer Sets for Detection of Backbone Junction Sites

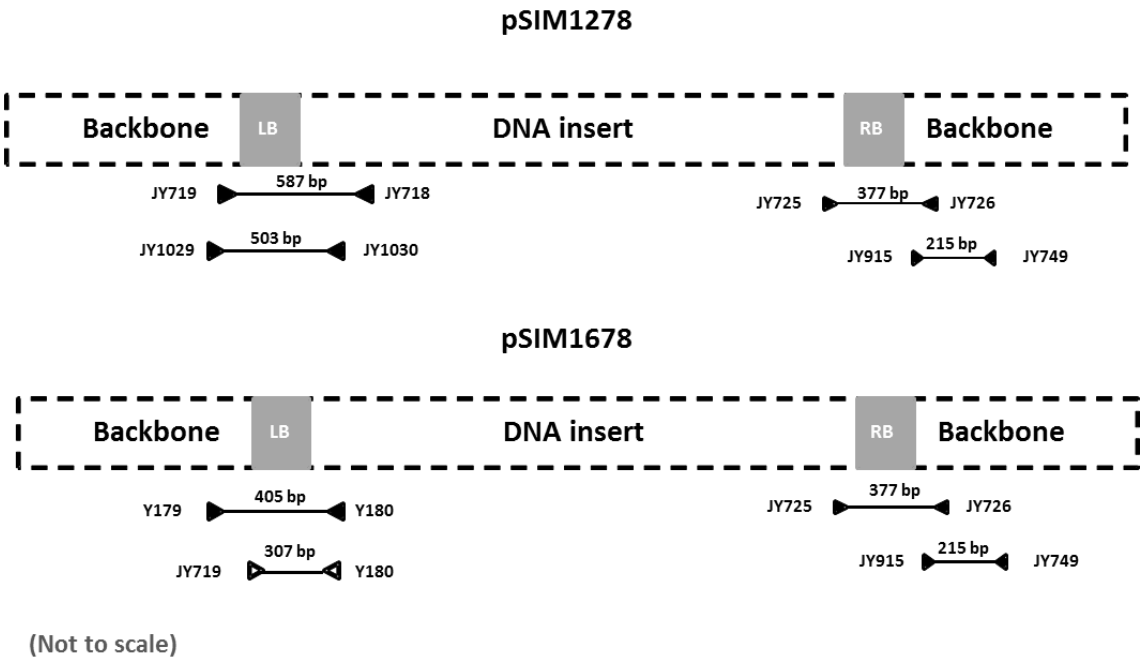
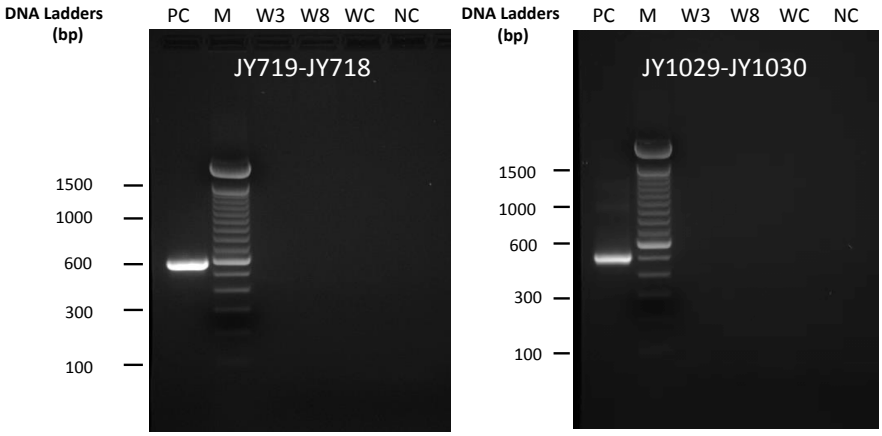
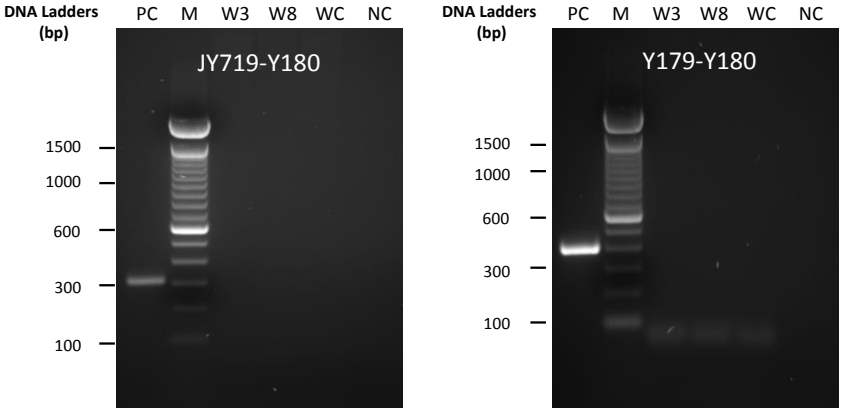


Figure 5-25. Confirmation of the Absence of Backbone DNA Junctions

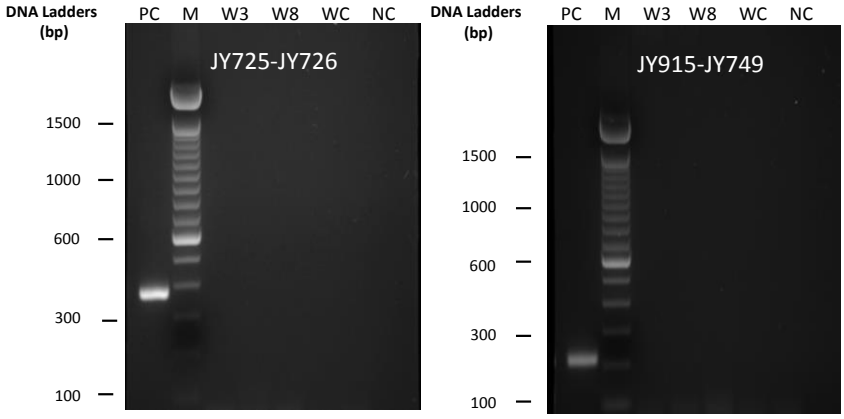
Detection of Left Border of the DNA insert and flanking backbone of pSIM1278



Detection of Left Border of the DNA insert and flanking backbone of pSIM1678



Detection of Right Border of the DNA insert and flanking backbone of pSIM1278 and pSIM1678



Ethidium bromide stained agarose gels for the PCR reactions using the primer sets indicated. PC = positive control, M = 100-bp DNA marker (Invitrogen), W3 = Additional event that was not submitted, W8 = Submitted Event, WT = Control Russet Burbank, NC = Negative control.

5.4.3 Conclusions: Absence of Plasmid Backbone

Collectively, our Southern blot and PCR analyses have shown that the Russet Burbank W8 event does not contain backbone from either plasmid used in the transformations.

5.5 Genetic stability of W8 over three generations

Bacterial T-DNAs are not always stable after insertion into a plant. The estimated instability rate (0.5-5.9×10^{-4}) is associated with meiosis (Müller *et al.* 1987; Conner *et al.* 1998), which is not relevant to potatoes as they reproduce vegetatively. Thus, DNA insertions are expected to be stable. Tubers rather than seeds were used to define subsequent generations since tubers are what are commercially planted.

Genetic stability was assessed using both molecular and phenotypic assays. The structure of the insert was shown to be stable using Southern blot analysis of genomic DNA isolated over three generations of W8 potatoes (G0 - G3), whereas the phenotypic stability was assessed by measuring polyphenol oxidase activity, in the second generation of field-grown tubers. This method shows visual evidence of PPO silencing after applying catechol to the cut surface of potatoes as shown in Chapter 10, Trait Efficacy. These studies were carried out to ensure that the desired genetic changes in W8 remained stable over multiple clonal cycles while maintaining the traits.

5.5.1 Molecular Evidence for Stability of the DNA Insert

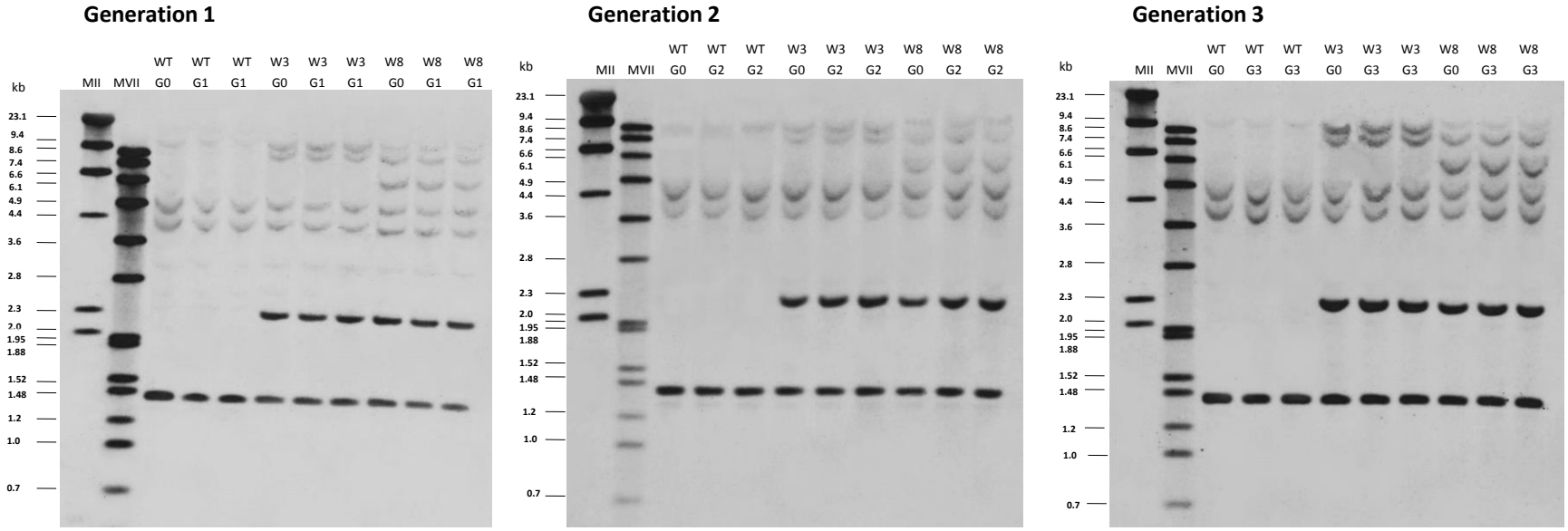
The stability of the DNA inserts was evaluated by comparing three successive clonal generations (G1, G2, and G3) to the original transformant (G0) using Southern blots. Stable DNA inserts are expected to maintain the same structure and thus produce the same digestion patterns over multiple generations of the plant. To test stability of the inserts in the W8 event, we compared its digestion pattern using two probes (GBS1 and AGP) that hybridize to regions of the inserts from both pSIM1278 and pSIM1678, and two probes (INV and VNT1) that are specific to the pSIM1678 insert. Since the DNA sequences these probes hybridize with are contained in the potato genome as well as within the DNA insert(s), both endogenous and insert-specific bands are expected in the Southern blots.

All genomic DNA samples were digested with the restriction enzyme, EcoRV, and hybridized with a probe specific to either AGP or GBS1. EcoRV was chosen for these studies as it digests within both inserts to provide a unique banding pattern with internal bands of predicted size in the pSIM1278 insert (e.g. 2.3 kb). The banding patterns between all samples of W8 were identical to each other for both probes (Figure 5-26). The multiple bands present in the Russet Burbank control are also found in W8, but W8 also contains bands corresponding to the pSIM1278 and pSIM1678 inserts. These bands are similarly consistent between all generations of W8 analyzed indicating genetic stability of both inserts.

A second analysis was performed using two probes specific to the pSIM1678 insert. For this analysis, genomic DNA samples were digested with the restriction enzyme, XbaI, and hybridized with VNT1 and INV probes. XbaI was chosen as the restriction enzyme for these studies as it digests the pSIM1678 internally and produces a band of known size (e.g. 4.6 kb for the INV probe). Again, both endogenous and insert-specific bands were detected with consistent banding patterns between the three generations analyzed (Figure 5-27).

Figure 5-26. Southern blot probing of EcoRV digestion

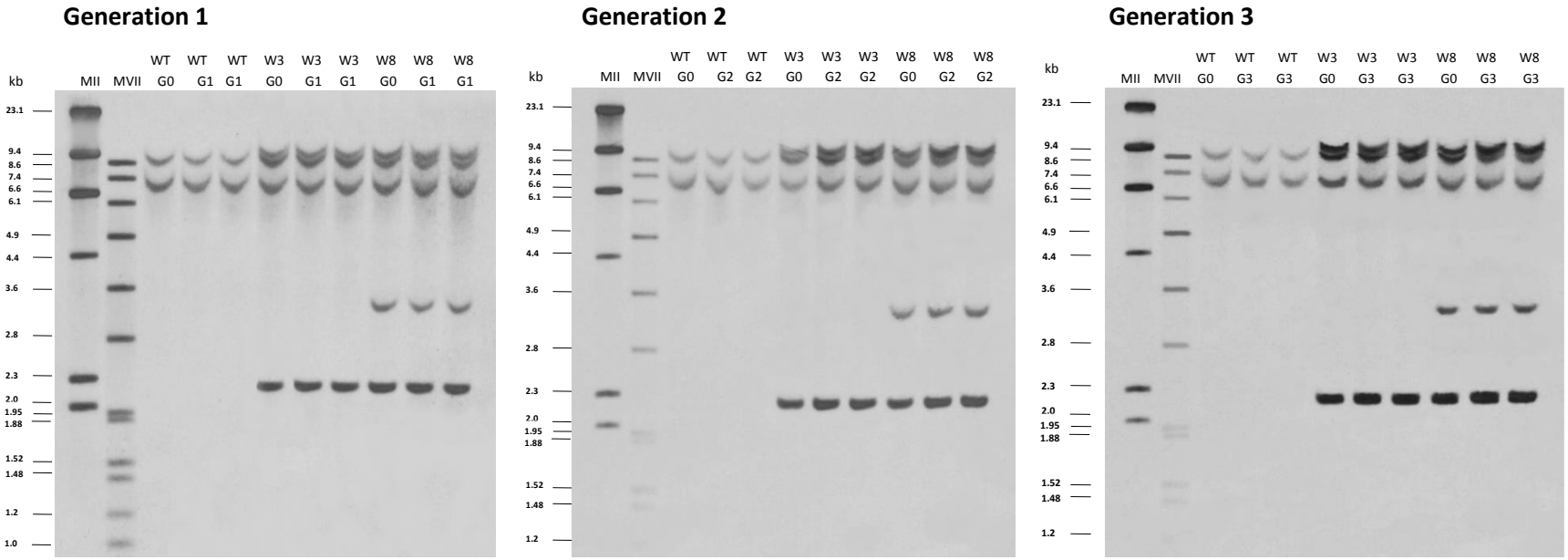
(A) AGP Probe



Southern blots of genomic DNA (3 µg) following digestion with EcoRV and probed for AGP sequence. Each blot compares the indicated generation of DNA from the initial transformant (G0). Russet Burbank untransformed controls (WT), W3 = additional event that was not submitted, W8 = submitted event. Genetic stability is established by the consistent digestion pattern for each line between the first generation (G0) and each subsequent generation (G1 - G3) within a set of gels.

Figure 5-26. Southern blot probing of EcoRV digestion (cont.)

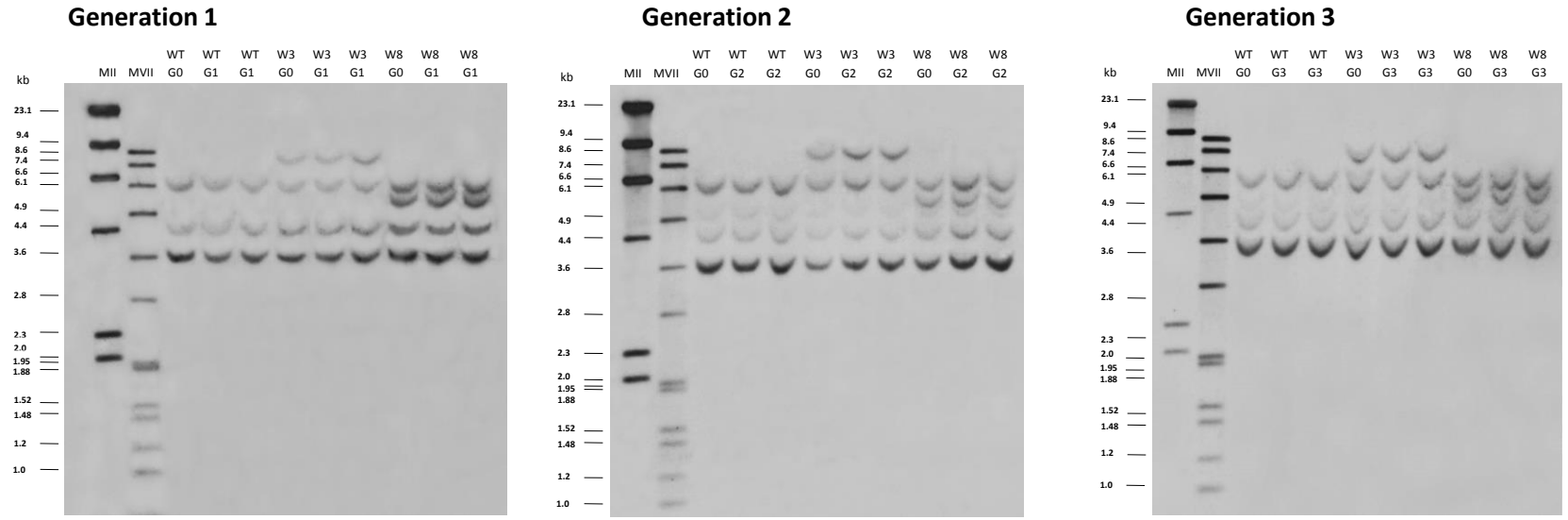
(B) GBS1 Probe



Southern blots of genomic DNA (3 µg) following digestion with EcoRV and probed for GBS1 sequence. Each blot compares the indicated generation of DNA from the initial transformant (G0). Russet Burbank untransformed controls (WT), W3 = additional event that was not submitted, W8 = submitted event. Genetic stability is established by the consistent digestion pattern for each line between the first generation (G0) and each subsequent generation (G1 - G3) within a set of gels.

Figure 5-27. Southern blot probing of XbaI digestion

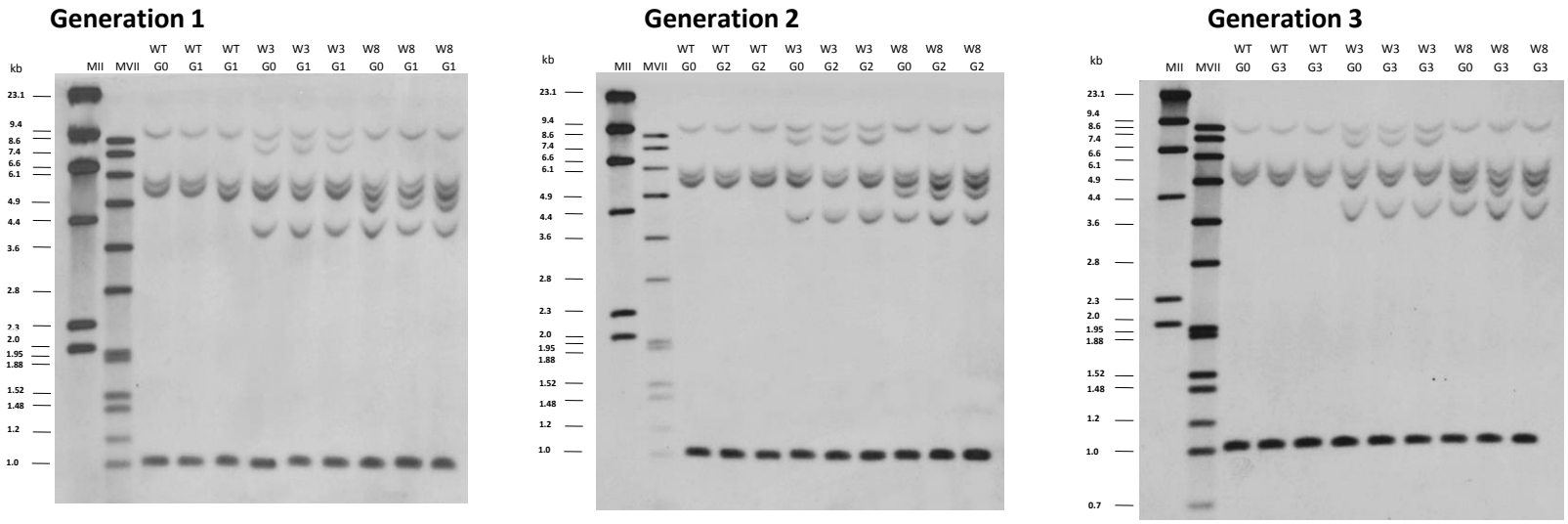
(A) VNT1 Probe



Southern blots of genomic DNA (3 µg) following digestion with XbaI and probed for *Rpi-vnt1* sequence. Each blot compares the indicated generation of DNA from the initial transformant (G0). Russet Burbank untransformed controls (WT), W3 = additional event that was not submitted, W8 = submitted event. Genetic stability is established by the consistent digestion pattern for each line between the first generation (G0) and each subsequent generation (G1 - G3) within a set of gels.

Figure 5-27. Southern blot probing of XbaI digestion (cont.)

(B) INV Probe



Southern blots of genomic DNA (3 µg) following digestion with XbaI and probed for *VInv* sequence. Each blot compares the indicated generation of DNA from the initial transformant (G0). Russet Burbank untransformed controls (WT), W3 = additional event that was not submitted, W8 = submitted event. Genetic stability is established by the consistent digestion pattern for each line between the first generation (G0) and each subsequent generation (G1 - G3) within a set of gels.

5.5.2 Phenotypic Evidence for Stability of the DNA Insert

Additional evidence for insert stability was obtained by measuring resistance to black spot in W8 potatoes using a catechol assay that measures PPO activity (see Chapter 10, Section 10.1.4 Black Spot for additional data and details of the assay). Black spot resistance is conferred by silencing of the *Ppo5* gene in tubers, which is being suppressed by a silencing cassette within the pSIM1278 insert. This assay provided phenotypic evidence for maintenance of a functional *Ppo5* silencing cassette in the Russet Burbank W8 event.

5.5.3 Summary of Genetic Stability Studies

The genetic and phenotypic analyses indicated the insertions arising from transformation of both pSIM1278 and pSIM1678 are stable over three generations. Given the demonstrated stability over three generations, it is likely that stability will be maintained during subsequent cycles of vegetative propagation. The catechol assay provides a convenient assay for monitoring stability as it is indicative of black spot tolerance, but also low asparagine formation because *Ppo5* and *Asn1* gene silencing are mediated by the same silencing cassette in the pSIM1278 insert. Although instability is unlikely and undesired, the result would be a loss of activity and reversion to wild-type, which would not trigger any biosafety issues as a result.

5.6 Characteristics of Transferred DNA and Gene Regulation

The DNA inserts introduced into W8 include three expression cassettes designed to partially silence up to five endogenous potato genes in a tissue-specific manner. Transcription of each cassette leads to the production of double-stranded RNA containing sequence derived from and specific to target potato genes, which are silenced using the cellular RNA interference (RNAi) pathway. Silencing of target genes leads to a reduction in acrylamide, black spot bruising, and accumulation of reducing sugars in W8 potatoes. A detailed description of the gene silencing cassettes, mode of action, and efficacy is described in Chapter 7: Characterization of Gene Silencing and Target Gene Expression.

The DNA insert derived from pSIM1678 contains an additional expression cassette consisting of the *Rpi-vnt1* gene. This gene is expressed under its native potato promoter and provides late blight resistance against *P. infestans*. A detailed description and characterization of *Rpi-vnt1* gene expression, VNT1 protein accumulation, safety and mode of action is described in Chapter 6: Characterization and Safety of the VNT1 Protein.

5.7 Event-Specific PCR

As part of our stewardship and identity preservation plan a method was developed to allow low-level detection of Russet Burbank W8 potatoes in plants and food products. The quantitative PCR (qPCR) method reliably detects the presence of W8 at concentrations of 0.2% with a 95% confidence interval (Appendix E: Detection of Russet Burbank W8 Using Real-Time PCR). The method has high specificity as it distinguishes between W8 and other events established through transformation with the same plasmids. The methods developed will be used to monitor plants and tubers in field and storage as part of quality management programs.

5.8 Summary of the Genetic Characterization of Event W8

The Russet Burbank W8 Event was transformed with two plasmids, pSIM1278 and pSIM1678. The DNA inserted from each transformation consisted solely of sequence targeted for insertion and did not contain any detectable plasmid backbone in the genome of W8. Transformation of each plasmid led to a single integration event in the genome. The sequence and structure of the DNA inserts and the genomic flanking regions were determined for both plasmids. The structure of the insert associated with the pSIM1278 plasmid was more complex than the original plasmid, but the duplicated silencing cassettes remained intact and under the control of tissue-specific promoters (Figure 5-4). The insert associated with the pSIM1678 plasmid was very similar to the input sequence, but contained a small deletion consisting of the left border and a small region of the *Rpi-vnt1* promoter (Figure 5-5). This small deletion in the promoter does not affect the gene's ability to confer late blight resistance (Chapter 10: Trait Efficacy). The structures of the DNA inserts were shown to be stable over three generations and a method of detecting low levels of W8 was developed. No biosafety concerns associated with the DNA inserts or the integration site were identified (See Chapter 9: Safety of Russet Burbank W8 Potato and Chapter 10: Trait Efficacy).

5.9 References

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6.0 Characterization and Safety of the VNT1 Protein

The VNT1 protein produced through introduction of the *Rpi-vnt1 gene* into the Russet Burbank Event W8 has been characterized and assessed for safety. A description of VNT1's origin, function, mode of action, and an assessment for toxins and allergens are presented along with its pattern of expression and protein concentration. The details of the materials and methods used in these studies are provided in Appendix A: Genetic, Molecular, and Biochemical Methods and Appendix B: Characterization of VNT1.

6.1 Identity and Function of the VNT1 Protein

Identity

Resistance proteins (R-proteins) such as VNT1 are signal transduction ATPases with homologs found in all domains of life (Leipe et al. 2004). They have a conserved domain structure consisting of a central nucleotide-binding domain flanked by either an N-terminal toll-like/interleukin-1 (TIR) or coiled-coil (CC) domain and a C-terminal leucine-rich repeat (LRR) (Takken and Goverse 2012). The LRR is the least conserved and has been hypothesized to function in effector recognition.

Using Simplot Innate™ Technologies, an *Rpi-vnt1* gene (accession: FJ423044.1) was transformed into the Russet Burbank potato variety after insertion into the pSIM1678 plasmid, resulting in late blight resistance. This allele is one of three identified in the wild potato species, *Solanum venturii*, and is identical to the *Rpi-phu1* gene from the related species, *S. phureja* (Sliwka et al. 2013). The VNT1 protein confers resistance to late blight with a relatively broad resistance spectrum against the plant pathogen *P. infestans,* a member of the oomycete class of fungus-like microorganisms. It is an 891 amino acid (Figure 6-1) CC-NB-LRR R-protein and a homolog of the Tm-22 tomato mosaic virus disease resistance protein (Foster et al. 2009).

Figure 6-1. Amino Acid Sequence of VNT1

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

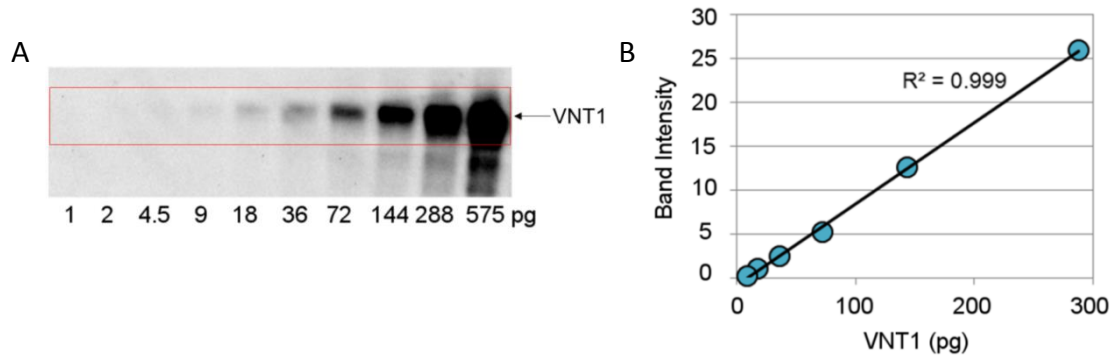
Expression of *Rpi-vnt1* in wild and cultivated potato confers broad-spectrum resistance to late blight caused by *P. infestans*. A critical layer of the plant defense system against pathogens relies upon recognition of pathogen-secreted effector proteins by resistance protein receptors (R-proteins). R-protein mediated immunity is part of a hypersensitive response that leads to destruction of infected plant tissue through programmed cell death (Moffett et al. 2002; Qi et al. 2012; Rairdan et al. 2008; Ayliffe et al. 1999; Tan et al. 2007; Morel and Dangl 1997). These R-proteins are tightly regulated and are maintained in an inactive state at low concentrations in the cell and are activated by their specific cognate ligands from the pathogen (Spoel and Dong 2012).

Specifically, following infection, *P. infestans* secretes an effector protein (AVR-VNT1), which is recognized by the R-protein, VNT1, expressed in potato. Consequently, VNT1 activates a signal transduction cascade that leads to localized plant cell death, which restricts growth and spread of the pathogen to the rest of the plant (Pel Dissertation 2010). Importantly, unlike Bt proteins, R-proteins do not confer pest resistance by directly targeting the pest or acting as toxins, but instead activate an immune response within the host plant.

6.2 Levels of the VNT1 protein in W8 Tissues

A number of VNT1 antibodies were generated and tested for sensitivity and specificity, and the antibody (5363) recognizing the peptide, FHSSKLPFGVWESKIL, of the LRR domain was chosen for detection and quantitative analyses. Recombinant VNT1 protein (VNT1-LRR domain) was purified from *E. coli* and used to determine the limit of detection and quantitation. As shown in Figure 6-1, antibody 5363 was sensitive enough to detect as little as 9 pg of purified VNT1 protein. For methods and supporting data refer to Appendix B: Characterization of VNT1.

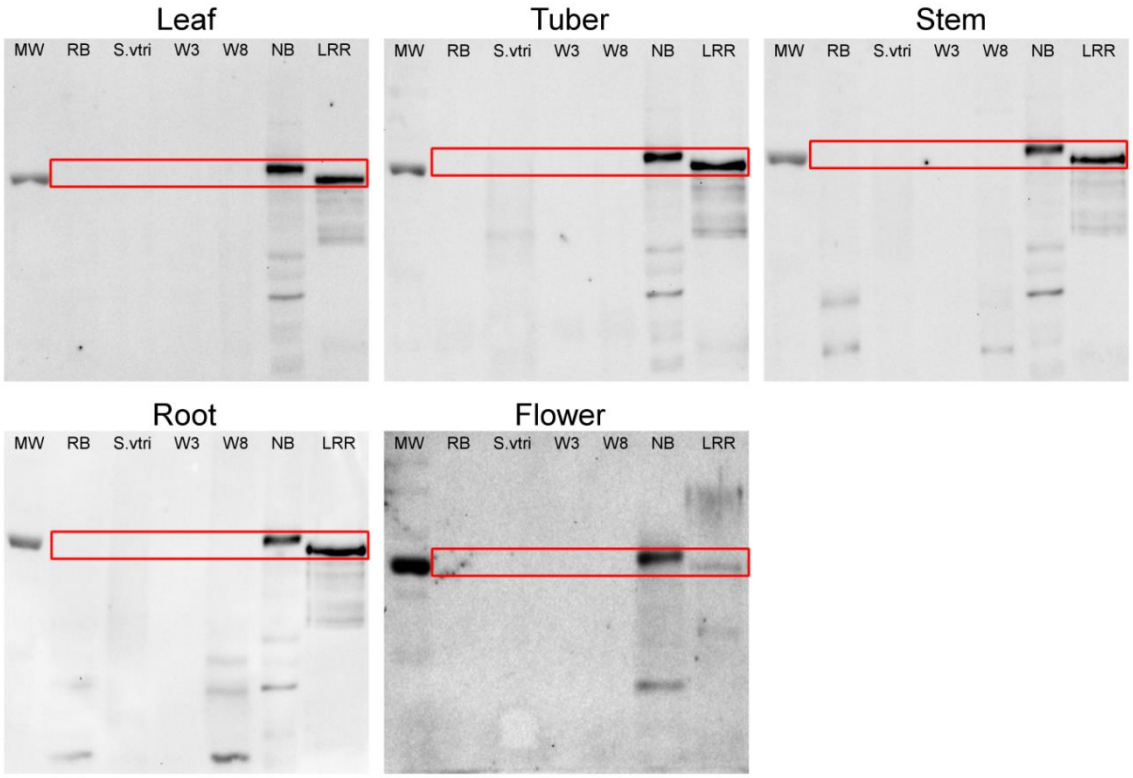
Figure 6-1. Limit of detection for anti-VNT1 antibody 5363



Limit of detection for anti-VNT1 antibody 5363. (A) Western blot analysis of a serial dilution of recombinant VNT1-LRR. (B) Quantitative curve showing linearity of western blot data.

Western blots using the sensitive antibody, 5363, were used to investigate the expression levels of VNT1 in various plant tissues. The VNT1 protein was undetectable in all tissues analyzed, indicating it was expressed at very low levels as expected (Figure 6-2).

Figure 6-2. Western blot analysis of VNT1 in potato tissues



Detection of VNT1 in Russet Burbank, *S. venturii*, W3, and W8 tissues. Proteins were extracted from each tissue and quantified by BCA assay. 320 ± 75 ng of total protein extracted from tissues from Russet Burbank (RB), *S. venturii* (S.vtri), W3, and W8 plants were loaded into each well of an SDS-PAGE gel. Western Blot analysis was performed using anti-VNT1 (5363) antibody. MW, molecular weight marker; NB, *N. benthamiana* expressed VNT1 (2.5 ng); LRR, *E. coli* expressed VNT1-LRR domain (2.5 ng). W3 = Additional event that was not submitted.

Given the low levels of VNT1 in the W8 tissue samples, we measured the limit of quantitation (LOQ) in samples isolated from those tissues to identify an upper bound on VNT1 expression levels. The results of those experiments are presented in Appendix B: Characterization of VNT1 and summarized here in Table 6-1.

Table 6-1. Limit of Detection, Quantification and Expression of VNT1 in Potato.

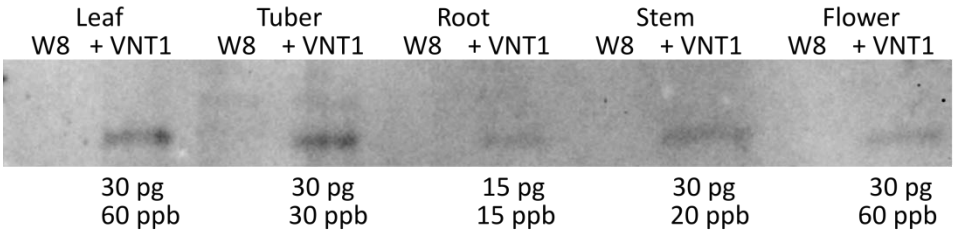
Tissue Type	LOD (pg)	LOQ (ng g^{-1} or ppb)	Russet Burbank (ng g^{-1} or ppb)	W8 (ng g^{-1} or ppb)
Leaf	9	60	< 60	< 60
Tuber	9	30	< 30	< 30
Stem	9	20	< 20	< 20
Root	9	15	< 15	< 15
Flower	9	60	< 60	< 60

VNT1 concentrations were calculated using ng g^{-1} dry weight basis (equivalent to parts per billion, ppb).

We found that the LOQ ranged between 15 and 60 ppb depending on the tissue sample. Western blot analysis was used to measure VNT1 concentration in protein extracts isolated from tissue where the amount of protein loaded was sufficient to allow detection of VNT1 at or above our measured LOQ (Figure 6-3). As shown, we were unable to detect VNT1 protein expressed in any tissues isolated from the Russet Burbank W8 event, but were able to detect VNT1 spiked in at concentrations similar to our reported LOQ.

Importantly, VNT1 was undetectable using an assay able to measure concentrations as low as 30 ppb in tubers, yet VNT1 expression was still able to provide resistance to late blight as described in Chapter 10: Trait Efficacy. In general, R-proteins are naturally low abundance and are estimated to be at concentrations as low as 18 ppt with low exposure levels in humans (3.6 ng protein/year) (Bushey, 2014). The concentration of VNT1 in tubers was minimally multiple orders of magnitude lower than has been determined for Bt protein in corn, soybean, cotton, and other crops deregulated by APHIS. In one example, the Cry1Ac protein in Bt soybean samples was expressed at mean levels of 25.4 ppm in leaves and 1.04 ppm in grain (Han 2012).

Figure 6-3. Quantitation of VNT1 in W8 tissue samples is below LOQ.
Spiked samples labeled as +VNT1.



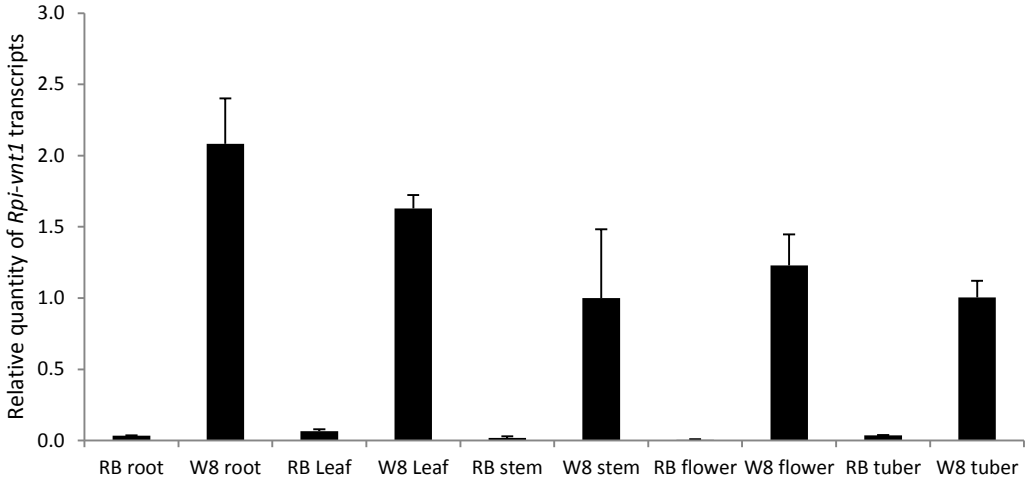
Western Blot analysis using antibody 5363 demonstrates that VNT1 is below detectable limits in all W8 tissues. Protein was extracted from 0.5 mg of leaf, 1 mg of tuber, 1mg of root, 1.5 mg of stem, and 0.5 mg flower. 30 pg of VNT1 were spiked into leaf, tuber, stem, and flower extracts and 15 pg of VNT1 were spiked into root extracts. LOQ values are shown in ppb.

6.3 *Rpi-vnt1* Gene Expression

Since the concentration of VNT1 protein was too low to detect using a highly sensitive antibody, quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) was used to verify expression of the *Rpi-vnt1* transcript in W8, which was not readily detectable by northern blot (data not shown). Total RNA was isolated from tissues of both Russet Burbank and the W8 event and subjected to quantitative RT-PCR using *Rpi-vnt1* specific primers (see Appendix A: Genetic, Molecular, and Biochemical Methods for details). Expression of *Rpi-vnt1* was normalized to a set of endogenous housekeeping genes, α-tubulin and elongation factor 1α, within each sample. Although expression was low in W8 tissue samples, it was considerably higher than in the Russet Burbank controls (Figure 6-4).

These data indicate that the *Rpi-vnt1* gene is transcribed in all W8 tissues analyzed, but are consistent with low levels of expression and protein accumulation in these potatoes.

Figure 6-4. *Rpi-vnt1* expression measured by RT-qPCR



Rpi-vnt1 expression measured by RT-qPCR. Each set of samples were analyzed in triplicate and normalized to a pair of endogenous genes (α-tubulin and elongation factor 1α). All analyses were performed using SYBR green dye with an associated melting curve analysis. RB (Russet Burbank); W8 (Event W8).

6.4 Assessment of VNT1 for Allergens and Toxins

Using a number of well-established bioinformatics tools, we performed a comprehensive analysis of possible toxins and allergens associated with the *Rpi-vnt1* gene (accession FJ423044.1) and the associated VNT1 protein sequence (Chapter 9: Safety of Russet Burbank W8 Potato). There was no significant sequence identity between ORFs within the *Rpi-vnt1* gene and known allergens or toxins, indicating VNT1 is not homologous with any known allergens or toxins. Thus, it is highly unlikely that introduction of this gene into Russet Burbank potatoes would represent a safety risk related to allergens or toxins.

6.5 Conclusions on VNT1 Protein Safety

Biochemical characterization and safety assessment of the VNT1 protein suggested a negligible risk associated with using VNT1 to confer late blight resistance to potato cultivars. The encoded VNT1 protein is native to *Solanum* species and provides protection against *P. infestans*, the causal agent of late-blight disease. In fact, the *Rpi-vnt1* (also called *Rpi-phu1*) gene has been introgressed into *S. tuberosum* (tetraploid potato varieties) from an interspecific cross between *Solanum phureja* and *Solanum stenotomum* (known as pinta boca in Peru and Bolivia). Both of these species are edible and research programs are underway in Europe to introgress *Rpi-phu1/Rpi-vnt1* in cultivated potatoes (Sliwka et al. 2010; 2013). Currently VNT1 represents only one of 68 distinct R-genes that have been characterized from wild *Solanum* species, which shows the breadth and importance of these genes to potato biology (Rodewald and Trognitz 2013).

Although we were able to confirm expression of the *Rpi-vnt1* gene in W8 plants, the protein concentration was below our limit of quantitation in all tissues tested, yet *Rpi-vnt1* conferred late blight resistance (Chapter 10: Trait Efficacy). The limit of quantitation in potato tubers was found to be 30 ppb. A bioinformatics assessment of possible allergens and toxins associated with the VNT1 protein did not identify any safety concerns.

Collectively, the ubiquitous nature of R-genes and their encoded R-proteins in *Solanum* species, including tomato and edible potatoes, lack of sequence identity to known toxins or allergens, mode of action not based upon inducing pest toxicity, and extremely low expression levels provides confidence in the safe use of potato varieties created to express VNT1.

6.6 References

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7.0 Characterization of Gene Silencing and Target Gene Expression

The DNA inserts introduced into W8 include three expression cassettes designed to partially silence as many as five endogenous potato genes in a tissue-specific manner. Transcription of each cassette leads to the production of double-stranded RNA containing sequence derived from and specific to the target potato genes, which are then silenced through the RNA interference (RNAi) pathway.

7.1 Gene Silencing History and Mode of Action

RNA interference (RNAi) refers to a cellular pathway used by plant and animal cells to regulate gene expression through destruction of selected or target RNAs within the cell. Mechanistically, enzymes within cells detect a unique, double-stranded form of RNA (dsRNA) and process it into small silencing RNAs (siRNA) that are then used to identify and specifically destroy RNAs containing complementary sequence (Chau and Lee 2007; Fire et al. 1998). This mechanism is particularly useful as part of the immune defense against invading organisms, such as viruses, many of which produce dsRNA as part of their normal replication cycle (Fusaro et al. 2006; Pumplin and Voinnet 2013). The utility of this mechanism is far reaching as it can be harnessed to selectively silence any gene within a cell without introduction of foreign sequence.

Traditional breeding practices have produced a number of cultivars that use RNAi to silence their own genes to produce desired traits (Petrick et al. 2013; Tuteja et al. 2004; Tuteja et al. 2009). Similarly, our Innate™ technologies have allowed us to create commercially desirable potatoes by using RNAi to specifically silence a set of target genes in the potato and reduce levels of enzymes responsible for quality related traits in tubers. These technologies allowed us to specifically silence the target genes in the desired plant tissue with minimal impact on the overall plant biology.

The expression of an inverted repeat is a regulatory system that has evolved naturally as a consequence of the duplication of genes and regulatory elements. One example relates to a 27-kb region of the soybean genome containing two perfectly repeated and inverted clusters of three chalcone synthase (*Chs*) genes (Tuteja et al. 2009). This results in the silencing of all *Chs* gene family members through the formation of double-stranded RNA. In this example, the double-stranded RNA initiates gene silencing, and is an example of naturally occurring inhibition of a metabolic pathway, which functions specifically in one tissue while allowing expression in other parts of the plant.

In order to create effective siRNA populations in plants, it is common to transcribe an extended inverted repeat sequence consisting of 100-500 nucleotides into a dsRNA precursor. The siRNAs generated generally consist of a mixture of sequences and sizes between 21 and 24-nts in length, where the 21-nt siRNA pool degrades mRNA and the 24-nt pool silences transcription in plants (Fusaro et al. 2006; Pumplin and Voinnet 2013).

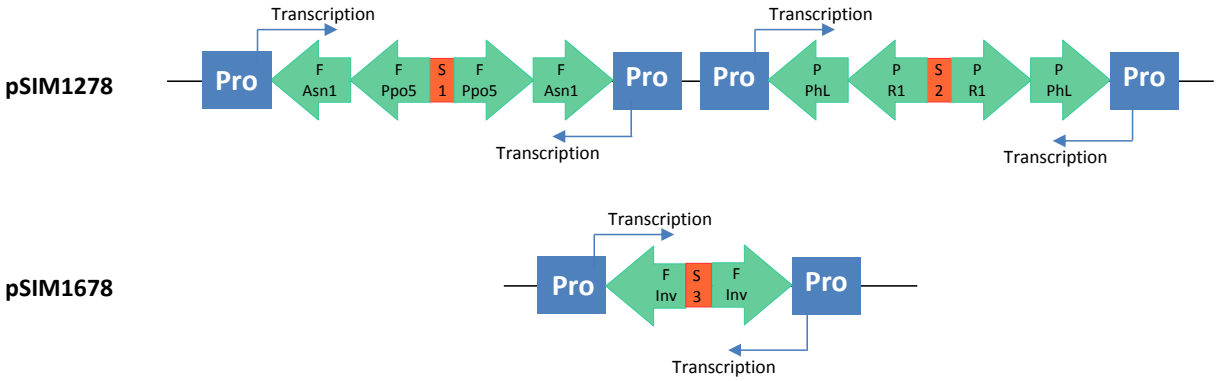
7.2 Gene Silencing Construct Design

Silencing was achieved by introducing inverted repeats containing sequences derived from the genes and promoters targeted for silencing. Although there are a number of parallel pathways involved in double-stranded RNA mediated silencing, transcription of these inverted repeats is thought to be processed by the cellular machinery involved in the viral defense (Fusaro et al. 2006). W8 potatoes contain three unique cassettes, which contain sequence from a total of five different potato genes. The pSIM1278 construct consists of two gene silencing cassettes (Figure 7-1, upper construct). One cassette

contains an inverted repeat of sequence from two genes, asparagine synthetase-1 (*Asn1*) and polyphenol oxidase-5 (*Ppo5*). The second cassette includes sequence from the promoters of the starch associated genes, *R1* (531-bp) and phosphorylase-L (*PhL*) (508-bp). The final cassette was introduced through the pSIM1678 construct, which includes an inverted repeat containing sequence from the vacuolar invertase (*VInv*) gene (Figure 7-1, lower construct).

All three silencing cassettes are regulated by the same set of well-characterized and tissue-specific promoters from the *Agp* and *Gbss* genes of potato, which are highly active in tubers compared with photosynthetically-active tissues and roots (Nakata et al. 1994; Visser et al. 1991). Therefore, expression and gene silencing was expected to be most effective in and largely limited to tubers.

Figure 7-1. Silencing Approach Using the DNA Inserts of pSIM1278 and pSIM1678



(Pro = functionally-active promoter, F =transcript fragment, S1 = spacer 1, P = promoter fragment, S2 = spacer 2, S3 = spacer 3)

7.3 Expression of Neighboring Genes

Previous studies on convergent transcription have shown that transcripts are of variable size but smaller than the distance between the two promoters, both in yeast (Prescott and Proudfoot 2002) and plants (Yan et al. 2006). In a study on convergent transcription-based silencing of the β-glucuronidase (*gus)* gene, no expression of neighboring genes was found (Yan et al. 2006).

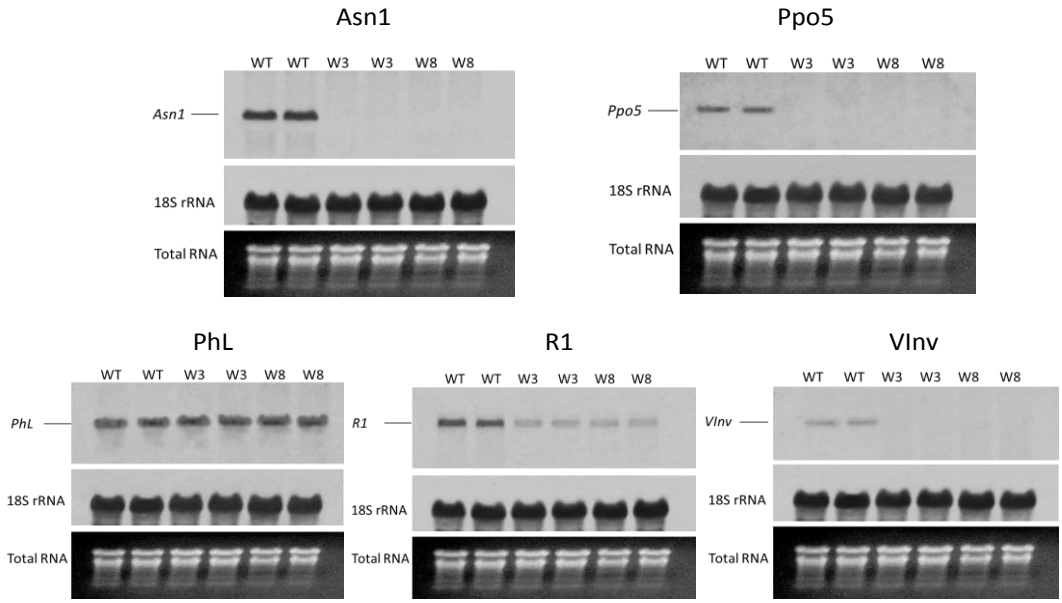
Although the possibility of rare read-through transcripts cannot be excluded, it is unlikely that such transcripts would interfere with the expression of neighboring genes because single stranded transcripts are ineffective in triggering gene silencing (Yan et al. 2006). Furthermore, studies on the expression of an antibiotic selectable marker gene next to a convergent transcription-based silencing construct demonstrated that this antibiotic gene was not affected by expression of the silencing construct (Yan et al. 2006). A complete bioinformatics analysis of open reading frames associated with the DNA inserts and flanking regions is discussed in Chapter 9: Safety of Russet Burbank W8 Potato, with additional details of the methods in Appendix C: Allergen and Toxin Assessment for Russet Burbank W8.

7.4 Silencing of Target Genes is Tissue Specific

The expression of all five target genes was characterized by northern blot analysis to determine the effectiveness of gene silencing from each cassette. Note that data for two events is presented in each of the figures in this chapter, but only event W8 is under consideration in this submission. Robust silencing of *Asn1*, *Ppo5*, and *VInv* was observed in tubers while silencing of *R1* was less effective (Figure 7-2). Silencing of *PhL* was considered ineffective as no measurable differences between control and W8 samples were observed. In other events with the same pSIM1278 construct, partial silencing of the promoters for *PhL and R1* in tubers was observed (Collinge and Clark 2013).

Previous studies have shown that *Ppo* gene silencing reduces the amount of associated protein to levels undetectable by western blot analysis (Llorente et al. 2011). Similarly, silencing of the *R1* gene diminished accumulation of a ~160kDa protein that is at least partially bound to starch granules (Lorberth et al. 1998).

Figure 7-2. Target Gene Expression in Tubers

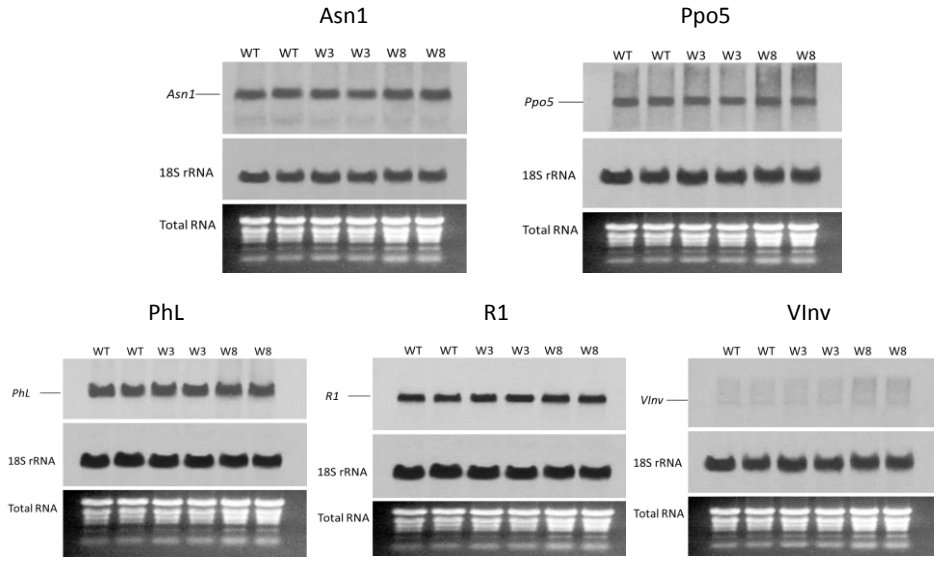


Northern blot analysis of total RNA (20 µg) isolated from tubers of field-grown plants for two events (W3 and W8) along with the Russet Burbank control (WT). Blots were hybridized with probes specific to the *Asn1, Ppo5, PhL, R1, and VInv* transcripts (upper panels). A probe specific to the internal control 18s rRNA (middle panels) and ethidium bromide stained total RNA (lower panels) were used as internal and loading controls. Each blot includes two independent biological replicates for each sample. W3 = Additional event that was not submitted.

We evaluated target gene expression in other plant tissues to determine the specificity of our gene silencing. Northern blot analysis was similarly performed on RNA isolated from leaves, stems, roots, and flowers from W8 and the Russet Burbank control. As shown in Figures 7-3, 7-4, and 7-5 there was no silencing of target genes in leaves, stems, or roots relative to the Russet Burbank controls. All transcripts were readily detectable, except those corresponding to the *VInv* gene, which was weakly expressed in all leaf and stem samples, including controls.

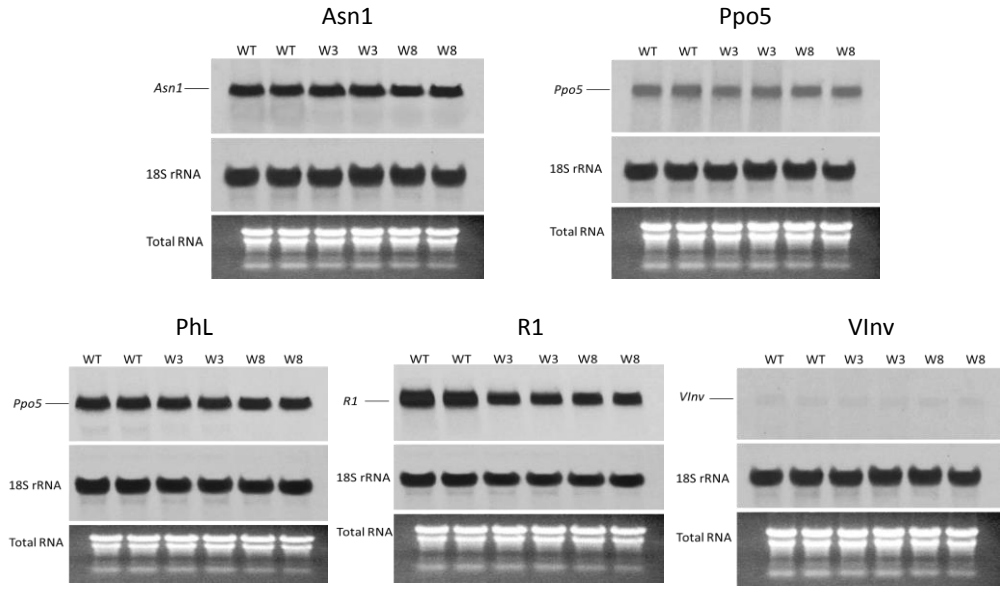
The only tissue other than tubers where some target silencing was observed was in the flower samples. These samples indicated some silencing of the *Asn1* transcript in W8 relative to the Russet Burbank controls, which may be due to some leaky expression in that tissue (Figure 7-6).

Figure 7-3. Target Gene Expression in Leaves



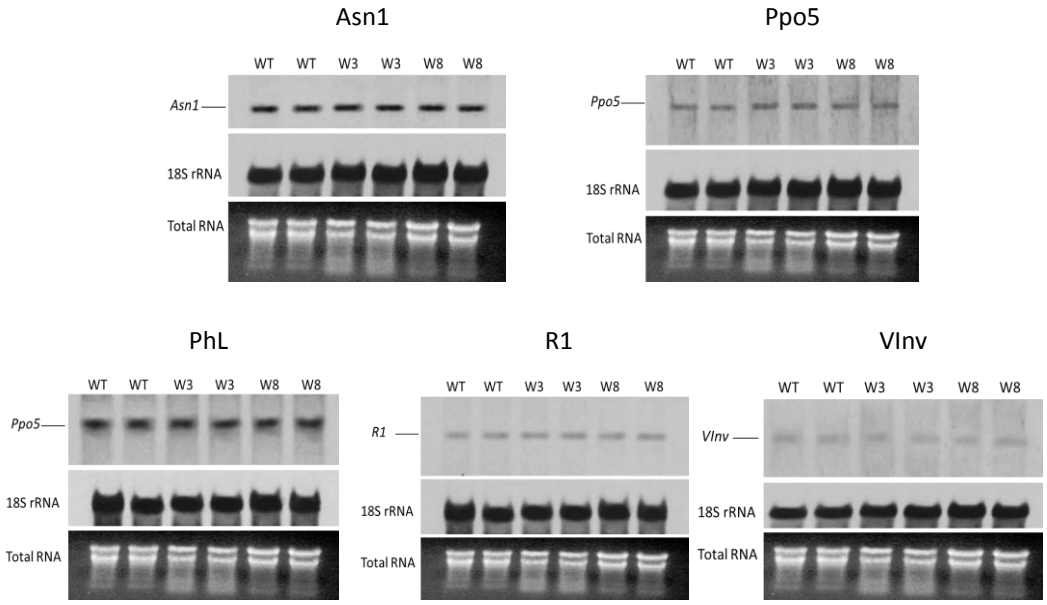
Northern blot analysis of total RNA (20 µg) isolated from leaves of field-grown plants for two events (W3 and W8) along with the Russet Burbank control (WT). Blots were hybridized with probes specific to the *Asn1, Ppo5, PhL, R1, and VInv* transcripts (upper panels). A probe specific to the internal control 18s rRNA (middle panels) and ethidium bromide stained total RNA (lower panels) were used as internal and loading controls. Each blot includes two independent biological replicates for each sample. W3 = Additional event that was not submitted.

Figure 7-4. Target Gene Expression in Stems



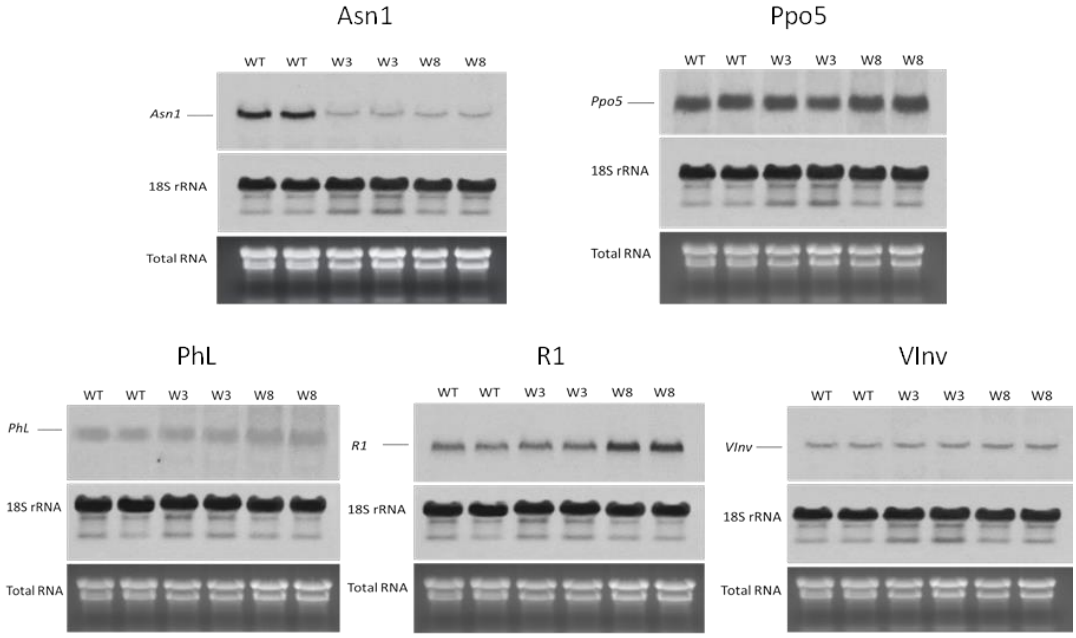
Northern blot analysis of total RNA (20 µg) isolated from stems of field-grown plants for two events (W3 and W8) along with the Russet Burbank control (WT). Blots were hybridized with probes specific to the *Asn1, Ppo5, PhL, R1, and VInv* transcripts (upper panels). A probe specific to the internal control 18s rRNA (middle panels) and ethidium bromide stained total RNA (lower panels) were used as internal and loading controls. Each blot includes two independent biological replicates for each sample. W3 = Additional event that was not submitted.

Figure 7-5. Target Gene Expression in Roots



Northern blot analysis of total RNA (20 µg) isolated from roots of field-grown plants for two events (W3 and W8) along with the Russet Burbank control (WT). Blots were hybridized with probes specific to the *Asn1, Ppo5, PhL, R1, and VInv* 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. Each blot includes two independent biological replicates for each sample. W3 = Additional event that was not submitted.

Figure 7-6. Target Gene Expression in Flowers



Northern blot analysis of total RNA (20 µg) isolated from flowers of field-grown plants for two events (W3 and W8) along with the Russet Burbank control (WT). Blots were hybridized with probes specific to the *Asn1, Ppo5, PhL, R1, and VInv* 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. Each blot includes two independent biological replicates for each sample. W3 = Additional event that was not submitted.

7.5 Summary of Gene Silencing and Target Gene Expression

Two of the three gene silencing cassettes introduced into Russet Burbank to generate the W8 event were very effective at silencing their target transcripts for RNAi-mediated silencing. These two constructs effectively silenced *Asn1*, *Ppo5*, and *VInv* in the tubers of W8. The specificity of silencing to the tubers indicates that few, if any, of the siRNA generated by the RNAi machinery spread to other tissues or that their levels were insufficient to invoke an RNAi response in those tissues. The only evidence for silencing outside of tubers was in flowers where lower levels of Asn1 were observed, yet the magnitude of change was much lower than in tubers. The promoter silencing strategy with *PhL* and *R1* had minimal effect, which was consistent with other events containing the same pSIM1278 construct (Collinge and Clark 2013).

As expected, the reduced expression of RNA transcripts associated with *Asn1*, *Ppo5*, and *VInv* were further corroborated as described in Chapter 10: Trait Efficacy. In addition, the compositional and agronomic data presented in Chapters 8: Compositional Assessment and 11: Agronomic Evaluation did not reveal any unexpected phenotypes that would be associated with significant off-target effects or unintended silencing. For instance, strong silencing of *Asn1* in tubers limits the accumulation of the amino acid asparagine as desired, whereas silencing of *Asn1* in leaves or stems might adversely affect growth and development, which was not the case. Thus, the RNAi response was both effective and specific and there is no indication that silencing these potato genes would affect weediness or other plant-pest characteristics.

7.6 References

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8.0 Compositional Assessment

The compositional assessment study evaluating proximates, vitamins, amino acids, minerals, and glycoalkaloids, was conducted on Russet Burbank Event W8 to 1) show equivalence to the untransformed control, 2) compare W8 to literature ranges, where applicable; and 3) show that there were no changes in potential toxins. Ultimately, results of the composition studies showed food safety equivalence between W8 and the control Russet Burbank.

8.1 Compositional Analysis Results

These analyses were conducted to confirm that composition of Event W8 remained within the normal levels for potato and would have equivalent food quality, feed quality, and safety when compared to untransformed potatoes**.** The compositional assessments determined the amounts of 1) proximates: protein, fat, ash, crude fiber, carbohydrate, calories, and moisture (Table 8-1); 2) vitamins: B3, B6, and C (Table 8-1); 3) minerals: copper, magnesium, and potassium (Table 8-1); and 4) total amino acids (Table 8-2). These assessments were conducted in W8, the control, and conventional reference varieties grown in potato-growing areas of the United States. A detailed list of reference varieties is shown in Table 8-5. For all the nutrients listed above, the goal was to determine if W8 contained equivalent amounts compared with the untransformed control at the time of harvest.

Table 8-1: Proximates, Vitamins, and Minerals in W8 and Control

Compound	Variety	Mean	P-value[1]	N	Range Min	Range Max	TI Low[2]	TI High[2]	CLR Low[3]	CLR High[3]
Protein (%)	W8	2.11	0.6444	32	1.83	2.58				
Protein (%)	Control	2.13	.	32	1.82	2.50	1.53	2.79	0.700	4.60
Fat (%)	W8	0.162	0.8616	32	0.100	0.250				
Fat (%)	Control	0.166	.	32	0.100	0.460	0	0.450	0.0200	0.200
Ash (%)	W8	0.958	0.8664	32	0.643	1.26				
Ash (%)	Control	0.951	.	32	0.717	1.49	0.461	1.41	0.440	1.90
Crude Fiber (%)	W8	0.469	0.2647	32	0.305	0.670				
Crude Fiber (%)	Control	0.438	.	32	0.305	0.660	0.190	0.740	0.170	3.50
Carbohydrates (%)	W8	16.5	0.0577	32	13.3	20.0				
Carbohydrates (%)	Control	17.2	.	32	14.5	19.4	13.2	22.1	13.3	30.5
Calories (kcal/100g)	W8	75.7	0.0599	32	63.7	89.4				
Calories (kcal/100g)	Control	78.8	.	32	68.8	87.7	63.5	97.9	80.0	110
Moisture (%)	W8	80.3	0.0652	41	76.9	83.3				
Moisture (%)	Control	79.7	.	41	77.6	82.0	75.0	83.4	63.2	86.9
Vitamin B3 (Niacin) (mg/100g)	W8	1.86	0.8651	32	1.41	2.30				
Vitamin B3 (Niacin) (mg/100g)	Control	1.84	.	32	1.43	2.48	0.768	2.86	0.0900	3.10
Vitamin B6 (mg/100g)	W8	0.120	**0.0019**	32	0.0960	0.150				
Vitamin B6 (mg/100g)	Control	0.132	.	32	0.111	0.150	0.0740	0.150	0.110	0.340
Vitamin C (mg/100g)	W8	26.7	**0.0040**	32	18.7	32.2				
Vitamin C (mg/100g)	Control	23.5	.	32	16.9	28.8	11.9	44.5	1	54.0
Copper (%)	W8	0.0695	0.4460	32	0.0500	0.120				
Copper (%)	Control	0.0724	.	32	0.0500	0.110	0.0210	0.130	0.0200	0.700
Magnesium (%)	W8	20.9	0.1472	32	17.3	29.1				
Magnesium (%)	Control	20.1	.	32	16.4	23.5	13.3	29.0	11.3	55.0
Potassium (%)	W8	427	0.9262	32	367	481				
Potassium (%)	Control	428	.	32	367	484	281	578	350	625

[1]P-values indicating significant differences with control are bold and underlined.
[2]TI = Tolerance Interval
[3]CLR = Combined Literature Range. Literature ranges are from, Lisinska and Leszczynski (1989); Rogan et al. (2000); Horton and Anderson (1992); Talburt and Smith (1987).
Many values fell below detection limits and were adjusted to the limit for statistical analysis.
. = Not applicable

Table 8-2: Total Amino Acids in W8 and Control

Compound	Variety	Mean	P-value[1]	N	Range Min	Range Max	TI Low[2]	TI High[2]	CLR Low[3]	CLR High[3]
Alanine (mg/100g)	W8	70.4	**0.0004**	32	58.0	87.7				
Alanine (mg/100g)	Control	63.8	.	32	41.3	75.2	38.9	95.1	39.2	95.2
Arginine (mg/100g)	W8	104	0.1463	32	83.7	157				
Arginine (mg/100g)	Control	98.4	.	32	62.5	119	52.9	142	70.0	138
Aspartic Acid (mg/100g)	W8	255	**<.0001**	32	189	331				
Aspartic Acid (mg/100g)	Control	454	.	32	278	636	203	686	339	738
Glutamic Acid (mg/100g)	W8	478	**<.0001**	32	389	583				
Glutamic Acid (mg/100g)	Control	310	.	32	232	373	185	482	292	604
Glycine (mg/100g)	W8	58.1	**0.0079**	32	45.7	70.6				
Glycine (mg/100g)	Control	52.8	.	32	40.8	62.4	38.5	76.3	1	97.5
Histidine (mg/100g)	W8	33.1	0.7008	32	26.2	41.6				
Histidine (mg/100g)	Control	32.7	.	32	24.2	42.7	18.4	46.8	13.3	46.9
Isoleucine (mg/100g)	W8	72.5	0.5519	32	60.0	95.3				
Isoleucine (mg/100g)	Control	71.2	.	32	47.9	86.9	46.9	98.4	52.5	95.3
Leucine (mg/100g)	W8	110	**0.0162**	32	81.4	137				
Leucine (mg/100g)	Control	102	.	32	72.1	123	67.2	149	68.5	138
Lysine (mg/100g)	W8	100.0	0.1590	32	83.6	118				
Lysine (mg/100g)	Control	95.7	.	32	73.2	115	63.7	136	68.7	137
Methionine (mg/100g)	W8	41.4	0.2150	32	34.2	51.6				
Methionine (mg/100g)	Control	39.9	.	32	29.8	49.4	24.7	52.0	9	128
Phenylalanine (mg/100g)	W8	81.9	0.7842	32	68.4	100				
Phenylalanine (mg/100g)	Control	81.2	.	32	54.5	94.8	55.5	115	55.2	109
Proline (mg/100g)	W8	63.1	**0.0399**	32	23.2	104				
Proline (mg/100g)	Control	52.9	.	32	11.8	72.9	11.0	127	35.5	146

[1]P-values indicating significant differences with control are bold and underlined.
[2]TI = Tolerance Interval
[3]CLR = Combined Literature Range. Literature ranges are from Talley et al. (1984); Rogan et al. (2000).
Many values fell below detection limits and were adjusted to the limit for statistical analysis.
. = Not applicable

Table 8-2 (continued): Total Amino Acids in W8 and Control

Compound	Variety	Mean	P-value[1]	N	Range Min	Range Max	TI Low[2]	TI High[2]	CLR Low[3]	CLR High[3]
Serine (mg/100g)	W8	74.0	**0.0175**	32	56.8	91.5				
Serine (mg/100g)	Control	68.7	.	32	49.1	80.1	48.5	94.0	50.0	102
Threonine (mg/100g)	W8	72.9	**0.0020**	32	56.0	91.5				
Threonine (mg/100g)	Control	65.3	.	32	46.7	79.2	41.8	95.2	43.6	85.5
Tryptophan (mg/100g)	W8	21.5	0.7800	32	17.4	26.8				
Tryptophan (mg/100g)	Control	21.9	.	32	16.1	26.8	13.5	29.1	11.4	28.2
Tyrosine (mg/100g)	W8	80.3	**<.0001**	32	68.8	95.1				
Tyrosine (mg/100g)	Control	65.2	.	32	47.3	77.7	39.3	96.5	45.7	94.2
Valine (mg/100g)	W8	112	0.2126	32	93.0	141				
Valine (mg/100g)	Control	108	.	32	73.6	127	67.7	143	75.2	145

[1]P-values indicating significant differences with control are bold and underlined.
[2]TI = Tolerance Interval
[3]CLR = Combined Literature Range. Literature ranges are from, Talley et al. (1984); Rogan et al. (2000).
Many values fell below detection limits and were adjusted to the limit for statistical analysis.
. = Not applicable

Compositional Analysis of Russet Burbank Event W8 at Harvest
Tubers of Event W8 were equivalent to those of the control except for the following observed changes:

- Event W8 tubers contained, on average, 0.12 mg/100g vitamin B6 as compared to the control, which contained 0.13 mg/100g ($p = 0.0019$). The mean for Event W8 was within the tolerance interval and combined literature range.

- Event W8 tubers contained, on average, 26.7 mg/100g vitamin C as compared to the control, which contained 23.4 mg/100g ($p = 0.004$). The mean for Event W8 was within the tolerance interval and combined literature range.

- Event W8 tubers contained, on average, 70.4 mg/100g total alanine as compared to the control, which contained 63.8 mg/100g ($p = 0.0004$). The mean for Event W8 was within the tolerance interval and combined literature range.

- Event W8 tubers contained, on average, 255.1 mg/100g total aspartic acid as compared to the control, which contained 477.8 mg/100g ($p < 0.0001$). The mean for Event W8 was within the tolerance interval and combined literature range. This was expected based on the intended reduction in asparagine.

- Event W8 tubers contained, on average, 477.8 mg/100g total glutamic acid as compared to the control, which contained 310.3 mg/100g ($p < 0.0001$). The mean for Event W8 was within the tolerance interval and combined literature range. This was expected based on the intended reduction in asparagine, which results in an increase in glutamic acid.

- Event W8 tubers contained, on average, 58.1 mg/100g total glycine as compared to the control, which contained 52.8 mg/100g ($p = 0.0079$). The mean for Event W8 was within the tolerance interval.

- Event W8 tubers contained, on average, 109.8 mg/100g total leucine as compared to the control, which contained 101.7 mg/100g ($p = 0.0162$). The mean for Event W8 was within the tolerance interval and combined literature range.

- Event W8 tubers contained, on average, 63.1 mg/100g total proline as compared to the control, which contained 52.9 mg/100g ($p = 0.0399$). The mean for Event W8 was within the tolerance interval and combined literature range.

- Event W8 tubers contained, on average, 74.0 mg/100g total serine as compared to the control, which contained 68.7 mg/100g ($p = 0.0175$). The mean for Event W8 was within the tolerance interval and combined literature range.

- Event W8 tubers contained, on average, 72.9 mg/100g total threonine as compared to the control, which contained 65.3 mg/100g ($p = 0.0020$). The mean for Event W8 was within the tolerance interval and combined literature range.

- Event W8 tubers contained, on average, 80.3 mg/100 g total tyrosine as compared to the control, which contained 65.2 mg/100g ($p < 0.0001$). The mean for Event W8 was within the tolerance interval and combined literature range.

The changes in vitamin B6, vitamin C, and total amino acids: alanine, aspartic acid, glutamic acid, glycine, leucine, proline, serine, threonine, and tyrosine, result in a very small modification in composition that is still standard for potato and will not significantly alter potential dietary intake levels. All values for Event W8 were within the tolerance interval or combined literature range and are thus still at normal levels for potatoes. It can be concluded that tubers of Event W8 are substantially equivalent to the control regarding their nutritional value for food and feed.

8.2 Glycoalkaloids

Results of Glycoalkaloid testing in Event W8

W8 and the control had mean glycoalkaloid ranges from 6.4 to 7.2 mg/100g, respectively (Table 8-3); all below the 20mg/100g safety limit described by Sinden (1987). Since the mean value for Event W8 was not significantly different from the control and was within the tolerance interval, it was concluded that glycoalkaloid levels in event W8 are no different from the Russet Burbank control.

This research confirmed that event W8 is as safe as controls and did not contain increased levels of natural glycoalkaloids, which are toxins commonly found in Solanaceous crops, including potato. The principal glycoalkaloids in potatoes are α-solanine and α-chaconine. For food safety purposes an upper limit for glycoalkaloid content of 20 mg per 100g of potato is generally accepted (Sinden 1987).

Table 8-3: Event W8 and Control Glycoalkaloids

Compound	Variety	Mean	P-value[1]	N	Range Min	Range Max	TI Low[2]	TI High[2]
Glycoalkaloids (mg/100g)	W8	7.2	0.1615	32	5.0	19.0	.	.
Glycoalkaloids (mg/100g)	Control	6.4	.	32	5.0	13.2	5.0	14.4

[1] P-values indicating significant differences with control are bold and underlined.
[2] TI = Tolerance Interval

8.3 Methods for Compositional Assessment

Field Trial Locations. Tubers for the compositional assessment were collected from Simplot Plant Sciences studies SPS-PAT-12-02 in 2012 and 13-02-SPS-ENV in 2013. The locations are summarized in Table 11-2 from Chapter 11: Agronomic Performance. Each combination of year, site, material, and replicate would represent one sample of six tubers in the compositional assessment.

Potato varieties and test events grown in field trials are summarized in Table 8-4. In 2012, only 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.

Table 8-4. Varieties in 2012 and 2013 Field Trials

Variety or Test	Type	Trait/Genotype	Seed Type[1]	Seed Source
Events in 2012 Field Trials				
Russet Burbank	Control	N/A	Greenhouse-grown Mini-Tubers	Simplot Plant Sciences - Boise, ID
W8	Test	pSIM1278+pSIM1678		
Events in 2013 Field Trials				
Russet Burbank	Control	N/A	NFT Mini-tubers	CSS Farms - Colorado City, CO
W8	Test	pSIM1278+pSIM1678		
Golden Sunburst	Reference	N/A		
Bintje	Reference	N/A		
Nicolet	Reference	N/A		
TX278[2]	Reference	N/A		
Red Thumb[2]	Reference	N/A		

[1]Greenhouse-grown mini-tubers were grown from tissue culture plantlets in the Simplot Plant Sciences greenhouse to produce nuclear seed. NFT mini-tubers were produced at CSS Farms in Colorado City, CO, using nutrient film technique to hydroponically produce a large number of seed.
[2]At WI-GRA, Red Thumb was used in place of TX278 due to a seed shortage.

Testing Laboratories. Analytical testing was conducted by Covance Laboratories, Inc. in Madison, WI or Greenfield, IN.

Sample preparation. Samples were obtained by randomly selecting 6 mid-sized tubers (at harvest) from each site and replicate (rep). Samples were powdered in an industrial blender with liquid nitrogen and stored at -70°C until analysis.

Protein. Protein was determined using the Kjeldahl method, approximating protein by multiplying Nitrogen by 6.25, as per Covance protocol PGEN_S:4 (AOAC 2005m; AOAC 2005n).

Fat. Fat was determined by Acid Hydrolysis using Covance protocol FAAH_S:7(AOAC 2005i; AOAC 2005j).

Ash. Ash levels were determined by Covance Laboratories using Covance protocol ASHM_S:5 (AOAC 2005e).

Crude Fiber. Crude fiber was determined by Covance Laboratories using Covance protocol CFIB_S:2 (AOAC 2005f).

Carbohydrates. Carbohydrate levels were determined by Covance Laboratories using Covance protocol CHO:6 (USDA 1973).

Calories. Total calories were determined by Covance Laboratories using Covance protocol CALC:4 (USDA 1975).

Moisture. Moisture levels were determined by Covance Laboratories using Covance protocol M100T100_S:4 (AOAC 2005c; AOAC 2005d).

Vitamin B3 (Niacin). Niacin was determined by Covance Laboratories using Covance protocol NIAP_S:11 **(**AOAC 2005k; AOAC 2005l).

Vitamin B6 (Pyridoxine). Pyridoxine was determined by Covance Laboratories using Covance protocol B6A_S:11 (AOAC 2005o; Atkin 1943).

Vitamin C. Vitamin C levels were determined by Covance Laboratories using protocol VCF_S:5 (AOAC 2005b).

Elements by ICP Emission Spectrometry. The minerals Copper (Cu), Magnesium (Mg),and Potassium (K) were determined by Covance Laboratories using Covance protocol (ICP_S:13) (AOAC 2005g; AOAC 2005h).

Tryptophan. Tryptophan levels were determined by Covance Laboratories using the Covance protocol TRPLC_S:3 (AOAC 2005a).

Total amino acids. Total amino acid levels were determined by Covance Laboratories using the Covance protocol TAALC_S:6 (Schuster 1988; Henderson 2000; and Barkholt and Jensen 1989).

Glycoalkaloids. Glycoalkaloid levels were determined by Covance Laboratories using protocol COID_S:2 (AOAC 2006).

8.4 Statistical Analysis for Compositional Assessment

Statistical Methods. The statistical analysis was performed by Simplot using SAS 9.3. All data were analyzed 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. 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 certain level of confidence, the range in which most values of a population will fall.

A step-wise approach was used to interpret any differences between event W8 and the control. First, statistical significance, p < 0.05, was determined for each attribute. If the p-value indicates no statistical

significance, then W8 is considered equivalent to the control. Next, if the p-value indicated statistical significance, mean values were compared with the tolerance intervals and combined literature range (Tables 8-6 and 8-7). If the means were within either the TI or CLR, they were considered within the normal range for potatoes.

A range of commercially-available, conventional varieties were selected for use in the tolerance interval calculation. They include varieties suitable for fresh use, for french frying, for chipping, and the conventional control. The following varieties were used to calculate tolerance intervals: Bintje, Golden Sunburst, Nicolet, Ranger Russet, Red Thumb, Russet Burbank, and TX278 (Table 8-5).

Table 8-5. Number of data points for each test, control and reference line

Line	N Per Attribute	Used in Tolerance Interval?
W8	32	No
Russet Burbank Control	32	Yes
Ranger Russet Control	12	Yes
Bintje	32	Yes
Golden Sunburst	30	Yes
Nicolet	32	Yes
Red Thumb	4	Yes
TX278	28	Yes
Total N	202	170 in Tolerance Interval

Table 8-6. Combined Literature Ranges (CLR) for Proximates, Vitamins, Minerals

Compound	Value	Units	Reference	Conversion Formula Used[1]	CLR	End Units
Protein low	0.70	%	Lisinska and Leszczynski 1989	NA	0.70	%
Protein high	4.60	%	Lisinska and Leszczynski 1989	NA	4.60	%
Fat low	0.02	%	Lisinska and Leszczynski 1989	NA	0.02	%
Fat high	0.20	%	Lisinska and Leszczynski 1989	NA	0.20	%
Ash low	0.44	%	Lisinska and Leszczynski 1989	NA	0.44	%
Ash high	1.90	%	Lisinska and Leszczynski 1989	NA	1.90	%
Crude fiber low	0.17	%	Lisinska and Leszczynski 1989	NA	0.17	%
Crude fiber high	3.50	%	Lisinska and Leszczynski 1989	NA	3.50	%
Carbohydrates low	13.30	%	Talburt and Smith 1987	NA	13.30	%
Carbohydrates high	30.53	%	Talburt and Smith 1987	NA	30.53	%
Calories low	70.00	kcal/100g	Horton and Anderson 1992	NA	80.00	kcal/100g
Calories high	551.00	kcal/100g dry	Horton and Anderson 1992	kcal/100g x 0.2	110.20	kcal/100g
Moisture low	63.20	%	Talburt and Smith 1987	NA	63.20	%
Moisture high	86.90	%	Talburt and Smith 1987	NA	86.90	%
Niacin (B3) low	0.18	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g	0.09	mg/100g
Niacin (B3) high	6.20	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g	3.10	mg/100g
Vitamin B6 (Pyridoxine) low	0.26	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g x 0.823	0.11	mg/100g
Vitamin B6 (Pyridoxine) high	0.82	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g x 0.823	0.34	mg/100g
Vitamin C low	1.00	mg/100g	Lisinska and Leszczynski 1989	NA	1.00	mg/100g
Vitamin C high	54.00	mg/100g	Lisinska and Leszczynski 1989	NA	54.00	mg/100g
Copper low	0.03	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g	0.02	mg/100g
Copper high	1.40	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g	0.70	mg/100g
Magnesium low	22.50	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g	11.25	mg/100g
Magnesium high	110.00	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g	55.00	mg/100g
Potassium low	700.00	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g	350.00	mg/100g
Potassium high	1250.00	mg/200g	Rogan et al. 2000	mg/200g x 0.5mg/100g	625.00	mg/100g

[1]0.2 = approximate conversion from dry weight to fresh weight (internal communication), 0.823 is the conversion factor from Pyridoxine free base to Pyridoxine HCl (internal communication)

NA = no conversion necessary because values came directly from literature.

Table 8-7. Combined Literature Ranges (CLR) for Total Amino Acids

Compound	Value	Units	Reference	Formula Wt (FW)	Conversion Formula Used[1]	CLR (mg/ 100g)
Alanine high	53.4	µmol/g	Talley et al. 1984	89.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	95.2
Alanine low	22.0	µmol/g	Talley et al. 1984	89.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	39.2
Arginine high	39.7	µmol/g	Talley et al. 1984	174.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	138.3
Arginine low	20.1	µmol/g	Talley et al. 1984	174.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	70.0
Aspartic acid high	1476	mg/200g	Rogan et al. 2000	NA	mg/200g x 0.5mg/100g	738.0
Aspartic acid low	677	mg/200g	Rogan et al. 2000	NA	mg/200g x 0.5mg/100g	338.5
Glutamic acid high	1207	mg/200g	Rogan et al. 2000	NA	mg/200g x 0.5mg/100g	603.5
Glutamic acid low	583	mg/200g	Rogan et al. 2000	NA	mg/200g x 0.5mg/100g	291.5
Glycine low	1	mg/100g	Lisinska and Leszczynski 1989	NA	NA	1
Glycine high	195	mg/200g	Rogan et al. 2000	NA	mg/200g x 0.5mg/100g	97.5
Histidine high	15.1	µmol/g	Talley et al. 1984	155.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	46.9
Histidine low	4.3	µmol/g	Talley et al. 1984	155.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	13.3
Isoleucine high	36.3	µmol/g	Talley et al. 1984	131.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	95.3
Isoleucine low	20.0	µmol/g	Talley et al. 1984	131.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	52.5
Leucine high	52.7	µmol/g	Talley et al. 1984	131.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	138.3
Leucine low	26.1	µmol/g	Talley et al. 1984	131.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	68.5
Lysine high	46.8	µmol/g	Talley et al. 1984	146.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	136.8
Lysine low	23.5	µmol/g	Talley et al. 1984	146.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	68.7
Methionine low	9	mg/100g	Lisinska and Leszczynski 1989	NA	NA	9
Methionine high	128	mg/100g	Lisinska and Leszczynski 1989	NA	NA	128
Phenylalanine high	32.9	µmol/g	Talley et al. 1984	165.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	108.7
Phenylalanine low	16.7	µmol/g	Talley et al. 1984	165.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	55.2
Proline high	63.6	µmol/g	Talley et al. 1984	115.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	146.4
Proline low	15.4	µmol/g	Talley et al. 1984	115.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	35.5
Serine high	48.6	µmol/g	Talley et al. 1984	105.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	102.2
Serine low	23.8	µmol/g	Talley et al. 1984	105.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	50.0
Threonine high	35.9	µmol/g	Talley et al. 1984	119.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	85.5
Threonine low	18.3	µmol/g	Talley et al. 1984	119.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	43.6
Tryptophan high	6.9	µmol/g	Talley et al. 1984	204.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	28.2
Tryptophan low	2.8	µmol/g	Talley et al. 1984	204.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	11.4
Tyrosine high	26.0	µmol/g	Talley et al. 1984	181.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	94.2
Tyrosine low	12.6	µmol/g	Talley et al. 1984	181.2	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	45.7
Valine high	61.9	µmol/g	Talley et al. 1984	117.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	145.0
Valine low	32.1	µmol/g	Talley et al. 1984	117.1	µmol/g x mol/10^6µmol x FW x 1000mg/g x 100g x 0.2	75.2

[1]0.2 is the approximate conversion from dry weight to fresh weight (internal communication)
NA = no conversion necessary because values came directly from literature.

8.5 Compositional Assessment Conclusions

This compositional assessment study, evaluating proximates, vitamins, minerals, amino acids, and glycoalkaloids was conducted on Russet Burbank Event W8 to 1) show equivalence to the untransformed control, and 2) compare W8 to conventional variety ranges and literature ranges, where applicable. Ultimately, results of this composition study showed food safety equivalence between the event and the untransformed Russet Burbank control. Because tubers of Event W8 are substantially equivalent to tubers from the control, the potatoes will be as safe as the Russet Burbank control for use as food and feed.

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9.0 Safety of Russet Burbank W8 Potato

The rationale for safety of Russet Burbank event W8 is based on the following evidence: 1) phenotype through agronomic studies, 2) compositional equivalence, 3) bioinformatics assessment for toxins and allergens, 4) glycoalkaloid testing, 5) dietary risk assessment of VNT1 protein, 6) low levels of VNT1 protein, 7) safety of nucleic acids, 8) literature review on gene silencing through RNAi, and 9) previous regulatory actions for plant incorporated protectants similar to the late blight resistance gene.

9.1 Phenotype through Agronomic Studies

Agronomic studies for W8 included complete assessments of phenotype at a total of 11 field trials during 2012 and 2013. Although some significant agronomic differences were observed between events and controls, most fell within the conventional variety range. For example, some differences in yield and tuber size were observed in W8, and these attributes will be investigated further in commercial trials. In addition, with the exception of the expected late blight resistance, W8 was not significantly different from the control when considering disease susceptibility. Thus, we concluded that there are no major differences in agronomic characteristics, susceptibility to diseases, yield and grading, and ecological interactions between the untransformed Russet Burbank variety and W8 that would contribute to increased weediness or plant pest potential. Results of these studies contribute to the evidence that W8 has similar agronomic and phenotypic traits to the Russet Burbank control and therefore W8 would be as safe as the controls.

9.2 Compositional Equivalence

The compositional assessment study, evaluating proximates, vitamins, minerals, amino acids, and glycoalkaloids was conducted on Russet Burbank W8 to 1) show equivalence to the untransformed control, and 2) compare W8 to conventional variety ranges and literature ranges, where applicable. Ultimately, results of this composition study showed food safety equivalence between W8 and the untransformed control. Because tubers of W8 are either statistically equivalent to tubers from the control or within the tolerance interval or combined literature range, the W8 potatoes will be as safe as their untransformed control for use as food and feed.

9.3 A Bioinformatics Approach to Allergen and Toxin Assessment

The bioinformatics approach to assessing toxins and allergens in W8 specifically investigates possible changes related to the DNA inserts and flanking regions. Other evidence that W8 would not contain new allergens or toxins comes from the compositional assessment as mentioned above in section 9.2: Compositional Equivalence. Based on the equivalent composition of W8 and the Russet Burbank control, additional allergies would not be expected.

Patatin is a storage glycoprotein with a molecular mass of about 40kDa (Racusen and Foote 1980) that displays lipase activity (Mignery et al. 1988). This protein has been given the allergen designation of Sol t 1 (Shewry 2003) and has been identified as the major allergen involved in allergic reactions to potatoes (Astwood et al. 2000). Some allergies have been detected in children as a result of patatin (Racusen and Foote 1980). Potato may elicit allergic responses when consumed as cooked food or in the handling of raw potatoes (Shewry 2003). However, allergic reactions to processed potatoes are considered to be very uncommon and have been reported for children only (De Swert et al. 2002; De Swert et al. 2007 Because potato protein naturally contains a relatively large proportion of patatin at about 40% of the

total soluble protein in potato tubers (Paiva et al. 1983, Park et al 1983, Shewry 2003, Bansal 2005), those who are allergic to potatoes would avoid them and changes to patatin levels would not be expected to alter that behavior. Although considered a mild allergen, to our knowledge, patatin levels are not used to screen new potato cultivars during breeding.

The scope of this study included a search of the DNA insert sequence and flanking regions for possible open reading frames (ORFs) followed by an assessment of potential allergens or toxins. A general consideration is that introduction of DNA into the plant's genome could result in disruption of a native gene or introduce sequence capable of expressing unexpected proteins that may act as allergens or toxins. A comprehensive bioinformatics analysis of each insertion site was performed to investigate this possibility (Appendix C: Allergen and Toxin Assessment).

BLAST searches indicated that there were no potato transcripts associated with the flanking regions of the inserts, and the available genome annotation lacked evidence of a known potato transcript associated with the insertion loci. A list of all ORFs present within the sequences of the inserts and their flanking regions were identified using the ORF Finder web application (Stothard 2000) available through the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/orf_find.html). The search parameters were defined to identify all ORFs with at least 19 amino acids located between any two contiguous stop codons regardless of whether a canonical start codon was present. This method identified a multitude of ORFs including 1) small ORFs naturally present in any DNA sequence, 2) those ORFs that specifically cover the junctions between native plant DNA and the inserted DNA (i.e. potential fusion proteins), and 3) the *Rpi-vnt1* gene (VNT1 protein) present in the pSIM1678 cassette.

To assess the potential for any of these ORFs to act as allergens, they were compared with known allergens from the repository available through the Allergy Resource Research Program (FARRP) via the University of Nebraska (available at http://www.allergenonline.org/databasefasta.shtml). The ORFs were analyzed to identify any 80 amino acid sequence within an ORF that possessed greater than 35% homology to a documented allergen or shared sequence identity over any 8 amino acid stretch. Only two allergen matches were identified following analysis of 240 ORFs.

One match was based upon an open reading frame containing the 8 amino acid sequence (LPLLLLLL), which is also present in an endochitinase protein (GI:3201547) from *Persea americana* (avocado). This polypeptide is just one of 319 distinct 8-mers present in the full-length endochitinase protein, and there is no evidence it contributes to the allergenicity as the LPLLLLLL peptide was not found in a database of epitopes (http://www.iedb.org/). This 8-mer is present in numerous proteins found in potatoes and humans. Thus, even if it was expressed in Russet Burbank W potatoes, it is highly unlikely to pose an allergenic risk. Collectively, these data suggest the peptide identified is a false positive and would not be a potential allergen, consistent with concerns previously expressed over the high false-positive rates associated with identifying 8-mer matches (Goodman 2008).

The second match (8-mer and 80-mer searches) corresponded to a vacuolar acid invertase protein (GI:18542113) present in tomatoes, which is also found in other *Solanum* species, such as potatoes. The presence of this partial ORF was by design in W8 potatoes as the vacuolar invertase (*VInv*) gene was the target of one of the introduced silencing cassettes. This silencing cassette will prevent expression of both the native INV protein and the ORF present in the pSIM1678 insert. Thus, silencing of the native vacuolar invertase gene may actually decrease the allergenic potential of Russet Burbank W8 potatoes relative to the control.

To assess the potential for these same ORFs to act as toxins, we performed BLAST searches against custom databases containing annotated protein toxins found in nature. We did not identify any significant matches (E-value < 1) for any of the ORFs.

In summary, using a number of well-established bioinformatics tools, we performed a comprehensive analysis of potential toxins and allergens associated with our DNA inserts and flanking regions, including the Late Blight Resistance Protein, VNT1. We did not identify any significant homology or identity between VNT1 and known allergens or toxins. Of the two matches identified from ORFs present in our DNA insert and flanking regions, neither appears to be significant, although one was associated with the *VInv* gene silencing cassette designed to limit expression of this gene and shown to be functional in tubers (see Chapter 7: Characterization of Gene Silencing and Target Gene Expression). If this sequence functioned as an allergen, the protein level would be reduced by the silencing of invertase resulting in even lower levels of a possible allergen. Overall, no allergen-related safety concerns were identified for the Russet Burbank W8 potato, and it is expected to be at least as safe as the Russet Burbank control.

9.4 Glycoalkaloid Testing

Potato plants naturally produce certain glycoalkaloids (solanine and chaconine) to defend against insects, disease, and predators which find those compounds toxic (Friedman 2006), and in response to environmental stress, such as physical damage and mishandling. Potato leaves, stems and shoots contain naturally higher levels of glycoalkaloids than potato tubers (Friedman and Dao 1990), but the relative tissue-specific levels vary depending on potato variety (Friedman et al. 1997). Most glycoalkaloids are present in the skin, or immediately below it, with gradually lower and lower levels of glycoalkaloids toward the center of the potato (Friedman and Dao 1990; Kozukue et al. 1987). Localized production of glycoalkaloids can also be found around eyes or sprouts (Friedman et al. 1997).

Sometimes, glycoalkaloid production is associated with a green color on the potato, typically when the tuber is exposed to light. This green color is because of the harmless light-induced production of chlorophyll. Depending on the variety, this green color may coincidentally correspond with increased glycoalkaloid production, but such color changes have not been found to reliably correlate with a higher level of glycoalkaloid content (Friedman 2006; Petersson et al. 2013; Friedman et al. 2003; Patil et al. 1971; and Edwards et al. 1998). Peeling potatoes and removing any green tissue or sprouts can reduce glycoalkaloid levels by 30-95% compared to the levels found in potatoes that remain unpeeled (Friedman and Dao 1990; Friedman et al. 2003; and Elzbieta 2012).

Glycoalkaloid Content in Commercial Varieties. The total glycoalkaloid content (TGA) of potato tubers varies widely. Values between 2 and 410 mg/kg FW were found (Lisinska and Leszczynski 1989), but in most cases the TGA concentration in whole tubers is between 10 and 150 mg/kg FW (van Gelder 1990). 95% of the total glycoalkaloids in potato tubers consists of a-chaconine (solanidine-glucose-rhamnose-rhamnose) and a-solanine (solanidine-galactose-glucose-rhamnose).

Most U.S. commercial potato varieties contain less than 120 parts-per-million (ppm) glycoalkaloids with ranges typically between 20-130 ppm on an unpeeled fresh weight basis (Friedman and Dao 1990; Friedman et al. 2003; and Sinden and Webb 1972). Whole russet potato varieties typically contain less than 60 ppm (Friedman and Dao 1990) which is far less than the accepted upper limit of 200 ppm (Friedman 2006; JECFA 1992; and JECFA 1993). Indeed, a joint World Health Organization and Food and Agriculture Committee on glycoalkaloid safety concluded that “the large body of experience with the consumption of potatoes, frequently on a daily basis, indicated that normal glycoalkaloid levels (20-100 mg/kg [=20-100 ppm]) found in properly grown and handled tubers were not of concern (JECFA 1993).

Bitterness and Glycoalkaloids. One sensory characteristic that correlates with glycoalkaloid levels is bitterness: potato varieties that are naturally bitter typically contain higher levels of glycoalkaloid than non-bitter varieties. Glycoalkaloid levels greater than 140 ppm are reported to taste distinctly bitter and levels greater than 200 ppm cause a burning sensation in the throat and mouth (Jansky 2010; Ross et al. 1978; Sinden et al. 1976; and Zitnak and Filadelfi 1985). The bitter taste and appearance of such potatoes makes it simple to exclude or set apart these types of potatoes from consumption and is one reason why the incidence of glycoalkaloid-related poisoning reports is low.

The main symptoms of glycoalkaloid illness are vomiting and diarrhea, but high acute consumption is necessary to produce such symptoms, *e.g*., 1.25mg/kg body weight (Hellenas et al. 1992; Mensinga et al. 2005; and Ruprich et al. 2009). For example, a 150 lb. (68 kg) person would need to eat about 1 lb. (425g) of peel-on potatoes having the maximum accepted levels of glycoalkaloids to show signs of illness. By contrast, he or she would have to consume as much as nine times that, *i.e*., 9 lbs. (3.8 kg), of peeled potatoes before such symptoms may arise.

Current Industry Practices Mitigate Risk. Notwithstanding the low levels of glycoalkaloids found in commercial varieties, appropriate pre- and post-harvest strategies can help further mitigate any potential health issues. Growers and processors follow storage and handling procedures to minimize glycoalkaloid production, such as: keeping tubers covered with soil while growing; harvesting mature tubers; discarding damaged, sunburned, or defective potatoes; peeling; minimizing mechanical injury; suppressing sprouting; and storing harvested tubers in the dark under appropriate conditions of temperature and humidity.

Glycoalkaloid testing of W8. Event W8 and the control had mean glycoalkaloid levels of 7.2 and 6.4 mg/100g (72 and 64 ppm), respectively; all well below the accepted safety limit of 20mg/100g (200 ppm) (Sinden 1987; Friedman 2006; JECFA 1992; and JECFA 1993). Since the mean value for Event W8 was not significantly different from the control and was within the tolerance interval, it was concluded that glycoalkaloid levels in event W8 are no different from the Russet Burbank control.

9.5 Dietary Risk Assessment of VNT1 protein

The similarity of the *Rpi-vnt1* gene sequence to disease-resistance (R) genes found in edible plant species was examined (Appendix D: Dietary Exposure to *Rpi-vnt1.1*). A Basic Local Alignment Search Tool (BLAST) homology search was conducted to identify edible plant species containing DNA sequences or amino acid sequences with similarity to the Potato Late Blight Resistance gene (*Rpi-vnt1)* sequence. Homology of *Rpi-vnt1* with tomato genes identified in the search was >90% with very low E-values, indicating significant homology with *Rpi-vnt1*.

The overall results of the nucleotide BLAST searches show that the *Rpi-vnt1* (FJ423044.1) gene shares greater than 90% sequence identity with tomato mosaic virus (ToMV) resistance genes. In addition, the VNT1 protein (ACJ66594.1) is greater than 75% homologous to ToMV Tm-2 proteins from *S. lycopersicum* (tomato) and *S. tuberosum* (potato) at the amino acid level. Taken together, results from the bioinformatics analyses suggest that genes highly similar to *Rpi-vnt1* at the nucleic acid and protein levels are present in the human diet as constituents of *S. tuberosum* L. (potato) and *S. lycopersicum* (tomato) / *Lycopersicon esculentum* (tomato, older nomenclature). Similar R-genes have been used in classical breeding for late blight resistance for over 50 years (Malcolmson and Black 1966).

Given the high degree of homology between *Rpi-vnt1* and R-proteins (e.g. ToMV Tm-2) present in tomato, existing dietary exposure to tomato varieties with the ToMV Tm-2 resistance gene could be

relied upon to establish the history of dietary exposure to the protein product of the *Rpi-vnt1* gene. Although a literature search did not yield information about the levels of ToMV resistance proteins in tomatoes or in diets, ToMV resistant tomato varieties and rootstocks are readily available and grown in the US for fresh market tomato production. Given the large per capita consumption of fresh market tomatoes in the United States, consumers have likely been exposed to tomatoes containing homologous ToMV resistant genes and associated proteins in their diets.

Widely consumed plant species that are reported to contain disease-resistance (R) genes include the Solanaceae family, *Zea mays* (maize), *Glycine max* (soybeans), *Oryza sativa* L. (rice), and *Triticum aestivum* L. (common wheat) (Bakker et al. 2011). Potato belongs to the Solanaceae family, which includes eggplant (*S. melongena* L.), pepper (*Capsicum annum* L.), and tomato (*S. lycopersicum* L.). In addition to these common cultivated crops, the *Rpi-vnt1* gene is identical to the *Rpi-phu1* gene (Rodewald and Trognitz 2013) found in some *S. phureja* species, a potato species consisting of many varieties which are currently consumed by indigenous people in South America.

In summary, the presence of similar or identical genes in frequently consumed tomatoes and potatoes that have not had negative impacts on human health establish a history of safe use.

9.6 Low levels of VNT1 protein

A sensitive method was developed for detection of the *S. venturii* derived R-protein, *Rpi-vnt1*, using polyclonal antibodies raised against VNT1 peptides and the purified VNT1-LRR domain. VNT1 confers strong resistance against *P. infestans*, the causal agent of potato late blight. The method is able to detect low picogram levels of VNT1 protein. Using immunoblot and quantitative western blot analyses, the VNT1 protein is below 30 ppb in potatoes transformed with the *Rpi-vnt1* gene.

A recent review discusses a need to consider alternative methods of safety assessment for intractable proteins, including R-proteins (Bushey et al. 2014). Intractable proteins are defined as those with properties that make it extremely difficult or impossible with current methods to express in heterologous systems, isolate, purify, or quantitate due to low levels. A specific example is given for the protein product of the *Rpi-blb1* gene, which also confers resistance to late blight. The limit of detection for the BLB1 protein was found to be 100 ppb, and the protein was not detectable using an ELISA method. The same authors recommend a weight of evidence approach, like we have presented here, to establish safety of this type of protein.

9.7 Safety of Nucleic Acids

In 2001, EPA established an exemption from the requirement for a tolerance for residues of nucleic acids that are part of a PIP (40 C.F.R. 174.507) under the Federal Food, Drug, and Cosmetic Act (FFDCA), noting that "[n]ucleic acids are ubiquitous in all forms of life, have always been present in human and domestic animal food and are not known to cause any adverse health effects when consumed as part of food" (66 Fed. Reg. 37817, July 19, 2001). FDA reached a similar conclusion, stating that nucleic acids were "generally recognized as safe" for purposes of FFDCA (57 Fed Reg. 22984, 22990, May 29, 1992).

9.8 Safety of Gene Silencing Methods

Many crops, including tomato, squash, soybean, papaya, potato, and plum, with traits that resulted from RNAi, have been deregulated by APHIS and evaluated for food safety by the FDA. In many of these

products, a small piece of RNA interferes with production of an enzyme, and thus influences a quality or nutritional trait. The Russet Burbank event W8 contains gene silencing cassettes for *Asn1, Ppo5, R1, PhL,* and *VInv*, all of which result in small RNAs that regulate gene expression. Such small RNA (sRNA), including siRNA, miRNA, and piRNA, in plants and animals are generally involved in regulating endogenous gene expression, repressing transposons, or targeting invading pathogens for destruction. The sRNA are ubiquitous in nature, including prokaryotes where sRNA have also been associated with the antiviral CRISPR pathway (Karginov and Hannon 2010). All of these pathways rely upon an RNase III endonuclease to process a double-stranded RNA (dsRNA) precursor into small effector RNAs that can be used to target RNA or DNA for modification or destruction. Due to bacterial colonization of our intestines and our daily diets of plants, animals, and fungi expressing their own spectra of sRNA, we are constantly exposed to a multitude of sRNA.

A publication by Chen-Yu Zhang's team claimed that a plant-derived miRNA had the potential to survive substantial obstacles to elicit a biological activity in the liver of humans and mice (L. Zhang et al. 2012). The implications of these findings led to a number of studies aimed at reproducing the author's study. However, these claims have not been substantiated (Dickinson et al. 2013; Snow et al. 2013; Witwer et al. 2013; Y. Zhang et al. 2012), and have been challenged by many experts in the field leading to self-correction of the scientific literature through publication of these numerous failed replication studies (Editorial 2013).

The results of the Zhang manuscript were central to the argument put forth by Jack Heinemann and colleagues in a communication calling for more rigorous safety testing of RNAi-based biotech products due to potential off-target effects of sRNA (Heinemann et al. 2013). The concerns of Heinemann and colleagues were thoughtfully considered by fellow scientists associated with the bi-national governmental regulatory agency, Food Standards Australia New Zealand (FSANZ), which evaluates food safety requirements for biotech foods. In their formal response, FSANZ concluded, "The weight of scientific evidence published to date does not support the view that small dsRNA in foods are likely to have adverse consequences for humans" (FSANZ 2013).

The history of safe use, complexity of the human GI tract, irreproducibility of the cited controversial manuscript (L. Zhang et al. 2012), our compositional and nutritional data, and the unique characteristics of Innate™ potatoes collectively establish these potatoes as safe for human consumption. In fact, there is no scientific rationale to suggest that sRNA present in GM-foods are any less safe than those naturally abundant and safely consumed in our current diets.

Stability through Microvesicles or Protein Complexes. Another possible mechanism to increase stability of siRNA would be for the plant to package them into microvesicles or apoptotic bodies or bind them to large protein or lipid-protein complexes. Plants are not thought to package cellular material within apoptotic bodies or microvesicles for destruction by other cells, as is done in animals. Instead, during programmed cell death, they concomitantly shrink their protoplasm while destroying cellular contents in an effort to contain a pathogen within the original cell structure to maintain structural integrity (van Doorn et al. 2011). A large amount of programmed cell death would thus be associated with sick plants that are not included in the food production process.

However, the biological activity of sRNA is linked to association with RNA induced silencing (RISC) complexes. A number of distinct cellular pathways exist in plants and animals for processing sRNA and executing their biological activities, where each pathway includes protein complexes that bind to longer dsRNA, siRNA duplexes, or the sRNA species (Pumplin and Voinnet 2013). These protein complexes are

considered critical for stabilizing sRNA as unincorporated sRNA (i.e. passenger strand) are more rapidly turned over. Turchinovich and colleagues found that the vast majority (>97%) of miRNAs identified in their culture media and plasma samples were not contained within vesicles, but were instead protected from degradation by a protein involved in the RISC complex (Turchinovich et al. 2011).
In summary, there is evidence that siRNA may be protected by association with RISC complexes within and outside of cells. However, since protein transport across cell membranes is highly regulated, these complexes may protect sRNA from degradation and prevent their indiscriminate uptake by human cells. The challenges of packaging sRNA have been realized by the pharmaceutical industry who have spent considerable amounts of time and effort attempting to develop techniques aimed at optimizing the stability, delivery, distribution, and pharmokinetics of sRNA for use as orally delivered therapeutics with limited success (Castanotto and Rossi 2009; Scaggiante et al. 2011). In fact, one of the groups that rebutted the work by the Zhang group, miRagen Therapeutics, could have benefitted from confirmation of those studies.

Uptake of sRNA in Animals. While genes in some simple organisms can be targeted through feeding upon organisms expressing double-stranded RNA (dsRNA), this is highly unlikely in higher organisms, such as humans. Humans have complex GI tracts that present numerous obstacles to the uptake of dietary RNA, have many more cells to prevent non-specific accumulation, and lack components of the RNAi pathway (e.g. RNA-dependent RNA polymerases) that could amplify and sustain a non-specific response (Petrick et al. 2013). In addition to the plethora of sRNA consumed through a normal diet, humans possess trillions of microbes within their digestive tracts that can both absorb and secrete their own sRNA, which have also been detected in human plasma samples (Wang et al. 2012).

Bioactivity of plant-derived sRNA in mammalian cells. The Lam lab investigated plants as a delivery system for siRNAs that could target viruses in consumers (Zhou et al. 2004), whereas the Lee lab explored the potential of using plants as an economical factory for production of siRNA (Chau and Lee 2007). These conflicting datasets are the only mammalian studies we are aware of that address bioactivity of plant sRNA in mammalian cells, but a study was performed in the model organism, *Caenorhabditis elegans*, which is a highly-sensitive system for inducing and detecting RNA interference activity. Consistent with the results of Chau et al., they did not find biological activity of plant-derived siRNA (Boutla et al. 2002; Chau and Lee 2007). Interestingly, they were able to induce an RNAi-mediated phenotype when injecting longer dsRNA derived from plants. These results may suggest the structure of plant siRNA are inconsistent with animal systems or that exogenous siRNA are much less efficient at inducing a biological phenotype than dsRNA being processed by the cell's own machinery. It remains unclear whether the very modest phenotype reported by the Lam group is dependent upon RNAi as they were treating cells with impure samples, including longer dsRNA that may have activated a cellular immune response.

Processing of dsRNA from inverted repeats in plants can produce multiple classes of sRNA, including 21-22 nt and 24 nt species. The 24 nucleotide population is especially unlikely to have RNAi activity in animals as they are not involved in degradation of target transcripts even in plants (Fusaro et al. 2006).

Summary of RNAi safety. In summary, humans consist of cells, tissues, and organs that remain homeostatic in the presence of varying diets consisting of abundant sRNA. It is highly unlikely that a sufficient quantity of these sRNA would survive the GI tract and accumulate in a given human cell resulting in a short-term, let alone a long-term, biological effect. In addition, the human body possesses a number of immune regulatory pathways dedicated to specifically detecting and destroying exogenous dsRNA as a means of protecting against foreign invaders.

Scientific rationale of the safety of orally ingested siRNA(s) derived from Innate™ potatoes. As described previously, the scientific literature does not support a model whereby sRNA present in consumed food pose a safety risk to humans following consumption (Petrick et al. 2013). In contrast there is a long record of safe consumption of sRNA within our natural diet. There are a number of important characteristics of our Innate™ potatoes and their use of RNAi:

- The Innate™ potatoes rely upon potato genomic DNA to initiate gene silencing using the plant's endogenous pathway. The inverted repeat sequence is derived from the sequence of the genes that are already being expressed in the potato.
- Many common potato preparation or cooking practices involve heating at high temperatures, which result in the conversion of asparagine with sugar into acrylamide, which has been associated with health concerns (Health Canada 2013). Innate™ potatoes use RNAi to reduce the accumulation of the precursor asparagine to limit acrylamide potential. Thus, Innate™ potatoes provide a consumer product with potentially enhanced safety.
- Processing of potatoes by consumers or the food industry involves treatments that are likely to limit the amount of sRNA in the final product. In addition to high temperature heating, treatments such as blanching, frying, dehydration, and freezing are commonly used, which lead to degradation and fragmentation of double-stranded genomic DNA. A similar fate is expected for sRNA as was shown in processed milk (Chen et al. 2010).
- The Innate™ potatoes under consideration do not target an evolutionarily conserved exogenous animal gene as might be the case when RNAi is used as a plant incorporated protectant. Since RNAi in Innate™ potatoes exclusively target plant genes, they are less likely to have adverse off-target effects in animals.
- We have performed rigorous compositional, nutritional, and agronomic analyses and have not observed any evidence of off-target effects in the plant where expression of sRNA was the highest and the potential for off-target effects greatest.

Numerous physiological barriers have impeded introduction of nucleic acid through oral uptake (O'Neill et al. 2011), and as noted previously, there is a long history of safe use associated with eating foods containing sRNA due to its ubiquitous presence in nature (Ivashuta et al. 2009; Jensen et al. 2013; Petrick et al. 2013). Mechanistic studies of a number of cultivars have shown plants selected for agronomic traits using conventional techniques are using RNA interference to silence their own genes through expression of inverted repeats (Della Vedova et al. 2005; Kusaba et al. 2003; Tuteja et al. 2004).

Comments presented to the EPA's Scientific Advisory Panel Public Meeting on RNAi technology as a pesticide, held January 28, 2014, included support for the safety of dsRNA by experts in the field (Mello 2014). Dr. Mello reported that oral uptake of dsRNA has proven unfeasible as a drug delivery route, thus unlikely to cause off-target effects when used for gene silencing in plants. He also reported that ingested RNA is rapidly metabolized in the gut where it is converted to nutrients, thus proposing that bioinformatics testing for similar sequences in humans would be unnecessary. Also, RNA is digested rapidly, suggesting that digestibility assays would be unnecessary for dsRNA.

In summary, we believe the history of safe use, the irreproducibility of the cited controversial manuscript (L. Zhang et al.2012), the submitted compositional and nutritional data, and the unique

characteristics of Innate™ potatoes collectively establish these potatoes as safe for human consumption.

9.9 Previous regulatory actions for plant incorporated protectants

The safety assessment of the Potato Late Blight Resistance Gene is based partly on previous regulatory actions for similar resistance genes that are considered plant incorporated protectants by EPA. For example, a rationale similar to that used for Coat Protein Gene of Plum Pox Virus (US EPA 2010) and Potato Leaf Roll Virus (PLRV) Resistance Gene (US EPA 2000) could be considered for safety of late blight resistance gene. Like both Coat Protein Gene of Plum Pox Virus and PLRV Resistance Gene, late blight resistance has been part of the food supply for many years without adverse effects. There is a long history of safe consumption of similar resistance genes throughout the food supply. Also, like the Coat Protein Gene of Plum Pox Virus and PLRV resistance, safety is based in part on a history of safe consumption of nucleic acids, and dietary prevalence of similar genes. The mechanism of action for both Plum Pox Virus and PLRV resistance resulted from gene silencing. In contrast, late blight resistance from *Rpi-vnt1* relies on synthesis of a protein, however, the VNT1 protein levels are too low to detect.

The EPA's Biopesticides Registration Action Document (BRAD) for the Coat Protein Gene of Plum Pox Virus (US EPA 2010) includes a summary of Data Waivers for Toxicology and the Environmental Assessment. Toxicology waivers were granted based on 1) "There is a long history of consumption of plant virus particles in food without any known toxicity or other deleterious health effects, and 2) Non-occupational exposure is minimal to non-existent since the gene is only expressed within plant tissue." The waivers for Non-target Organisms included a rationale that *Prunus* species are difficult to breed with domestic or wild relatives and result in few hybrids with low vigor and fertility. Also, there is a long history of consumption of plant viruses in foods consumed by animals without deleterious effects or evidence of toxicity. Exposure to nontarget aquatic species or terrestrial insects, including honey bees, is not expected since the gene is only expressed within the plant genome. Even if the plum pox virus coat protein was produced in plum, the coat protein does not match known toxins or allergens.

EPA's approval of the Potato Leaf Roll Virus Resistance Gene also included data waivers for toxicology and the environmental assessment. The justification for waivers for toxicology was "The long history of consumption of virus-infected plants without any reports in the scientific literature of toxicity or other harm caused to the general population are sufficient to support the registration of the active ingredient potato leaf roll virus resistance gene (also known as orf1/orf2 gene)." Waivers of the data requirements for the environmental assessment were based on "the long history of virus-infected food plants as part of the domestic animal food supply without any reports of adverse effects." Also, the environmental fate studies were waived because of the lack of toxicity/pathogenicity associated with the active ingredient and lack of significant levels of expression. Requirements for nontarget insects, fish, and other wildlife were waived because "the lack of toxicity/pathogenicity and mitigating label language for aquatic exposure result in minimal to non-existent risk to wildlife."

Like the plum species, *Prunus domestica*, potatoes are difficult to breed because they are tetraploid, highly heterozygous and subject to inbreeding depression. The most popular potato variety in the US, Russet Burbank, is sterile. Potatoes will not readily breed with other varieties or wild relatives, and in the unlikely case where that could occur, true seeds are unlikely to survive, since only the potato tubers are typically kept for "seed".

9.10 Conclusions: Safety of Russet Burbank W8 Potato

In summary, we present a rationale for the safe use of W8 potatoes containing the Potato Late Blight Resistance Gene. Among the supporting evidence are the phenotypic data collected in a robust field trial program at 11 sites over two crop years. From those studies, a compositional assessment shows the W8 potatoes substantially equivalent to Russet Burbank controls. Open reading frames associated with the DNA inserts, including the late blight resistance gene itself, were screened for allergen and toxin potential, but no safety concerns were identified. The W8 potatoes were tested for glycoalkaloids and found no different from the Russet Burbank controls and well below the accepted safety limit. Similarity of the *Rpi-vnt1* to other gene sequences in related species was assessed and greater than 90% homology discovered between this late blight resistance gene and the Tomato Mosaic Virus resistance gene (ToMV Tm-2) identified in tomatoes, which has been widely bred into tomato varieties in the fresh market and is consumed by humans.

Among the risk considerations is the presence of the VNT1 protein, and although effective at controlling late blight, levels in potato tubers are below our quantitation limit of 30 ppb. As noted in Section 9.7, nucleic acids have long been considered safe under the FFDCA by EPA and FDA. An in depth review is included on the topic of the safety of using RNA interference for gene silencing. Regulatory actions supporting safe use of the Coat Protein Gene of Plum Pox Virus and Potato Leaf Roll Virus Resistance Gene provide additional evidence of the safety of W8.

Accordingly, based on the results of the studies presented here, there is no reason to believe that the VNT1 protein will have any impact to human health through allergenicity or toxicity, or any environmental impacts to non-target mammals, birds, fish, or insects due to the low expression and lack of toxic effects of the protein. There is negligible risk of environmental contamination and no persistence in the environment because of the low expression of the VNT1 protein in potato tubers. The prevalence of similar resistance genes throughout edible crops suggests that extremely low levels of similar proteins are widespread in nature and unlikely to pose risk to human health, non-targets or the environment.

Based on the foregoing rationale for safety, the J.R. Simplot Company respectfully requests that it should be granted deregulated status for Russet Burbank event W8 containing the Potato Late Blight Resistance Gene.

9.11 References

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10.0 Trait Efficacy

Introduction

Russet Burbank Event W8 was created using Innate™ technologies in order to address the need of the potato industry to improve quality by increasing resistance to late blight, reducing expression of the enzyme responsible for black spot, reducing acrylamide and lowering levels of reducing sugars. In the following discussion, we provide evidence for the efficacy of these traits in Russet Burbank Event W8. Results of the trait efficacy studies are presented first, with methods grouped together following the results.

10.1 Composition and Field Results

10.1.1 Potato Free Amino Acids Results

W8 tubers contained less free asparagine, but more aspartic acid, glutamine, and glutamic acid than the control (Table 10-1). This change was expected because of silencing *Asn1* and is linked to a reduced acrylamide-forming potential. The mean values of asparagine, aspartic acid, glutamine, and glutamic acid for W8 were all within the tolerance intervals and therefore considered within the normal range for potatoes. A review of the biosynthetic pathway for glutamine and asparagine in Figure 10-1 illustrates how a reduction in asparagine could lead to increases in these other amino acids.

Table 10-1. Potato Free Amino Acids at Harvest

Compound	Variety	LSMean	P-value[1]	N	Range		TI[2]	
					Min	Max	Min	Max
Asparagine (mg/100g)	W8	87.8	**<.0001**	41	62.1	140	.	.
Asparagine (mg/100g)	Burbank	300	.	41	198	469	60.0	482
Aspartic Acid (mg/100g)	W8	45.2	**0.0032**	41	32.8	63.9	.	.
Aspartic Acid (mg/100g)	Burbank	40.4	.	41	27.9	52.7	12.6	72.8
Glutamine (mg/100g)	W8	252	**<.0001**	41	203	335	.	.
Glutamine (mg/100g)	Burbank	139	.	41	95.7	198	23.3	260
Glutamic Acid (mg/100g)	W8	51.3	**<.0001**	41	27.9	75.5	.	.
Glutamic Acid (mg/100g)	Burbank	40.8	.	41	26.0	72.0	10.2	80.3

[1]P-values indicating significant differences between W8 and control are in bold and underlined.
[2]TI = Tolerance Interval
. = Not applicable

Figure 10-1. Biosynthesis of GLN and ASN in plants

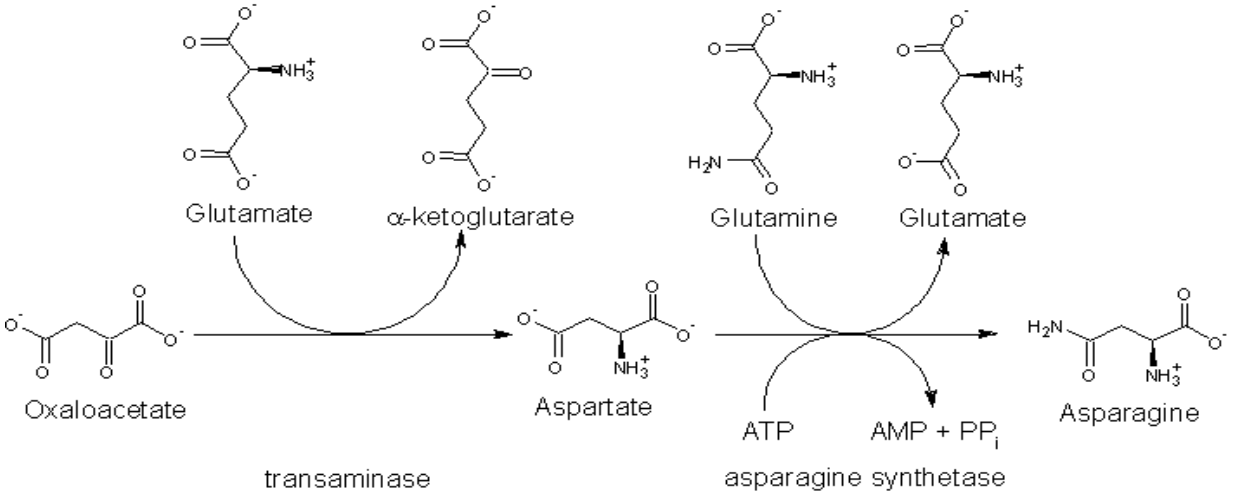


Figure 10-1 is from New World Encyclopedia, http://www.newworldencyclopedia.org/entry/Asparagine.

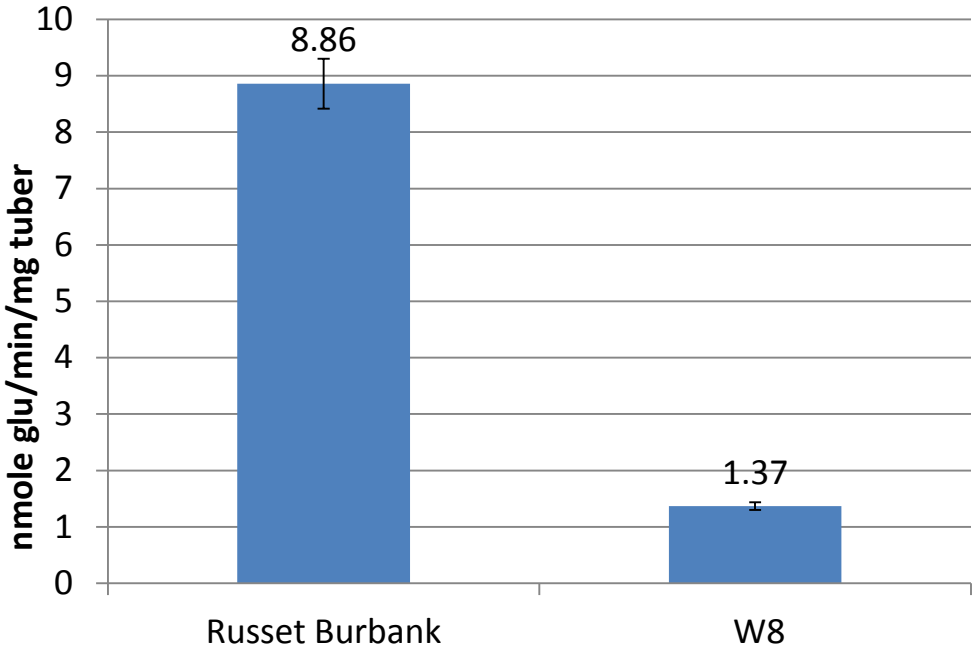
10.1.2 Reducing Sugars and Invertase Silencing

The W8 event contains expression cassettes that could lower levels of reducing sugars in tubers by multiple mechanisms. Through the transformation with pSIM1278, we introduced a silencing cassette for the promoters of the starch associated gene (*R1*) and the phosphorylase-L gene (*PhL*) whereas transformation with pSIM1678 introduced a silencing cassette for the invertase gene (Ye et al. 2010). Together, these traits function by slowing the conversion of starch and sucrose to reducing sugars (glucose and fructose). Although silencing of *R1* and *PhL* resulted in lowered levels of reducing sugar when analyzed at one month after harvest (Collinge and Clark 2013), the major reduction in reducing sugar appears to be related to invertase silencing. Overall benefits of silencing *R1, PhL, and VInv* include improved quality, especially relating to color control, and thus contributing to the desired golden brown colors required by most french fry or chip customers. Also, the reducing sugars react with amino acids, such as asparagine, to produce Maillard products including acrylamide.

Invertase Activity

The *VInv* gene silencing cassette in pSIM1678 results in decreased levels of vacuolar invertase, an enzyme which converts sucrose into glucose and fructose. When levels of invertase are decreased in potatoes, reducing sugars glucose and fructose remain at low levels during storage while sucrose increases, especially when held below typical storage temperatures of 46 - 48°F for french fry potatoes. Before testing for invertase activity, tubers were stored at 39°F for one month. Three replicates for each of W8 and Russet Burbank control were used for the assay which was measured by the accumulation of glucose. W8 showed an 85% reduction in vacuolar invertase activity in cold-stored tubers compared to the control (Figure 10-2). The reduced vacuolar invertase activity in W8 tubers is associated with reduced RNA accumulation from the *VInv* gene (Chapter 7: Characterization of Gene Silencing and Target Gene Expression, Figure 7-2) and lower levels of reducing sugars glucose and fructose (Tables 10-2, 10-3).

Figure 10-2. Enzymatic Activity of Invertase in W8 and Russet Burbank Tubers



Potato tubers were stored at 4°C for 30 days. Three independent tubers of each variety were used for the assay. The activity was measured by the accumulation of glucose in units of nmol·min^{-1}·mg tuber^{-1}.

Changes in Reducing Sugars and Sucrose

Long-term cold storage is necessary to maintain an adequate supply of high quality potatoes for year-round processing into french fries and potato chips, but also leads to cold-induced sweetening (CIS). CIS causes unwanted side effects in potato products processed at high temperatures including flavor changes, unwanted dark colors and elevated amounts of acrylamide. Vacuolar acid invertase (*VInv)* is an enzyme that is critically important in the CIS process in increasing the amount of glucose and fructose in tubers stored at very low temperatures (Zrenner et al. 1996). W8 has suppressed expression of the *VInv* gene and therefore has reduced glucose and fructose levels in cold storage and less CIS compared to the Russet Burbank control. In order to demonstrate efficacy of the traits leading to lowered reducing sugar, field-grown tubers of W8 and the untransformed control were analyzed at harvest and at normal (46°F) and cold (38°F) storage temperatures.

Reducing sugars, glucose plus fructose, and the non-reducing sugar, sucrose, were tested in W8 at the time of harvest and then after 3, 6, and 9 months of storage. Two different storage temperatures were used, 46˚F, which is typically used for Russet Burbank potatoes destined for frozen french fries, and 38˚F, a lower temperature enabled by silencing *VInv,* possibly allowing for better quality without high levels of reducing sugars. At harvest and all storage time points and temperatures, W8 tubers contained lower levels of reducing sugars compared with the control (Tables 10-2 and 10-3). The decrease in reducing sugars was expected from silencing the *VInv* gene. All sugar values for W8 at the time of harvest were within the tolerance interval, indicating compositional equivalence to the controls.

Table 10-2. Potato Sugar Levels at Harvest and After Storage at 46°F

Timing	Variety	LS Mean	P-value[1]	N	Range		Tolerance Interval	
					Min	Max	Min	Max
Fructose + Glucose (mg/100g)								
Fresh	W8	38.4	**0.0002**	41	9.68	106	1.00	616
	Control	146	.	41	14.0	406		
Month 3	W8	122	**0.0056**	9	54.1	210	1.00	616
	Control	483	.	9	298	598		
Month 6	W8	116	**<.0001**	9	20.8	310	1.00	616
	Control	261	.	9	153	459		
Month 9	W8	106	**0.032**	9	79.7	160	1.00	616
	Control	224	.	9	105	372		
Sucrose (mg/100g)								
Fresh	W8	395	**<.0001**	41	161	775	1.00	503
	Control	241	.	41	113	558		
Month 3	W8	651	**<.0001**	9	520	738	1.00	503
	Control	148	.	9	56.2	228		
Month 6	W8	202	**0.0021**	9	177	229	1.00	503
	Control	97.6	.	9	80.1	144		
Month 9	W8	146	**<.0001**	9	105	201	1.00	503
	Control	56.9	.	9	44.8	77.3		

[1]P-values indicating significant differences between W8 and control are in bold and underlined.
. = Not applicable

Table 10-3. Potato Sugar Levels When Stored at 38°F

Timing[1]	Variety	LSMean	P-value[2]	N	Range		TI[3]	
					Min	Max	Min	Max
Fructose + Glucose (mg/100g)								
Month 6	W8	91.7	**0.0002**	3	83.7	97.4	1.00	616
	Control	640	.	3	590	726		
Month 9	W8	151	**<.0001**	3	102	188	183	616
	Control	754	.	3	703	788		
Sucrose (mg/100g)								
Month 6	W8	963	**<.0001**	3	945	986	1.00	503
	Burbank	182	.	3	138	206		
Month 9	W8	645	**<.0001**	3	598	714	1.00	503
	Burbank	152	.	3	137	163		

[1]Testing occurred at 6 and 9 months only because the purpose of the study was to evaluate long term storage at 38°F.
[2]P-values indicating significant differences between W8 and control are in bold and underlined.
[3]TI = Tolerance Interval
. = Not applicable

All W8 tubers contained more sucrose (Table 10-2 and 10-3) than control samples at harvest and after multiple storage time points at both 38˚F and 46˚F. The increase in sucrose was expected from silencing the *VInv* gene, thus inhibiting the conversion of sucrose into the reducing sugars glucose and fructose. The mean levels of sucrose for W8 were greater than the upper boundary of the tolerance interval when potatoes were stored at 38˚F for 6 or 9 months and after 3 months at 46°F. The sucrose values exceeded the tolerance interval because invertase silencing resulted in reduced conversion of sucrose to the

reducing sugars glucose and fructose. When levels of invertase are decreased in potatoes, reducing sugars glucose and fructose remain at low levels during storage while sucrose increases. Therefore, sucrose may exceed typical levels and ideally glucose and fructose may be lower than in control potatoes.

The net result of silencing the *VInv* gene in W8 is lower levels of reducing sugars and higher levels of sucrose observed at the time of harvest and throughout the storage period of up to 9 months. Reducing sugars in W8 increase with storage time, but remain consistently lower than the Russet Burbank control. Much lower levels of reducing sugars were observed in W8 compared with controls when stored at 38˚F, suggesting that lower temperature storage could be feasible for W8. In all cases, significant decreases in reducing sugars are coupled with higher levels of sucrose. It would be expected that lower temperature storage results in less shrink from respiration, but also would reduce losses from disease.

10.1.3 Acrylamide

Reduced asparagine levels in W8 (Table 10-1) result from the intended silencing of the asparagine synthase-1 gene (*Asn1*). Lowered asparagine, fructose, and glucose levels lead to an overall reduction of acrylamide in processed potato products because they are reactants in the formation of acrylamide. In order to demonstrate the efficacy of reducing acrylamide, field-grown tubers of W8 and the control were analyzed at harvest and at normal (46°F) and cold (38°F) storage temperatures.

At the time of harvest, french fries made with W8 tubers contained 85% less acrylamide than the control (Table 10-4). When potatoes were stored throughout nine months at 46˚F, acrylamide levels in W8 were 78 to 83.7% lower than control Russet Burbank (Table 10-4). Acrylamide levels in W8 potatoes were somewhat higher after storage at 38˚F for 6 to 9 months (Table 10-5), but consistently much lower than controls. These significantly lower acrylamide levels in W8 were expected from silencing the *Asn1* and *VInv* genes, thus reducing the reactants free asparagine and reducing sugars. Similar reductions in reducing sugars and acrylamide were reported by Zhu et al. (2014).

Table 10-4. French Fry Acrylamide Levels (ppb) at Harvest and After Storage at 46°F

Timing	Compound	Variety	LSMean (ppb)	P-value[1]	Percent Reduction[2]	N	Range (ppb)		TI[3] (ppb)	
							Min	Max	Min	Max
Fresh	Acrylamide	W8	75.3	**<.0001**	85.0	41	32.7	185	10.0	1035
		Burbank	503	.	.	41	229	971		
Month 3	Acrylamide	W8	86.1	**<.0001**	80.9	9	74.5	94.3	10.0	599
		Burbank	450	.	.	9	393	514		
Month 6	Acrylamide	W8	68.3	**0.0011**	83.7	9	50.4	96.2	10.0	688
		Burbank	420	.	.	9	330	528		
Month 9	Acrylamide	W8	115	**0.0013**	78.2	9	90.7	156	10.0	1047
		Burbank	528	.	.	9	429	740		

[1]P-values indicating significant differences between W8 and control are in bold and underlined.
[2]Percent Reduction in Acrylamide is relative to control Russet Burbank at same storage time.
[3]TI = Tolerance Interval
. = Not applicable

Table 10-5. French Fry Acrylamide Levels at Harvest and After Storage at 38°F

Timing[1]	Compound	Variety	LSMean (ppb)	P-value[2]	Percent Reduction[3]	N	Range (ppb)		TI[4] (ppb)	
							Min	Max	Min	Max
Month 6	Acrylamide	W8	203	**<u><.0001</u>**	86.2	3	199	207	1155	1792
		Burbank	1473	.	·	3	1450	1500		
Month 9	Acrylamide	W8	212	**<u><.0001</u>**	90.8	3	201	234	761	3839
		Burbank	2300	.	·	3	2160	2380		

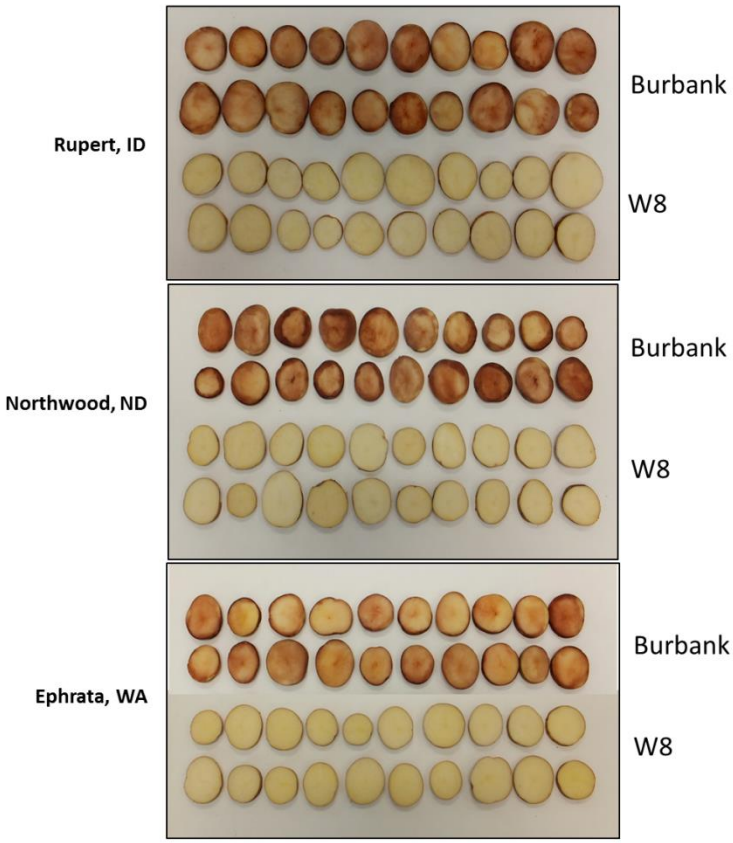
[1]Testing occurred at 6 and 9 months only because the purpose of the study was to evaluate long term storage at 38°F.
[2]P-values indicating significant differences between W8 and control are in bold and underlined.
[3]Percent Reduction in Acrylamide is relative to control Russet Burbank at the same storage time.
[4]TI = Tolerance Interval
. = Not applicable

10.1.4 Black Spot

Black spot is a discoloration affecting potato tubers and is a result of leakage of polyphenol oxidase (PPO) from damaged plastids into the cytoplasm. In the cytoplasm, the enzyme oxidizes polyphenol, which then form dark precipitants. In order to reduce black spot in W8, the potato *Ppo5* gene, was silenced. To demonstrate efficacy of this trait, an indirect method was used to test for black spot tolerance based on applying catechol to the cut surfaces of tubers.

Black Spot Analysis Results
Results of the catechol assay to determine PPO activity are presented in Figure 10-3. All W8 tubers remained unchanged after addition of catechol. All Russet Burbank control tubers turned darker in color after addition of catechol. These results indicate that W8 tubers are more resistant to black spot than tubers of the control, and support the efficacy of the reduced black spot trait.

Figure 10-3. Catechol Assay for Polyphenol Oxidase Activity

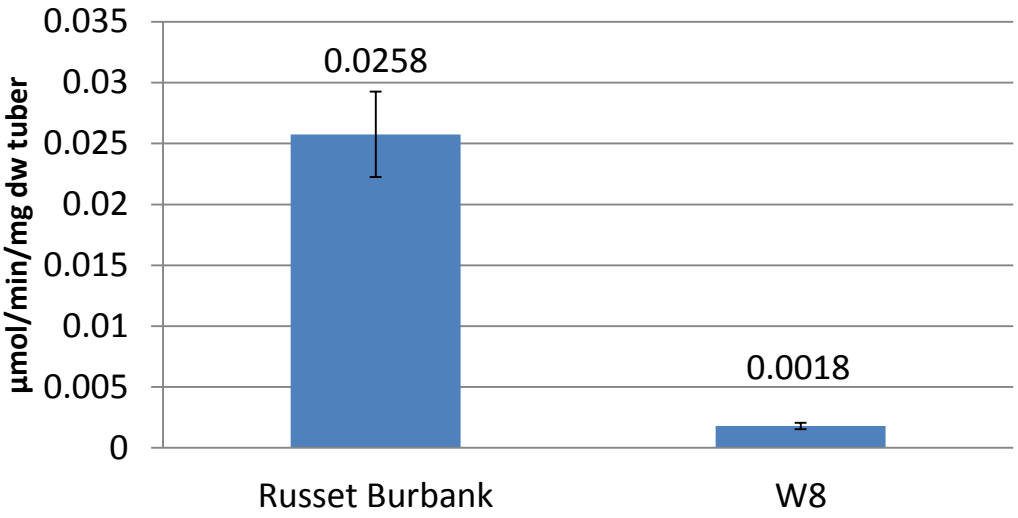


Enzymatic Assay of PPO Activity in W8

Additional tests were conducted to show that reductions in *Ppo5* could be measured by enzymatic assay. An enzymatic assay shows that W8 has a 90% decrease in PPO activity (0.025 µmol·min^{-1}·mg dw tuber^{-1}), compared to the Russet Burbank control (Figure 10-4).

The reduced activity in W8 tubers was intentional and associated with reduced black spot through silencing of the *Ppo5* gene (Chapter 7: Characterization of Gene Silencing and Target Gene Expression, Figure 7-2).

Figure 10-4. PPO Activity in Russet Burbank and W8 Tubers



PPO Activity in Russet Burbank and W8 Tubers. Conversion of L-DOPA to dopachrome was monitored over time by measuring A_{474nm}. $\Delta A_{474nm} \cdot min^{-1}$, which was converted to units of $\mu mol \cdot min^{-1} \cdot mg\ dw\ tuber^{-1}$.

10.1.5 Late Blight Resistance

The Potato Late Blight Resistance Gene has been added to W8, successfully conferring late blight resistance. To demonstrate the efficacy of late blight resistance, studies were conducted by inoculating both foliage and tubers. Tests with both foliage and tubers were quantitative trials conducted to demonstrate the efficacy of late blight resistance in W8 to common strains in the U.S. (Table 10-6). The foliage or tubers were inoculated with a set amount of inoculum so that W8 and the control would be treated equally. In the foliar tests, the inoculum was applied in late July or August and the plants were evaluated based on % foliar infection. These tests were conducted by Pennsylvania State University, Michigan State University, and by an independent researcher familiar with potato diseases in northern Idaho.

Tests of late blight resistance were conducted on tubers supplied by Simplot to scientists at Michigan State University. Tubers were evaluated by comparing % tuber infection in W8 and the control potatoes after being inoculated.

Table 10-6. Inoculated Late Blight Study Information

Year of Study	Study	Site State	Trial Type	Material Tested	Trial Design[1]	N per entry	Inoculation Date	Inoculum Genotype
2012	Tubers	WA	Lab	W8, Control	RCB, 3 Reps	4	Lab Assay	US-22 and US-8
2013	Foliar	MI	Field	W8, Control, TX278[2], Bintje[2], Nicolet[2], Golden Sunburst[2]	RCB, 4 Reps	12	7/26/2013	US-22 and US-23
2013	Foliar	PA	Field	W8, Control, TX278[2], Bintje[2], Nicolet[2], Golden Sunburst[2]	RCB, 4 Reps	12	8/8/2013	US-23
2013	Foliar	ID	Field	W8, Control, TX278[2], Bintje[2], Nicolet[2], Golden Sunburst[2]	RCB, 4 Reps	12	8/1/2013	US-8

[1]RCB=Randomized Complete Block design. Number of blocks was equal to the number of reps.
[2]Reference varieties used as a part of the conventional variety range calculation.

Foliar Late Blight Test Results

At the end of the trial period, the last rating from each site is summarized in Table 10-7 to show the effect of the potato late blight resistance gene on foliar resistance in W8. Each trial site had different strain inoculum that included US8, US22, or US23, depending on the strains that were found in that area.

Table 10-7. Percent Foliar Late Blight Infection at Last Rating

Variety	Mean Percent Foliar Late Blight Infection	P-value[1]	CVR[2]
Control	58.3	**<.0001**	18.8 - 100
W8	0.50	.	.

[1]P-values indicating significant differences between W8 and control are in bold and underlined.
[2]CVR = Conventional variety range. The range of mean values of conventional varieties.
. = Not applicable

A significant reduction in percent foliar late blight infection was detected for W8 compared to the Russet Burbank control (Table 10-7). This supports the conclusion that the potato late blight resistance gene confers resistance in W8 and is efficacious in the foliage.

Tuber Late Blight Results

Tuber infection rate determined by percent infection is summarized in Table 10-8.

Table 10-8. Percent Tuber Late Blight Infection

Isolate	Variety	Mean Percent Late Blight Tuber Infection	P-value[1]
US-22	Control	100.0	.
US-22	W8	51.0	**<0.0001**
US-8	Control	67.0	.
US-8	W8	21.1	**<0.0001**

[1]P-values indicating significant differences between W8 and control are in bold and underlined.
. = Not applicable

A significant reduction in percent late blight infection in tubers was detected for W8 compared to the control (Table 10-8) for both US-22 and US-8 isolates.

10.2 Conclusions on Trait Efficacy

Significant decreases in free asparagine and reducing sugars in W8 contribute to substantial reductions in acrylamide in french fries compared with the control. In addition, silencing of invertase significantly lowers reducing sugars, potentially allowing for long term storage of potatoes at colder temperatures such as 38˚F. An enzymatic assay for vacuolar invertase activity confirmed a reduction of 85% in tubers of W8 compared to the control. Ultimately, results of this study demonstrate the efficacy of the constructs to lower reducing sugars, free asparagine, and acrylamide at harvest and after storage at normal and cold temperatures for at least 9 months.

Samples of W8 tubers from multiple field trial sites all showed strong resistance to black spot as demonstrated through the catechol assay. In addition, the enzymatic assay showed a 90% reduction in PPO activity in tubers from W8 compared to the control.

Quantitative tests with late blight inoculum on tubers and foliage support the conclusion that the Potato Late Blight Resistance Gene confers strong resistance in both foliage and tubers of W8. Surprisingly, the information summarized in Table 11-5 Stressor Observations described in Chapter 11: Agronomic Evaluations, showed more late blight reported in W8 than controls. However, these observations are subjective rather than quantitative data dependent on the principal investigators professional opinion. The quantitative studies described in this chapter and the disease specificity studies described in Section 11.4 Disease Susceptibility Assessments should be far more definitive than the stressor observations.

The results of the studies with Russet Burbank Event W8 show the expected late blight resistance, along with reductions in black spot, reducing sugars, and acrylamide. Significant reductions in reducing sugars and acrylamide were observed at harvest and sustained throughout long term storage at both 38˚F and 46˚F. Lastly, activity for enzymes associated with the *VInv* and *Ppo5* genes are reduced as expected from gene silencing.

10.3 Analytical Methods for Trait Efficacy and Composition

10.3.1 Analytical Methods for Free Amino Acids, Sugars, Acrylamide

Sample Source, Preparation, and Analysis

Tubers were analyzed at harvest and after 3, 6, and 9 months of storage to determine the efficacy of the traits leading to decreased reducing sugars and acrylamide at harvest and throughout storage at normal and cold temperatures. Tubers for compositional analyses were collected from all sites in 2012 and 2013 at harvest. Tubers for compositional analyses at 3, 6, and 9 months at normal storage temperature of 46˚F (normal) were collected from all 2012 sites and tubers at the cold temperature storage temperature of 38°F (cold) were collected from Grant County, Washington. These locations have been summarized in Table 8-1 of the Compositional Analyses Chapter. Samples were obtained by randomly selecting 6 mid-sized tubers at harvest and 3 mid-sized tubers at 3, 6, or 9 months of storage from each replicate of W8 and control. Samples were powdered in an industrial blender with liquid nitrogen and stored at -70°C until analysis. For acrylamide testing, twenty pound samples (combined across replicates) of the potatoes were processed into french fries prior to analysis, using standard practices.

Analytical testing was conducted by Covance Laboratories, Inc. in Madison, WI or Greenfield, IN. Free amino acid levels were determined by Covance Laboratories using the Covance protocol FAALC_S:6 (Schuster 1988; Henderson 2000; and Barkholt and Jensen 1989). Sugar levels were determined by Covance Laboratories using High Performance Anion Exchange Chromatograph (HPAEC) equipped with a Pulsed Amperometric Detector (PAD) and following Covance protocol SWET_S:9 (Lilla et al. 2005; Dionex Technical Note 20). Acrylamide levels were determined by Covance Laboratories in Greenfield, IN using the Covance protocol ACMS_GRN_S:4 (FDA 2003; Musser 2003; FDA 2003).

Statistical Methods Free Amino Acids, Sugars, Acrylamide
The statistical analysis was performed by using SAS 9.3.

Data were analyzed using the following linear mixed model:

$$Y_{ij} = \alpha_i + \gamma_j + \varepsilon_{ij}$$

- α = mean of treatment (fixed)
- γ = rep (random)
- ε = residual random error

Where α_i denotes the mean of the i^{th} treatment (fixed effect), γ_j denotes the random rep effect, and ε_{ij} denotes the residual random error.

The tolerance intervals were calculated to contain, with 95% confidence, 99% of the values in the population. Although tolerance intervals are presented for attributes such as sugars, acrylamide, and asparagine, the primary purpose in presenting these data was to demonstrate trait efficacy. Therefore, mean values can be compared with the natural variability predicted by the TI, however, rather than equivalence, the purpose is to address quality issues such as lowering acrylamide and reducing sugars.

A step-wise approach was used to interpret any differences between event W8 and the control. First, statistical significance, $p < 0.05$, was determined for each attribute. If the p-value indicates no statistical significance, then W8 is considered equivalent to the control. Next, if the p-value indicated statistical significance, mean values were compared with the tolerance intervals (Table 10-9). If the means fell within the TI, they were considered within the normal range for potatoes.

A range of commercially-available, conventional varieties were selected for use in the tolerance interval calculation. They include varieties suitable for fresh use, for french frying, for chipping, and the conventional control. The following varieties were used to calculate tolerance intervals: Bintje, Golden Sunburst, Nicolet, Ranger Russet, Red Thumb, Russet Burbank, and TX278 (Table 8-5 in Compositional Assessment Chapter).

10.3.2 Black Spot Analysis by Catechol Assay

An indirect method to test for black spot tolerance, the polyphenol oxidase activity assay, was used to demonstrate trait efficacy. In this assay, two slices each from 10 tubers of W8 and the control were tested (Table 10-9). To conduct this polyphenol oxidase assay, 1-ml catechol (25 mM in 50 mM MOPS, pH6.5) was pipetted onto the cut surfaces of randomly chosen tubers for W8 and the control. The PPO-dependent development of a dark brown precipitate was assessed after 20 minutes.

Table 10-9. Origin of Tubers from 2013 Field Trials for PPO Assay

Site Code	Material Tested	Trial Design[1]
Minidoka County, ID	W8, Control	RCB, 4 reps
Grant County, WA	W8, Control	RCB, 4 reps
Grand Forks County,ND	W8, Control	RCB, 4 reps

[1]RCB=Randomized Complete Block design. Number of blocks was equal to the number of reps.

10.3.3 Foliar Late Blight Resistance Test Methods

Late Blight Field Trial Locations

During 2013, plants of W8 and the control were grown at several locations detailed in Table 10-6. With the exception of fungicide applications, normal pest control and maintenance practices consistent with potato production for the area were used to produce the crop. The entire trial was treated with the same agronomic inputs and pesticide and fertilizer applications to ensure uniformity from pre-season through harvest. Any fungicide treatment did not affect *P. infestans* infection and was discussed with and approved by study coordinator before application. **Plot Inoculation and Foliar Infection Rating**
Susceptible spreader rows were inoculated with *P. infestans* after row closure. Plots were inoculated with zoospores at a concentration of 8-12x10^4 sporangia/mL. Details on inoculation at each site are listed in Table 10-6. After inoculation, plots were irrigated regularly to induce a humid environment and facilitate spread of disease (Personal Communication with Joe Coombs, Michigan State University).

Foliar evaluations were carried out by agronomists and scientists with experience in evaluation of late blight infection in the field. Foliar infection of plots was analyzed approximately every week after the first symptoms of infection by estimating the percentage of foliar area affected. Observations were made until plant senescence conditions progressed enough to possibly interfere with disease ratings. The last foliar rating at each site is analyzed in this report to determine the effect of the potato late blight resistance gene on foliar late blight resistance in W8 (Personal Communication with Joe Coombs, Michigan State University).

Statistical Methods for Foliar Late Blight Test

The statistical analysis was performed by using SAS 9.3.

Data were analyzed 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. 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.

10.3.4 Tuber Late Blight Resistance Test Methods

Tuber Late Blight Inoculation Methods

In order to analyze tuber late blight resistance, samples of the event and control were obtained by randomly selecting 15 mid-sized tubers from each field replicate from the 2012 field site in Grant County, WA (described in Table 11-2). Four replicates of five tubers for each event and control were tested with *P. Infestans* genotypes US-22, US-8, and a negative control (water) for a total of 60 tubers of each event and control being tested for disease resistance. Tuber late blight development was evaluated using whole tuber sub–peridermal inoculation. All tubers were washed in distilled water to remove soil. The tubers were then surface-sterilized by soaking in 2% sodium hypochlorite solution for 4 hours. Tubers were dried in a controlled environment with forced air ventilation at 5950 l min^{1} at 15^{o}C in dry air (30% relative humidity) for four hours prior to inoculation. To inoculate the tubers, a single plug (4 mm diameter) was removed from the surface of the test tuber at the apical end about 1 cm from the dominant sprout to a maximum depth of 1 cm and a plug of mycelium plus agar about 5 x 4 mm (length x diameter) was placed into the cavity and the tuber tissue plug replaced. The wound was sealed with petroleum jelly. Each sub-peridermal inoculation contained about 2 x 10^{-5} sporangia per plug. Negative control tubers were inoculated with cold (4^{o}C) sterile distilled H_2O. The non-inoculated control tubers were inoculated with an agar plug. After inoculation, tubers were placed in the dark in sterilized covered plastic crates and returned to controlled environment chambers [Percival Incubator (Model I-36LLVL, Geneva Scientific, LLC, PO Box 408, Fontana, WI)]. The chambers were set at 10^{o}C and 95% humidity and the sample tubers were incubated for 40 days until evaluation (Personal Communication with Dr. Willie Kirk, Michigan State University).

Tuber Late Blight Infection Rating

Tubers were evaluated visually for percent late blight disease infection. Twenty tubers were cut longitudinally from apical to basal ends and the amount of internal and external late blight disease was assessed on a 0-100% percent scale for each tuber (Personal Communication with Dr. Willie Kirk, Michigan State University).

Statistical Methods for Tuber Late Blight Test

Data were analyzed using the following linear mixed model:

$$Y_{ij} = \alpha_i + \gamma_j + \varepsilon_{ij}$$

- α = mean of treatment (fixed)
- γ = rep (random)
- ε = residual random error

Where α_i denotes the mean of the i^{th} treatment (fixed effect), γ_j denotes the random rep effect, and ε_{ij} denotes the residual random error. A significant difference was established with a p-value < 0.05.

10.3.5 Enzymatic Assays for PPO and Invertase

PPO Assay

Tubers were ground under liquid N_2 to a fine power and stored at -80 °C. Protein was extracted by vortexing ~100 mg of powdered material in Tricine buffer (pH 7.5) followed by centrifugation @ 21,100 x g. The protein extracts from Burbank control lines were diluted 1:10 to maintain activity within a linear range. L-DOPA conversion to dopachrome was monitored spectrophotometrically by measuring $A_{474\ nm}$ at 8 sec intervals over a 10 min time course. The $\Delta A_{474nm} \cdot min^{-1}$ was converted to moles$\cdot min^{-1}$ using ε = 3700 $M^{-1} \cdot cm^{-1}$ for L-DOPA (Behbahani et al. 1993). Rates were determined by non-linear regression fitting to a single exponential using Sigma Plot 8.02 (SPSS Science, Inc.). All experiments were done with three biological replicates. L-DOPA conversion to dopachrome was monitored over time by measuring A_{474nm}. $\Delta A_{474nm} \cdot min^{-1}$ then converted to units of $\mu mol \cdot min^{-1} \cdot$mg dw $tuber^{-1}$.

Invertase Activity Assay

One gram of tuber samples was ground in liquid nitrogen to a fine powder. Approximately 500 mg of sample was transferred to a tube containing 1 ml of extraction buffer (50 mM HEPES-KOH, pH 7.5, 5 mM MgCl2·6H2O, 1 mM EDTA, 1 mM EGTA, 0.1% (w/v) Triton X-100, 10% (w/v) glycerol, 5 mM dithiothreitol, and 1 mM phenylmethlsulfonyl fluoride). Crude extracts were centrifuged at 15,000 rpm for five minutes and 1 ml of supernatant was desalted using PD MidiTrap G-25 columns (GE Healthcare) and eluted in 1.5 mls of extraction buffer. The extracts were shaken for 90 min rapidly enough to generate foaming to minimize the activity of invertase inhibitors (Brummell et al. 2011). 20 µl of protein extract was added to a tube containing 60 ul of reaction buffer (133 mM Suc and 26.7 mM Na-acetate, pH 4.7) and incubated at 30^{o}C for 60 minutes (Bhaskar et al. 2010). Reactions were stopped by the addition of 8 µl of 1M Na-phosphate, pH 7.4 and heating samples at 97^{o}C for 3 minutes. Controls were first inactivated by heating samples at 97^{o}C for 3 minutes followed by the addition of 8 µl of 1M Na-phosphate, pH 7.4 and 60 µl of reaction buffer. Glucose formed was measured using the MyQubit Amplex Red Glucose Assay (Life Technologies) following the manufactures instructions. Activity was expressed as nmol glu min^{-1} mg^{-1} protein.

10.4 References

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11.0 Agronomic Evaluation

11.1 Agronomic Performance

The purpose of the agronomic trials was to confirm that Russet Burbank Event W8 has an equivalent phenotype compared to the control Russet Burbank, when grown at multiple locations representing the major areas for potato production in the US, including Russet Burbank. Observations throughout the growing season allowed for a thorough assessment of (1) growth, (2) disease and pest susceptibility, and (3) tuber yield and quality. The field assessments confirmed that W8 has the intentionally incorporated new traits and maintains all the benefits of the conventional Russet Burbank parent variety. These assessments also made it possible to ensure that addition of the DNA insert did not result in unintended effects associated with weediness or pest-like characteristics. Results of the studies are presented first followed by detailed descriptions of the test methods.

11.1.1 Typical Agronomic Practices

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 Russet Burbank potato production is given in Table 11-1. W8 and untransformed control received identical inputs and treatments within each site. The trial sites selected for the agronomic evaluations were different agricultural zones and represented the main production areas for potatoes in the US, including Russet Burbank.

Table 11-1. Example of Agronomic Inputs for Russet Burbank Potatoes

Planting Date	April 1 to May 10
Planting Rate	15,000 - 18,000 seed pc or 17 – 23 cwt/A
Row Spacing	34-36” between rows
Seed Spacing	10-12” within row
Fertilizer	For 600 cwt/A yields and optimum soil test levels: 250 lb N-100 lb P_2O_5- 330 lb K_2O/ acre
Yield/Plant	2-4 lb
Yield/Acre	400-700 cwt/A
Harvest Date	September 1 to October 15

11.1.2 Field Trial Locations

During 2012 and 2013, plants of W8 and the Russet Burbank control were grown at several locations representing the major production areas for potatoes in the US including Russet Burbank. Specific details regarding the field trial sites are summarized in Table 11-2. All field trials were conducted in accordance with applicable USDA APHIS notification requirements at 7 CFR 340.3.

Table 11-2. Field Trial Locations

Year	USDA Notification #	Site State	Site County	Material Tested	Trial Design[1]	Rows x Planted Tubers/ Rep	Seed Type	Sticky Traps Deployed	Regional Specifics
2012	12-066-102n	ID	Canyon	W8, Control	RCB, 3 reps	4x20	Mini-tubers	Y	Typical for Southwest Idaho, an important potato-growing region in the Northwest that produces about 120 million cwt/year, mainly for the french fry industry. Careful management is needed to limit or prevent heat-associated agronomic issues.
2012	12-121-101n	ID	Minidoka	W8, Control	RCB, 3 reps	4x20	Mini-tubers	N	
2013	13-079-106n	ID	Canyon	W8, Control, Nicolet, Bintje, TX278, Golden Sunburst	RCB, 4 reps	2x20	NFT Mini-tubers	Y	
2013	13-072-112n	ID	Minidoka	W8, Control, Nicolet, Bintje, TX278, Golden Sunburst	RCB, 4 reps	6x20	NFT Mini-tubers	Y	Typical for Southern Idaho, an important potato-growing region that produces about 120 million cwt/year, mainly for the french fry industry, with harvests in Fall. The growing season is relatively short because of cooler temperatures.
2012	12-066-102n	WA	Grant	W8, Control	RCB, 3 reps	4x20	Mini-tubers	N	Typical for Washington, a state in the Northwest that produces about 85 million cwt/year, mainly for the french fry industry, with harvests in Fall. Ideal growing conditions give rise to very high yields per acre.
2013	13-072-112n	WA	Grant	W8, Control, Nicolet, Bintje, TX278, Golden Sunburst	RCB, 4 reps	6x20	NFT Mini-tubers	Y	
2013	13-072-112n	ID	Jerome	W8, Control, Nicolet, Bintje, TX278, Golden Sunburst	RCB, 4 reps	2x20	NFT Mini-tubers	Y	Typical for Southern Idaho, an important potato-growing region that produces about 120 million cwt/year, mainly for the french fry industry, with harvests in Fall. The growing season is relatively short because of cooler temperatures.
2013	13-072-112n	WA	Adams	W8, Control, Nicolet, Bintje, TX278, Golden Sunburst	RCB, 4 reps	2x20	NFT Mini-tubers	N	Typical for Washington, a state in the Northwest that produces about 85 million cwt/year, mainly for the french fry industry, with harvests in Fall. Ideal growing conditions give rise to very high yields per acre.

[1]RCB=Randomized Complete Block design

Table 11-2 continued. Field Trial Locations

Year	USDA Notification #	Site State	Site County	Material Tested[1]	Trial Design[1]	Rows x Planted Tubers/ Rep	Seed Type	Sticky Traps Deployed	Regional Specifics
2013	13-079-107n	ND	Grand Forks	W8, Control, Nicolet, Bintje, TX278, Golden Sunburst	RCB, 4 reps	6x20	NFT Mini-tubers	Y	Typical for North Dakota, a Midwest state that produces about 20 million cwt/year, mainly for the french fry industry, with harvests in Fall. The Red River Valley is the 3rd largest potato growing region in the US.
2013	13-072-112n	MN	Sherburne	W8, Control, Nicolet, Bintje, TX278, Golden Sunburst	RCB, 4 reps	2x20	NFT Mini-tubers	N	Typical for Minnesota, a Midwest state that produces about 20 million cwt/year for the french fry and chip industry, with harvests in the Fall. Sherburne county produces about 35-40% of the potatoes grown in Minnesota.
2013	13-079-102n	WI	Adams	W8, Control, Nicolet, Bintje, Red Thumb, Golden Sunburst	RCB, 4 reps	2x20	NFT Mini-tubers	N	Typical for Wisconsin, a Midwest state that 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.

[1]RCB=Randomized Complete Block design

11.1.3 Field Performance and Tuber Assessment Results

Summaries of evaluations of agronomic characteristics, yield and grading characteristics, and ecological interactions of W8 and controls grown in 2012 and 2013 are shown in Tables 11-3, 11-4, and 11-5. Overall, the results confirm that there are no major differences between W8 and the control with respect to these characteristics.

Agronomic Characteristics

The agronomic characteristics for W8 and the control are shown in Table 11-3. No statistically significant differences were detected between W8 and the control for final emergence, stems per plant, or vine desiccation. For early emergence, W8 was lower than the control (39.5 vs 61.5 %), for plant vigor, W8 was less vigorous than the control (3.0 vs 3.7), and for plant height, W8 was shorter than the control (40.4 vs 45.1 cm). All of the values for W8 for which differences were detected were within the CVR.

Although W8 showed delayed emergence, lower vigor, and was shorter than the control, these characteristics do not indicate increased plant pest potential in W8 compared to the control.

Table 11-3. Agronomic Characteristics

Characteristic	Variety	N	Mean	P-Value[1]	SD[2]	CVR[3]	
Early Emergence (%)	Control	41	61.5	.	14.8	0.0	93.1
	W8	40	39.5	**0.0001**	23.3		
Final Emergence (%)	Control	41	87.1	.	10.7	10.6	98.1
	W8	40	80.3	0.2060	20.2		
Stems Per Plant (#)	Control	39	1.7	.	0.7	1.0	3.1
	W8	36	1.7	0.8868	0.7		
Plant Vigor (1-5 Scale)	Control	37	3.7	.	0.9	2.0	5.0
	W8	35	3.0	**0.0065**	0.8		
Plant Height (cm)	Control	41	45.1	.	14.0	31.8	71.6
	W8	38	40.4	**0.0098**	12.5		
Vine Desiccation (%)	Control	37	44.0	.	29.3	3.8	100.0
	W8	35	35.5	0.1471	30.7		

[1]P-values in bold and underlined indicate statistically significant differences.
[2]SD = standard deviation.
[3]CVR = Conventional variety range. The range of mean values of conventional varieties.
. = Not applicable

Yield and Grading Results

The yield and grading characteristics of W8 and the control are shown in Table 11-4. There were no statistically significant differences detected for total yield, US#2 yield, tubers per plant, tubers <4 oz, tubers 4-6 oz, tubers 6-10 oz, tubers >14 oz, specific gravity, fry 1, fry 3, fry 4, and total internal defects. Compared to the control, W8 had fewer tubers in the 10-14 oz group (14.0 vs. 19.0 %), fewer fry strips with high sugar (1.4 vs. 11.0 %), fewer strips with sugar ends (3.3 vs. 19.7 %), and more strips with light color defined as fry 0 (94.9 vs. 76.5%), and fewer scored as fry 2 (1.7 vs. 9.9%). All of the values for W8 for which differences were detected were within the CVR.

W8 had fewer large tubers than the control, which could be associated with lower yield, however yield was not significantly different. Other characteristics, such as fewer sugar ends and high sugar fries and more fries with lighter fry color are positive indicators of potato and fry quality and are an expected result of invertase silencing. Zhu et al. (2014) also reported much fewer sugar ends in Russet Burbank potatoes in which vacuolar invertase was silenced. None of these differences in size or quality would indicate increased plant pest potential.

Table 11-4. Yield and Grading Characteristics

Characteristic	Variety	N	Mean	P-Value[1]	SD[2]	CVR[3]	
Total Yield (cwt/a)	Control	41	445.7	.	149.7	135.6	733.2
	W8	38	417.9	0.4080	165.4		
US#2 Yield (cwt/a)	Control	41	375.7	.	147.5	118.9	653.7
	W8	38	312.2	0.0506	144.1		
Tubers Per Plant (#)	Control	41	7.8	.	2.5	2.5	19.5
	W8	38	8.4	0.4657	3.2		
Tubers <4 oz (%)	Control	9	8.2	.	5.3	.	.
	W8	9	15.9	0.2434	6.9		
Tubers 4-6 oz (%)	Control	41	18.5	.	8.6	4.6	41.1
	W8	41	20.5	0.3148	7.1		
Tubers 6-10 oz (%)	Control	41	31.1	.	8.7	15.3	41.2
	W8	41	28.6	0.3435	8.2		
Tubers 10-14 oz (%)	Control	41	19.0	.	8.4	1.0	26.8
	W8	41	14.0	**0.0071**	8.1		
Tubers >14 oz (%)	Control	41	14.3	.	16.9	0.0	45.5
	W8	41	8.6	0.0554	11.0		
Specific Gravity	Control	41	1.077	.	0.0	0.7	1.2
	W8	41	1.073	0.8058	0.0		
High Sugar (%)	Control	41	11.0	.	16.9	0.0	84.8
	W8	41	1.4	**0.0337**	7.3		
Sugar Ends (%)	Control	41	19.7	.	18.5	0.0	52.6
	W8	41	3.3	**<.0001**	6.5		
Fry 0	Control	32	76.5	.	30.9	0.0	100.0
	W8	32	94.9	**0.0272**	19.4		
Fry 1	Control	32	2.9	.	10.5	0.0	29.7
	W8	32	1.5	0.6142	6.2		
Fry 2	Control	32	9.9	.	18.9	0.0	28.4
	W8	32	1.7	**0.0173**	7.3		
Fry 3	Control	32	1.9	.	3.7	0.0	23.1
	W8	32	0.8	0.4899	3.4		
Fry 4	Control	32	6.3	.	14.8	0.0	79.7
	W8	32	1.0	0.2316	5.9		
Total Internal Defects (%)	Control	41	1.6	.	2.5	0.0	15.5
	W8	41	1.2	0.7205	2.2		

[1]P-values in bold and underlined indicate statistically significant differences.
[2]SD = standard deviation.
[3]CVR = Conventional variety range. The range of mean values of conventional varieties.
. = Not applicable

Insect, Disease, and Abiotic Stressor Assessments

The insect, disease, and abiotic stressor evaluations for W8 and the control are shown in Table 11-5. Insect, disease, and abiotic stressors were reported by the principal investigators based on their professional opinion. Reported stressors varied depending on which stressors were present or expected to be present. In particular, the disease stressor evaluations for late blight and early blight should be considered secondary to the quantitative studies described in Chapter 10: Trait Efficacy and the disease specificity studies described in Section 11.4: Disease Susceptibility Assessments. However, the stressor observations are useful because they provide an opportunity to assess environmental interactions across a broad range of stressors and locations.

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

0 = stressor was not present,
1 = symptoms were observed,
2 = moderate symptoms were present, and,
3 = severe symptoms were observed.

Stressor evaluations were intended to be categorical and were not statistically analyzed. The range of ratings for W8 and the control were compared for each observation and an observed difference occurred when the range of W8 did not overlap with the range of the control. Three differences were noted between W8 and the control during 92 individual observations of seven insect stressors. Eight differences were noted between W8 and the control during 95 individual observations of disease stressors. Three differences between W8 and the control were observed during 69 observations of the eight abiotic stressors. Observed differences in stressor evaluations between W8 and the control are detailed below in Table 11-5. In two disease stressor observations out of twenty one, W8 had more late blight symptoms than controls. However, multiple replicated field trials with inoculated strains of late blight confirmed significant late blight resistance in W8 compared with the Russet Burbank control as tested by expert plant pathologists at Michigan State University (Chapter 10: Trait Efficacy). The small number of observed differences and the lack of trends across sites provide a weight of evidence that supports a conclusion of no altered environmental interactions of W8 compared to the control.

Table 11-5. Stressor Observations in W8 and the Control

Stressor	Total Observations	Observations Without Differences	Observations With Differences	Differences[1]
Insect Stressors				
Aphid	30	30	0	-
Colorado Potato Beetle	32	30	2	Minidoka County, ID Obs. 1: W8 = 0-1; Ctrl = 0-0; Ref = 0-1 Adams County, WI Obs. 1: W8 = 0-0.5; Ctrl = 0-0; Ref = 0-1
Grasshopper	2	2	0	-
Leaf Hopper	8	8	0	-
Loopers	7	6	1	Minidoka County, ID Obs. 3: W8 = 0-1; Ctrl = 0-0; Ref = 0-2
Psyllids	11	11	0	-
Stink Bugs	2	2	0	-
Insect Totals	92	89	3	-
Disease Stressors				
Black Dot	2	2	0	-
Black Leg	5	5	0	-
Early Blight	29	25	4	Grant County, WA (2) Obs. 2: W8 = 0-0; Ctrl = 2-2; Ref = 0-3 Obs. 3: W8 = 0-1; Ctrl = 2-3; Ref = 0-3 Jerome County, ID Obs. 3: W8 = 1-2; Ctrl = 1-1; Ref = 0-3 Adams County, WA Obs. 2: W8 = 0-2; Ctrl = 0-1; Ref = 0-3
Fusarium	1	1	0	-
Grey Mold	2	2	0	-

[1]Stressor evaluations were intended to be categorical and were not statistically analyzed. The range of ratings for W8 and its control (Ctrl) were compared for each observation and an observed difference occurred when the range of the variety was outside the range of the control. The range of values observed in conventional reference varieties (Ref) provide values common to potatoes.

- = no differences observed. Obs. 1 = early season. Obs. 2 = mid season. Obs. 3 = late season.

Rating scale: 0 = stressor was not present, 1 = symptoms were observed, 2 = moderate symptoms were present, and, 3 = severe symptoms were observed.

Table 11-5. continued. Stressor Observations in W8 and the Control

Stressor	Total Observations	Observations Without Differences	Observations With Differences	Differences[1]
Disease Stressors continued				
Late Blight	21	19	2	Jerome Co., ID Obs. 2: W8 = 1-2; Ctrl = 1-1; Ref = 0-2 Adams Co., MI Obs. 1: W8 = 0-1; Ctrl = 0-0; Ref = 0-2
Leaf Roll Virus	3	3	0	-
Psyllids	2	2	0	-
Rhizoctonia	3	3	0	-
Sclerotinia	3	3	0	-
Stem Rot	3	3	0	-
Verticillium	8	6	2	Canyon County, ID Obs. 2: W8 = 0-1; Ctrl = 0-0; Ref = 0-3 Grant County, WA Obs. 3: W8 = 2-3; Ctrl = 0-2; Ref = 0-3
White Mold	13	13	0	-
Disease Totals	95	87	8	
Abiotic				
Cold Stress	7	7	0	-
Drought	13	13	0	-
Excessive Moisture	5	5	0	-
Hail	2	2	0	-
Heat Stress	25	23	2	Canyon County, ID Obs. 2: W8 = 0-1; Ctrl = 0-0; Ref = 0-2 Minidoka County, ID Obs. 1: W8 = 0-2; Ctrl = 0-1; Ref = 0-2
Nutrient Imbalance	6	5	1	Adams County, WI Obs. 2: W8 = 0-0.5; Ctrl = 0-0; Ref = 0-2
Sun Scald	3	3	0	-
Wind Damage	8	8	0	-
Abiotic Stress Totals	69	66	3	

[1]Stressor evaluations were intended to be categorical and were not statistically analyzed. The range of ratings for W8 and its control (Ctrl) were compared for each observation and an observed difference occurred when the range of the variety was outside the range of the control. The range of values observed in conventional reference varieties (Ref) provide values common to potatoes.

- = no differences observed. Obs. 1 = early season. Obs. 2 = mid season. Obs. 3 = late season.

Rating scale: 0 = stressor was not present, 1 = symptoms were observed, 2 = moderate symptoms were present, and, 3 = severe symptoms were observed.

Arthropod Abundance

The results of the arthropod abundance monitoring are presented in Tables 11-6 and 11-7. No significant differences were observed between W8 and the control for any of the arthropods collected. Because there were no damsel bugs found in control samples, there is no p-value available for a comparison between damsel bug abundance in W8 and the control. The presence of 0.3 damsel bugs, on average in W8 compared to an average of 0.0 damsel bugs present in the control could be seen as a significant difference even though this comparison lacks a p-value. In both W8 and control, the presence of damsel bugs, one of the beneficial insects, was very low. The overall lack of differences in 15 of 16 insect types observed supports a conclusion that the environmental interactions of Russet Burbank Event W8 potatoes were not altered as a result of the introduction of the biotechnology-derived traits compared to conventional potatoes. While these data do not show differences in overall arthropod fitness, the fact that there are few differences between Russet Burbank Event W8 and the control show that the insertions of the traits do not have an impact on abundance within the potato agro-ecosystem.

Table 11-6. Beneficial Arthropod Abundance in Field Trials

Arthropod	Variety	N	Average Abundance	P-value[1]	SD[2]
Spiders	Control	112	2.9	.	3.9
	W8	112	3.3	0.4045	4.4
Big-eyed Bugs	Control	15	0.6	.	1.0
	W8	15	0.7	0.9142	1.4
Lacewings	Control	85	2.4	.	3.3
	W8	85	2.3	0.8100	3.8
Ladybird Beetles	W8	132	10.0	.	16.9
	Control	132	9.5	0.3068[3]	17.5
Minute Pirate Bugs	Control	95	4.1	.	10.2
	W8	95	4.0	0.8608[3]	8.1
Damsel Bug	Control	4	0.0	.	0.0
	W8	4	0.3	NA	0.5
Syrphid Flies	Control	70	1.6	.	3.8
	W8	70	1.5	0.8152	2.7
Tachinid Flies	Control	28	0.6	.	1.1
	W8	28	0.9	0.4334	1.7
Trichogramma Wasps	Control	98	12.3	.	35.3
	W8	98	11.0	0.9609[3]	29.3

[1]P-values in bold and underlined indicate statistically significant differences.

[2]SD = standard deviation.

[3]P-values derived from log-transformed data due to unequal variance in the data.

. = Not applicable

Table 11-7. Pest Arthropod Abundance in Field Trials

Arthropod	Variety	N	Average Abundance	P-value[1]	SD[2]
Aphids	Control	132	54.6	.	68.5
	W8	132	68.0	0.9622[3]	96.2
Flea Beetles	Control	57	3.1	.	10.4
	W8	57	2.8	0.8565	9.8
Potato Leafhoppers	Control	82	47.5	.	247.8
	W8	82	52.6	0.8793[3]	257.8
Psyllids	Control	129	5.4	.	7.0
	W8	129	6.6	0.6458[3]	9.5
Potato Tuberworm	Control	12	0.8	.	1.2
	W8	12	0.5	0.6561	0.8
Click Beetle	Control	4	0.0	.	0.0
	W8	4	0.0	1.0000	0.0
Tarnished Plant Bugs	Control	94	2.6	.	4.6
	W8	94	2.9	0.5133	4.3

[1]P-values in bold and underlined indicate statistically significant differences.
[2]SD = standard deviation.
[3]P-values derived from log-transformed data due to unequal variance in the data.
. = Not applicable

11.2 Conclusions: Agronomic Characteristics

Results of these agronomic trials confirmed that W8 had an equivalent phenotype compared to the control when grown at multiple locations representing the major areas for potato production in the US. Observations throughout the growing season demonstrated equivalence of growth, disease and pest susceptibility, and tuber yield and quality. The field assessments confirmed that W8 has the intentionally incorporated new traits and maintains all the benefits of the conventional Russet Burbank parent variety.

Phenotypes that could indicate enhanced weediness or plant pest potential are any characteristic where the test products were more vigorous than controls. Although there were significant agronomic differences, none of them would signal enhanced survival compared with control and, all fell within the conventional variety range. For example, at the time plants were observed for early emergence, fewer W8 plants had emerged than the control. However, when final emergence data were collected, W8 was no different from the control.

W8 had fewer large tubers than the control, which could be associated with lower yield; however yield was not significantly different. Other characteristics, such as fewer sugar ends, fewer high sugar fries, and more fries with lighter fry color are positive indicators of potato and fry quality and are an expected result of invertase silencing. None of these differences in size or quality would indicate increased plant pest potential. No differences were observed for 237 out of 256 insect, disease, or abiotic stressors. The 19 differences that were observed were not consistent across all sites or years. In cases where efficacy data was conflicting, specifically late blight, we are confident that the data from the quantitative disease assays should be considered as the primary source for this information.

Thus, we concluded that no biologically meaningful differences that would contribute to increased weediness or plant pest potential were observed in W8 for any of the agronomic characteristics, yield and grading characteristics, or ecological interactions such as plant-insect interactions, plant-disease interactions, and plant interactions with abiotic stressors. In addition the information provided by the broad range of reference varieties and the data available from the literature further confirms the lack of biologically meaningful differences and lack of weediness or plant pest potential.

11.3 Agronomic and Phenotypic Methods

This summary includes agronomic evaluations of W8 and the Russet Burbank control at geographically distinct sites that represent most of the main production areas for potatoes destined for french fry production in the US, including Russet Burbank. All agronomic trials were conducted under Biotechnology Quality Management System (BQMS) standards and USDA-APHIS compliance. The agronomic evaluations relate to both field observations and tuber assessments, both at harvest and during storage.

Varieties grown in field trials are summarized in Table 11-8. 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.

Table 11-8. Varieties in Field Trials

Variety	Type	Trait/Genotype	Seed Type[1]	Seed Source
Varieties in 2012 Field Trials				
Russet Burbank	Control	N/A	Greenhouse-grown Mini-Tubers	Simplot Plant Sciences - Boise, ID
W8	Test	pSIM1278+pSIM1678		
Varieties in 2013 Field Trials				
Russet Burbank	Control	N/A	NFT Mini-tubers	CSS Farms - Colorado City, CO
W8	Test	pSIM1278+pSIM1678		
Golden Sunburst	Reference	N/A		
Bintje	Reference	N/A		
Nicolet	Reference	N/A		
TX278[2]	Reference	N/A		
Red Thumb[2]	Reference	N/A		

[1]Greenhouse-grown mini-tubers were grown from tissue culture plantlets in the Simplot Plant Sciences greenhouse. NFT mini-tubers were produced at CSS Farms in Colorado City, CO, using nutrient film technique.
[2]At Adams County, WI, Red Thumb was used in place of TX278.

11.3.1 Agronomic Trial Experimental Design

The experiments were established in a randomized complete block design (RCBD). The RCBD is typical for the evaluation of new potato varieties and events. In 2012, each plot consisted of four rows approximately 20 feet long, each planted with 20 mini-tubers, 12" apart. There were three replicates at each site. The mini-tubers were either mechanically or hand planted to a depth of 2-3 inches. In 2013, three sites had plots that consisted of six rows and five sites had plots that consisted of two rows. Each row was approximately 20 feet long, planted either mechanically or by hand with 20 mini-tubers, 12" apart and to a depth of 2-3 inches, as in 2012. There were four replicates at each site in 2013.

Agronomic Trial Seed

For the 2012 field trials, greenhouse grown mini-tubers for W8 and the control were planted. Seed for the field trials was grown in Simplot Plant Sciences greenhouse. Seed piece size varied and to minimize within site variability, the largest seed pieces were planted at Canyon County, ID, medium sized pieces were planted at Minidoka County, ID, and the smallest pieces were planted in Grant County, WA.

For the 2013 field trial evaluations, minitubers were produced using nutrient film technology (NFT) mini-tubers for W8 and the control. This seed was grown at CSS Farms in Colorado City, Colorado. Plants from tissue culture were used to grow mini-tubers using NFT, which refers to seed that is hydroponically propagated 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.

11.3.2 Phenotypic Assessments

The phenotypic characteristics evaluated are listed in Table 11-9.

Table 11-9. Characteristics Evaluated

Characteristic	Evaluation Timing[1]	Description	Units/Scale
Early Emergence	Early season	Count of emerged plants	Percent based on number of seed pieces planted
Final Emergence	Early season	Count of emerged plants	Percent based on number of seed pieces planted
Insect Stressors	Early, mid, & late season	Visual estimate of damage	0 to 3 scale: 0=not present, 1=slight, 2=moderate, 3=severe
Disease Stressors	Early, mid, & late season	Visual estimate of damage	0 to 3 scale: 0=not present, 1=slight, 2=moderate, 3=severe
Abiotic Stressors	Early, mid, & late season	Visual estimate of damage	0 to 3 scale: 0=not present, 1=slight, 2=moderate, 3=severe
Stems Per Plant	Early season	Count of stems per plant	Count
Plant Vigor	Mid season	Visual estimate of vigor	1 to 5 scale: 1=poor vigor, 3=average vigor, 5=excellent vigor
Plant Height	Mid season	Plant height of 10 non-systematically selected plants per plot measured from the soil surface to the top of the uppermost leaf	Average height (cm)
Vine Desiccation	Late season	Visual estimate of percent natural vine desiccation (prior to chemical or mechanical vine desiccation)	Percent
Total Yield	Yield	Total yield including all tubers	Hundredweight per acre (cwt/a)
US#2 Yield	Yield	Yield of tubers meeting or exceeding the US#2 standard (consists of potatoes or usable pieces which meet the following requirements: similar varietal characteristics, moderately firm, not seriously misshapen, free from freezing, disease, or insect damage, not less than 1 1/2 inches in diameter or usable pieces not less than 4 ounces in weight.)	Hundredweight per acre (cwt/a)
Tubers Per Plant	Yield	Average count of tubers per plant from one entire row per plot	Count
Tubers <4 oz.[2]	Grading	Tubers weighing <4 oz.	Percent by weight
Tubers 4-6 oz.	Grading	Tubers weighing 4-6 oz.	Percent by weight
Tubers 6-10 oz.	Grading	Tubers weighing 6-10 oz.	Percent by weight
Tubers 10-14 oz.	Grading	Tubers weighing 10-14 oz.	Percent by weight
Tubers >14 oz.	Grading	Tubers weighing >14 oz.	Percent by weight
Specific Gravity	Grading	Tuber sample weight in air/(weight in air - weight in water)	Specific gravity ratio
High Sugar	Grading	Color rating of fried strips based on Munsell Color Chart for french fried potatoes	Percent of tubers with fry strips which, when compared with the Munsell Color Chart for french fried potatoes, has on the darkest side a predominate color of a number 3 or darker.
Sugar Ends	Grading	Color rating of the end of fried strips based on Munsell Color Chart for french fried potatoes	Percent of tubers with fry strips which has an end ¼ inch long or longer on the darkest side of the strip, for the full width of the strip, testing number 3 color or darker when compared with the Munsell Color Chart for french fried potatoes.
Fry 0, Fry 1, Fry 2, Fry 3, Fry 4	Grading	Color rating of fried strips based on Munsell Color Chart for french fried potatoes	Percent of tubers with fry strips which, when the predominate color of the darkest side is compared with the Munsell Color Chart for french fried potatoes, is determined to be a color reading of 0, 1, 2, 3, or 4.
Total Internal Defects	Grading	Tubers showing evidence of defects including hollow heart, vascular necrosis, brown center, internal discoloration, insect, internal brown spot, nematode, and other internal defects	Percent

[1]Early season observations were made when emergence was complete (about 30-80 days after planting). Midseason observations were made during the early bloom stage (about 60-100 days after planting). Late season observations were made during the crop senescence stage (about 100-140 days after planting).

[2]Data on tubers <4oz. were obtained in 2012 only. In 2013, tubers <4 oz. were grouped with all unusable tubers because tubers <4 oz. are not taken into account when calculating US#2 yield.

Early emergence (at approximately 50% emergence) and final emergence (at complete emergence) were evaluated by determining the number of plants that emerged out of 20 mini-tubers planted in the middle two rows of each plot (in the case of two-row plots, “middle rows” refer to both rows). Emergence rates vary among conventional varieties and are dependent on both the physiological age of the “seed” and environmental factors such as temperature and moisture levels.

Stems per plant were evaluated by counting the number of stems of 10 plants in the middle rows of each plot.

At approximately mid-season, plant vigor was visually assessed in the middle rows of each plot using the following 1 to 5 comparative scale based on the principal investigators’ experience of the potato varieties being grown:

1 = less vigor – plants are less vigorous than the varietal average
2 = intermediate to 1 and 3
3 = normal vigor - plants are similar in vigor to the varietal average
4 = intermediate to 3 and 5
5 = more vigor - plants are more vigorous than the varietal average

At approximately mid-season, plant height was measured in centimeters from the soil surface at the top of the hill, to the top of the uppermost leaf of 10 non-systematically selected plants per plot from the middle rows in each plot.

At approximately late season, vine desiccation was assessed by visually estimating the percent of vines desiccated in the middle rows of each plot prior to chemical or mechanical vine desiccation.

11.3.3 Insect, Disease, and Abiotic Stressors

Each plot was evaluated at approximately early season, mid-season, and late season for the presence of insects, diseases, and abiotic stressors using a 0 to 3 rating scale described in Table 11-9. Examples of common potato disease and insect symptoms can be found in Table 11-10. An observation is defined as looking for a specific insect, disease, or abiotic stressor during a point in time. Even if no stressors were present, zeroes were recorded because the stressors were looked for and comparisons can be made between the event and the conventional control.

Table 11-10. 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	Causes cankers
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.

Arthropod Abundance

At sites with arthropod collection, Pheromone-free sticky traps were deployed in every plot during the 2012 and 2013 field seasons in order to monitor arthropod abundance. Sticky traps were deployed at canopy height, collected approximately every two weeks (every week in 2012) and shipped to the University of Arkansas for identification and enumeration. Arthropods were broken into 2 groups: beneficial which is defined as an arthropod that has characteristics that are beneficial to potatoes, such as those that consume pests; and pest which is defined as an arthropod that has characteristics that may be damaging to potatoes, such as vectoring diseases.

11.3.4 Tuber Grading and Yield

Tuber Grading

Tubers were harvested during early fall. At harvest, all tubers from one row of each plot were transported to the State of Idaho Department of Agriculture Grading Facility in Caldwell, ID. The grading methods employed were identical to those used to grade commercial potatoes intended for the production of fries.

Size profiles were determined by weighing tubers from the sample sorted by size. The size groups included tubers <4 oz (in 2012 only), tubers 4-6 oz, tubers 6-10 oz, tubers 10-14 oz, and tubers >14 oz. Potatoes greater than six-ounces produce optimal french fries and allow the french fry processor to meet most customer specifications at the highest potential recovery.

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 the event to the conventional control.

High Sugar and Sugar Ends

Fried potato strips were prepared by cutting several (10-20) tubers into approximately 3/8 inch strips. The center strip was selected from each tuber and fried in cooking oil for three minutes at 375 F. The color of the fried strips was compared to a USDA Munsell color chart. High sugar is the percentage of tubers with fry strips which, when compared with the Munsell Color Chart for french fried potatoes, has on the darkest side a predominate color of a number 3 or darker. Sugar ends is the percentage of tubers with fry strips which has an end ¼ inch long or longer on the darkest side of the strip for the full width of the strip, testing number 3 or darker color (USDA AMS 1969). Fry 0 – Fry 4 is the percent of tubers with fry strips which, when the predominate color of the darkest side is compared with the Munsell Color Chart for french fried potatoes is determined to be a color reading of 0-4, respectively. Therefore, the reported numbers are the percentage of Fries that score 0, 1, 2, 3, or 4 on the Munsell chart.

Yield and Defects

Total yield was determined by weighing each single row sample and US#2 yield was determined by subtracting the weight of undersize and unusable tubers. Tubers per plant was determined by counting the total number of tubers in each single-row sample and dividing by the total number of plants in the sample row.

Tubers were checked for internal defects which included hollow heart, vascular necrosis, brown center, internal discoloration, insect, internal brown spot, nematode, and other internal defects. Total internal defects represent the percentage of tubers affected by any internal defect.

11.3.5 Statistical Methods for Agronomic Studies

The statistical analysis for agronomic, grading, stressor, and arthropod abundance data was performed by Simplot using SAS 9.3. All data were analyzed 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. In the arthropod abundance data, a Welch unpaired t-test was used due to the unequal variances of the data across different locations (McDonald 2009). To address the unequal variance, the p-values were derived from log-transformed data for Ladybird Beetles, Minute Pirate Bugs, Trichogramma Wasps, Aphids, Potato Leafhoppers, and Psyllids.

Interpretation of the Results

A step-wise approach was used to interpret any differences between event W8 and the control. First, statistical significance, $p < 0.05$, was determined for each attribute. If the p-value indicates no statistical significance, then W8 is considered equivalent to the control. Next, if the p-value indicated statistical significance or if a p-value was not present, the mean value of W8 was compared to the conventional variety range (CVR), which represents the minimum and maximum mean values of all conventional varieties included in the experiments. If the value for W8 was within the CVR, it was concluded that W8 exhibited characteristics within the natural variation of potatoes and that the difference was unlikely to be biologically meaningful or indicative of increased plant pest potential. If the value of W8 was outside the CVR, further consideration was given to the difference in the context of agronomic impact and increased plant pest potential.

The CVR for the agronomic, yield, and grading characteristics used the range of mean values from one conventional control at three sites during 2012, four conventional reference varieties at eight sites during 2013, one conventional control at eight sites during 2013, and a second conventional control at five sites during 2013 (Table 11-8).

11.3.6 BQMS Quality Management Systems

All agronomic trials were conducted according to USDA Biotechnology Quality Management System (BQMS) standards and were USDA-APHIS compliant. Compliance with the applicable USDA requirements described in 7 CFR part 340 included:

(1) Providing details on the introduced DNA in the submitted Design Protocol.
(2) Confirming that plants and tubers are free of *Agrobacterium.*
(3) Monitoring eight critical control points (CCPs) in the process of transporting, evaluating, harvesting, storing, and eliminating potatoes that are no longer needed. The associated documents were developed in collaboration with APHIS/BQMS and include: (1) Record of Transport indicating the USDA movement permit number, (2) Record of Storage and Inventory Change, (3) Record of Planting, (4) Record of Spatial Isolation, (5) Record of Harvest, (6) Record of Processing, (7) Record of Post-Harvest Monitoring, which commits to monitoring trials for the establishment of volunteers during the two years following an experiment, and (8) Record of Corrective Action.

Both internal and external audits were conducted to ensure adherence to the guidelines of BQMS. The BQMS guidelines were followed to ensure that regulated plants or tubers were not lost or mixed with other plants or tubers during the conduct of the study. After the studies, all unused regulated material was devitalized and discarded as prescribed in the J.R. Simplot Company Design Protocol which was approved by USDA APHIS. Internal audits were conducted by third party auditors that are approved by APHIS for the BQMS program. The external audits were conducted by USDA auditors. In addition, many

of the regulated field trials referenced in this chapter were inspected by USDA inspectors and no compliance infractions were received.

11.3.7 Survival in Fields

Any potatoes that produce tubers, including all varieties such as Russet Burbank can persist in fields for several years after planting, including W8 and the Russet Burbank control. In most Northern areas, potatoes are rotated with other crops which minimizes the survival in subsequent years and the chance of accidental mixing in subsequent potato plantings.

After conducting field trials, we routinely look for potatoes in the field the following year. We have not found any potatoes to persist in test plots containing W8 from 2012 trials after monitoring and destroying volunteers. Fields will be checked in 2014 for 2012 and 2013 field trials and in 2015 for 2013 field trials, as potatoes have a 2-year volunteer monitoring cycle. These field trials consisted of small plots that are carefully monitored by expert researchers and represent a well-controlled environment.

11.4 Disease Susceptibility Assessment

As part of the agronomic assessment, quantitative assays demonstrated W8 has resistance to late blight without effecting susceptibility or resistance to other diseases. Resistance to late blight in W8 is conferred by the Potato Late Blight Resistance Gene (*Rpi-vnt1*) which expresses the VNT1 protein. This protein, with 891 amino acids, induces resistance to late blight with a broad spectrum against *P. infestans* isolates (Foster et al. 2009). Also, researchers have investigated the relationship between polyphenoloxidase (PPO) and disease resistance (Valentines et al. 2005; Li and Steffens 2002; Hakimi et al. 2006); with some proposing that enhanced PPO may increase resistance to disease, while others claim that reduced PPO could increase resistance. Considering that some evidence exists for a relationship between PPO and diseases and that the VNT1 protein is directly related to disease resistance, we chose to test W8 and the Russet Burbank control for trait specificity by testing for resistance to 3 common potato foliar diseases, one common potato stem and stolon disease, and 6 common potato tuber diseases.

The purpose of these studies was to evaluate the susceptibility of W8 to common foliar, stem, stolon, and tuber diseases in order to determine if W8 has altered disease susceptibility other than the intended increased resistance to late blight. These were quantitative assays, conducted by inoculating with the specific pathogens, tested by pathologists with expertise in these types of studies and analyzed using statistical methods. These studies address the environmental safety issues associated with the spread of plant diseases. All disease trials were conducted according to USDA Biotechnology Quality Management System (BQMS) standards and were USDA-APHIS compliant.

11.4.1 Disease Study Locations

Diseases that were evaluated affect a variety of tissues and studies were designed using the most relevant susceptible parts of the plant. Tests on foliage were conducted for *Atlernaria alternata* (early blight), *Alternaria solani* (brown leaf spot), and *Botrytis cinerea* (botrytis leaf spot). Stolons and stems, from field grown potato plants were tested for resistance to *Rhizoctonia solani.* Additionally, field grown tubers were tested for resistance to *R solani* (black scurf), *Fusarium sambucinum* (dry rot), *Phytophthora erythroseptica* (pink rot), *Pythium ultimum* (pythium leak), *Pectobacterium carotovora* (soft rot), and *Streptomyces scabies* (common scab). Details of each disease specificity trial are listed in Table 11-11.

Table 11-11. 2012 and 2013 Disease Study Details

Year	Study	USDA Notification #	Site State	Site County	Material Tested	Trial Design[1]	Rows x Planted Tubers/ Rep	Seed Type	Regional Specifics
2013	Early Blight, Brown Leaf Spot, Botrytis Leaf Spot	13-079-108n	MI	Ionia	W8, Burbank Control, TX278[2], Bintje[2], Nicolet[2], Golden Sunburst[2]	RCB, 4 reps	2X5	NFT Mini-tubers	Typical for Michigan, a Midwest state that 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	*Rhizoctonia solani*	13-079-108n	MI	Ionia	W8, Burbank Control, TX278[2], Bintje[2], Nicolet[2], Golden Sunburst[2]	RCB, 4 reps	1X10	NFT Mini-tubers	Typical for Michigan, a Midwest state that 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.
2012	Dry Rot, Pink Rot, Pythium Leak, Soft Rot- Tubers	12-066-102n	WA	Grant	W8, Burbank Control	RCB, 3 reps	4x20	Greenhouse-grown Mini-tubers	Typical for Washington, a state in the Northwest that produces about 85 million cwt/year, mainly for the french fry industry, with harvests in Fall. Ideal growing conditions give rise to very high yields per acre.
2013	Common Scab	13-079-108n	MI	Montcalm	W8, Burbank Control, TX278[2], Bintje[2], Nicolet[2], Golden Sunburst[2]	RCB, 4 reps	1X5	NFT Mini-tubers	Typical for Michigan, a Midwest state that 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.

[1]RCB=Randomized Complete Block design

[2]Reference varieties used as a part of the conventional variety range calculation.

11.4.2 Disease Susceptibility Assessment Results

Symptoms of diseases caused by *Atlernaria alternata*, *Alternaria solani, Botrytis cinerea, Rhizoctonia solani, Fusarium sambucinum*, *Phytophthora erythroseptica*, *Pythium ultimum*, and *Pectobacterium carotovora* were not significantly different between W8 and the control (Table 11-12). For all these diseases, we would expect a similar response in W8 as in the Russet Burbank controls.

Common scab caused by *Streptomyces scabies* was significantly different between W8 and the control for mean percent coverage of tubers and mean severity of lesion type. W8 had less scab than the control as measured by mean percent coverage and lesions were less severe than the control (Table 11-12). Altered response to *S. scabies* was not expected in W8, but would be considered a positive finding if future experience confirms such resistance. Also, this unexpected observation of resistance to *S. scabies* would not enhance the plant pest potential of W8.

Overall, the results of the quantitative disease studies showed that W8 had similar responses to most potato diseases as the Russet Burbank control, with the exception of increased resistance to *P. infestans* as shown in Chapter 10: Trait Efficacy. The stressor observations discussed in Section 11.1.3 showed few differences between W8 and the control for the same diseases that were quantitatively evaluated, including early blight (Alternaria), Rhizoctonia, and Fusarium. In total, the majority of the evaluations of all diseases assessed in the stressor observations showed no difference between W8 and the control in terms of susceptibility or resistance.

Table 11-12. Disease Susceptibility Assessment Results

Pathogen Test and Data	Variety	Mean Value	P-Value[1]	CVR[2]	
Foliar Assessment					
Early Blight and Brown Leaf Spot (Mean Percent Infection)	Control	7.5	.	0.5	17.5
	W8	0.0	0.1316		
Botrytis Leaf Spot (Mean Percent Infection)	Control	5.00	.	3.75	5.00
	W8	5.00	1		
Stem Infection Assessment					
Rhizoctonia solani (Mean Percent Infection)	Control	27.5	.	0.0	80.0
	W8	45.0	0.4114		
Stolon Infection Assessment					
Rhizoctonia solani (Mean Percent Infection)	Control	37.5	.	20.7	57.5
	W8	54.0	0.1499		
Tuber Assessment					
Rhizoctonia solani (Mean Severity of Infection)	Control	0.20	.	0.2	0.9
	W8	0.50	0.5254		
Rhizoctonia solani (Mean Percent Incidence of Infection)	Control	2.5	.	2.5	8.75
	W8	5.0	0.5776		
Tuber Dry Rot (Mean Percent Infection by Area)	Control	15.0	.	.	.
	W8	18.6	0.3404		
Pink Rot (Mean Percent Infection)	Control	35.0	.	.	.
	W8	45.0	0.1639		
Pythium Leak (Mean Percent Infection)	Control	75.0	.	.	.
	W8	75.0	.		
Soft Rot Infection (Mean Percent Weight)	Control	57.4	.	.	.
	W8	47.9	0.1715		
Common Scab (Mean Percent Coverage of Tuber)	Control	2.65	.	2.48	4.58
	W8	1.90	**0.034**		
Common Scab (Lesion Type, Table 11-13)	Control	3.18	.	3.18	5.95
	W8	1.35	**<.0001**		

[1]P-values in bold and underlined indicate statistically significant differences.
[2]CVR = Conventional variety range. The range of mean values of conventional varieties.
Data in this table are from 2013 field trials in Montcalm County, MI.
. = Not applicable

11.5 Conclusions: Disease Susceptibility

Out of 3 foliar diseases, 1 stem and stolon disease, and 6 tuber diseases analyzed, W8 showed no difference in susceptibility compared to the control with the exception of a reduction in tuber coverage and severity of common scab caused by *S. scabies*. This unexpected difference in *S. scabies* susceptibility will be monitored in future field and storage studies to determine whether this pattern persists in commercial production. Overall, these data support the claim that the *Ppo5* silencing and the *Rpi-vnt1* genes are not linked to altered disease susceptibility in foliage, stolons, stems, and tubers. The results provide evidence of no increased plant pest potential in W8 and also show specificity to the Potato Late Blight Resistance Gene, *Rpi-vnt1*. Thus, we concluded that no biologically meaningful differences that would contribute to increased weediness or plant pest potential were observed in W8 for altered susceptibility or resistance to the majority of potato diseases other than late blight when compared to the Russet Burbank control.

11.6 Disease Susceptibility Methods

The following assessments were conducted to determine if the insertion of the late blight resistance trait altered disease susceptibility of other common potato diseases:

Foliar *Alternaria alternata*, *Alternaria solani*, and *Botrytis cinerea*

The trial was planted at the Michigan State University Horticultural Experimental Station in Clarksville, MI, as a RCBD consisting of four replicates. Each plot was five feet in length and consisted of two rows of five plants. Plots were irrigated as needed with sprinklers and were hilled immediately before sprays began. Potato late blight was prevented from movement into the plots from adjacent plots inoculated with *P. infestans* with weekly fungicide applications that prevent late blight infection but do not affect *A. alternaria*, *A. solani*, or *B. cinerea*. Plots were rated visually for percentage foliar area affected by *A. alternaria*, *A. solani*, and *B. cinerea* on August 30, September 5, and September 10, 2013. *A. alternaria* and *A. solani* developed slowly during August and untreated controls reached about 15% foliar infection by September 10. *B. cinerea* developed slowly during August and untreated controls reached about 5% foliar infection by September 10 (Personal Communication with Dr. Willie Kirk, Michigan State University).

***Rhizoctonia solani* in Stems, Stolons, and Tubers**

The trial was planted at the Michigan State University Horticultural Experimental Station in Clarksville, MI as a RCBD consisting of four replicates of ten plants per plot. Each plot was one row and ten feet in length. A five foot unplanted alley separated single-row beds form other trials. Four plants per plot were harvested 90 days after planting and the percentage of stems and stolons with greater than 5% of the total surface area affected by *R. solani* infection were counted. The rest of the plots were harvested on October 7, 2013, and potatoes were stored for 35 days in the dark at 50°F. After 35 days of storage, 20 tubers from each plot were assessed for *R. solani* incidence (%) and severity. Severity of *R. solani* was measured as an index calculated by counting the number of tubers (n = 20) falling into each class 0 = 0; 1 = 1 - 5; 2 = 6 - 10; 3 = 11 - 15; 4 >15% surface area of tuber covered with sclerotia. The number in each class was multiplied by the class number and summed. The sum was multiplied by a constant to express as a percentage. Indices of 0 - 25 represent 0 - 5%; 26 - 50 represent 6 - 10%; 51 - 75 represent 11 - 15% and 75 - 100 >15% surface area covered with sclerotia. *R. solani* symptoms were mild in this trial because this was the first year that potatoes had been planted at that site and the seed tubers were clear of black sclerotia at planting. Despite this, some symptoms developed on stems, stolons and tubers harvested and stored (Personal Communication with Dr. Willie Kirk, Michigan State University).

***Fusarium sambucinum* in Tubers**

Samples of W8 and the control were obtained by randomly selecting mid-sized tubers across field replicates from the 2012 field site in Grant County, WA. Four replicates of five tubers each for W8 and the control were tested with *F. sambucinum* inoculum and a negative control (water). All tubers were washed in distilled water to remove soil. The tubers were surface-disinfected for 10 min in 0.5% sodium hypochlorite and rinsed twice in sterile water. The tubers were then surface-sterilized by soaking in 2% sodium hypochlorite solution for four hours. Tubers were dried in a controlled environment with forced air ventilation at 5950 L min^{-1} at 15^{o}C in dry air (30% relative humidity) for four hours prior to inoculation. Tubers were injected with 20 µl of a conidial suspension (approximately 10^{6} conidia/ml as determined by a hemocytometer) of the *Fusarium* isolate grown on PDA for 7 days. Negative control tubers were each injected with 20 µl sterile distilled water. After inoculation, tubers were placed in the dark in sterilized covered plastic crates and returned to controlled environment chambers [Percival Incubator (Model I-36LLVL, Geneva Scientific, LLC, PO Box 408, Fontana, WI)]. The chambers were set at

10°C and 95% humidity and the sample tubers were incubated for 30 days until evaluation (Personal Communication with Dr. Willie Kirk, Michigan State University).

After 30 days of incubation, tubers were cut in half through the point of inoculation and evaluated for the development of symptoms typical of potato dry rot: dry, necrotic areas discolored light to dark chocolate brown on the tuber surface. Tubers were subdivided into 4 replications of 5 tubers and placed in a boxes arranged in a randomized complete block design. To assess the virulence level of the *Fusarium* isolates, images of the symptomatic areas on the inoculated tuber surfaces were generated from a method adapted from Niemira et al. described below. The freshly-cut tuber sections were placed on a piece of glass (30 cm X 40 cm X 2 mm) with the cut tuber surface facing down. A ruler was placed underneath the glass, and used for measurements during image analysis. The glass and ruler were transferred to a flatbed scanner (HP Scan-Jet 4c; Hewlett Packard Co., Houston, TX) for image processing. Scanner control software (DeskScan II Version 2.4; Hewlett Packard Co.) generated an image of the cut tuber surfaces against a black background. The image files created were first loaded into Adobe Photoshop CS3 (Version 10.01, 2007, Adobe Systems Incorporated, Pittsburg, PA) from which the lesions were selected on each image and "painted" white using the "fill" tool. The images were then loaded into SigmaScan Pro 5 Version 1987-1999, (SPSS Inc., Chicago, IL) to determine the area of the lesion. The lesion length and width (mm) were measured to calculate the dimension and area (mm^2) of the lesions, and calibrated to convert image pixels to a unit of measurement using the ruler within the image. The measurement "fill' was then adjusted to a threshold so that the lesion was a lighter color than the rest of the tuber surface. The area of the lesion was then measured according to the SigmaScan manufacturer's protocol (Personal Communication with Dr. Willie Kirk, Michigan State University).

***Pythium erythroseptica* and *Pythium ultimum* in Tubers**

Samples of W8 and the control were obtained by randomly selecting 80 mid-sized tubers across field replicates from the 2012 field site in Grant County, WA. Four replicates of five tubers for W8 and the control were tested with *P. erythroseptica, P. ultimum,* and a water negative control. All tubers were washed in distilled H_2O to remove soil. The tubers were each surface-disinfested for 10 min in 0.5% sodium hypochlorite and rinsed twice in sterile water. The tubers were then surface-sterilized by soaking in 2% sodium hypochlorite solution for 4 hours. Tubers were dried in a controlled environment with forced air ventilation at 5950 l min^{-1} at 15°C in dry air (30% relative humidity) for four hours prior to inoculation. Tubers were immersed in a water/oospore/sporangial suspension (approximately 10^4 propagules/ml) for 24 hours; control tubers were immersed in distilled water for a corresponding period. After inoculation, tubers were placed in the dark in sterilized covered plastic crates and returned to controlled environment chambers [Percival Incubator (Model I-36LLVL, Geneva Scientific, LLC, PO Box 408, Fontana, WI)]. The chambers were set at 10°C and 95% humidity and the sample tubers were incubated for 30 days until evaluation (Personal Communication with Dr. Willie Kirk, Michigan State University).

Tubers were cut longitudinally into four slices and evaluated for presence of symptoms and/or signs of the target pathogens. Tubers with symptoms or signs of the individual disease were counted and disease incidence determined as percentage of symptomatic tubers relative to the total number of tubers in each replicate (Personal Communication with Dr. Willie Kirk, Michigan State University).

***Pectobacterium carotovora* in Tubers**

Samples of W8 and the control were obtained by randomly selecting 40 mid-sized tubers across field replicates from the 2012 field site in Grant County, WA. Four replicates of five tubers for W8 and the control were tested with *P. carotovora* and a negative control (water). All tubers were washed in

distilled H_2O to remove soil. The tubers were then surface-sterilized by soaking in 2% sodium hypochlorite solution for 4 hours. Tubers were dried in a controlled environment with forced air ventilation at 5950 l min^{-1} at 15°C in dry air (30% relative humidity) for four hours prior to inoculation. The washed, surface-sterilized tubers were inoculated by a sub-peridermal injection of a bacterial suspension of $1x10^{10}$ ml (delivering about 1000 bacterial cells per inoculation) with a hypodermic syringe and needle at the apical end of the tuber about 1 cm from the dominant sprout to a maximum depth of 1 cm. The control tubers were inoculated with cold sterile distilled water. The wound was sealed with paraffin wax. After inoculation, tubers were placed in the dark in sterilized covered plastic crates and returned to controlled environment chambers [Percival Incubator (Model I-36LLVL, Geneva Scientific, LLC, PO Box 408, Fontana, WI)]. The chambers were set at 10°C and 95% humidity and the sample tubers were incubated for 40 days until evaluation (Personal Communication with Dr. Willie Kirk, Michigan State University).

Tubers were weighed prior to inoculation and the weight recorded on a spreadsheet and written on the tuber surface. The tubers were subdivided into four replications of five tubers and placed in a boxes arranged in a randomized complete block design. After incubation, the site of the inoculation was cut longitudinally and the rotted area removed with a stream of water. The remaining tubers tissue was weighed and the percentage tissue lost was calculated (Personal Communication with Dr. Willie Kirk, Michigan State University).

***Streptomyces scabies* in Tubers**

A trial was planted in the Potato Common Scab Disease Nursery at Michigan State University. The field was inoculated with *S. scabies* from aggressive Michigan isolates and was cultivated for high disease pressure for six years. Potatoes are grown every year in this field and organic matter is added to promote disease development. The trial was planted as a randomized complete block design consisting of four replications of five plants per plot. *S. scabies*-susceptible potato varieties were used as markers between plots. Standard cultivation practices were used for field preparation, planting, and field maintenance under irrigation conditions. The plots were harvested with a one-row digger and laid on top of the soil for evaluation of disease severity. A pathology rating scale was used to evaluate *S. scabies*. Each tuber was rated separately for lesion type and severity of coverage. From each plot, 10-20 tubers were scored based on the rating scale described in Table 11-13 (Personal Communication with Joe Coombs, Michigan State University).

Table 11-13. Scab Severity Rating Scale

Rating	Lesion Type	Percent Coverage (%)
0	No scab lesions	0
0.5	Brown-like lesions (small, star-shaped)	1
1	Superficial lesions, discrete.	1-2
2	Superficial lesions, coalescing	2.1-5
3	Raised lesions, discrete	5.1-10
4	Raised lesions, coalescing	10.1-25
5	Pitted lesions, discrete	25.1-50
6	Pitted lesions, coalescing	>50

11.6.1 Statistical Methods for Disease Studies

The statistical analysis for disease studies was performed by Simplot using SAS 9.3. All data were analyzed using the following linear mixed model:

$$Y_{ij} = \alpha_i + \gamma_j + \varepsilon_{ij}$$

- α = mean of treatment (fixed)
- γ = rep (random)
- ε = residual random error

Where α_i denotes the mean of the i^{th} treatment (fixed effect), γ_j denotes the random rep effect, and ε_{ij} denotes the residual random error.

A significant difference was established with a p-value < 0.05. 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.

Interpretation of the Results
For each comparison between W8 and the control, the following logic was employed to assess the results. First, when p-values were available, statistical significance was determined for each comparison. If the p-value indicated no statistical significance, it was unlikely that there was a difference that would be biologically meaningful or indicative of increased plant pest potential. Next, if the p-value indicated statistical significance or if a p-value was not present, the mean value of W8 was compared to the conventional variety range (CVR), which represents the minimum and maximum mean values of all conventional varieties included in the experiments. If the value for W8 was within the CVR, it was concluded that W8 exhibited characteristics within the natural variation of potatoes and that the difference was unlikely to be biologically meaningful or indicative of increased plant pest potential. If the value of W8 was outside the CVR, further consideration was given to the difference in the context of agronomic impact and increased plant pest potential.

The CVR for the field disease specificity studies used the range of mean values from three conventional controls and four conventional reference varieties at one site per study during 2013.

11.7 Field Test Reports

The J.R. Simplot Company has adhered to USDA compliance regulations by submitting the field test reports for each notification (Table 11-14).

Table 11-14. Field release notifications for agronomic and disease susceptibility studies

USDA Reference Number	Field Trial Year	Effective Start and Expiration Date	Release Sites (by State) Covered by Notification	Field Test Report Submitted (Yes or No)
12-066-102n	2012	3/28/2012 - 4/2/2013	ID, WA	Yes
12-121-101n	2012	4/30/2012 – 4/30/2013	ID	Yes
13-079-106n	2013	3/29/2013 – 4/8/2014	ID	No
13-072-112n	2013	4/2/2013 – 4/2/2014	ID, WA, MN, PA	No
13-079-107n	2013	4/10/2013 – 4/10/2014	ND, ID	No
13-079-102n	2013	4/10/2013 -4/10/2014	WI	No

11.8 Conclusion on Agronomic Performance, Yield and Grading, and Disease Susceptibility

The purpose of the agronomic trials was to confirm that Russet Burbank Event W8 has an equivalent phenotype compared to the Russet Burbank control, when grown at multiple locations representing the major areas for potato production in the US including Russet Burbank.

Results of the trials confirmed that W8 had an equivalent phenotype compared to the control. Phenotypes that could indicate enhanced weediness or plant pest potential are any characteristic where the test products were more vigorous than controls. Although there were significant agronomic differences, none would signal enhanced survival compared with control. For example, at the time plants were observed for early emergence, the percent of W8 plants emerged was less than the control. However, when final emergence data were collected, W8 was no different from the control.

W8 had fewer large tubers than the control, which could be associated with lower yield; however yield was not significantly different. Other characteristics, such as fewer sugar ends and high sugar fries and more fries with lighter fry color are positive indicators of potato and fry quality and are an expected result of invertase silencing. None of these differences in size or quality would indicate increased plant pest potential.

Out of 3 foliar diseases, 1 stem and stolon disease, and 6 tuber diseases analyzed, W8 showed no difference in susceptibility compared to the control with the exception of a reduction in tuber coverage and severity of common scab caused by *S. scabies*. If W8 has lower susceptibility to common scab, it would not likely be associated with increased plant pest potential. Overall, these data support the claim that the *Ppo5* silencing and the *Rpi-vnt1* genes are not linked to altered disease susceptibility in foliage, stolons, stems, and tubers. The results provide evidence of no increased plant pest potential in W8 and also show specificity to the Potato Late Blight Resistance Gene, *Rpi-vnt1*.

Thus, no differences were observed that would contribute to increased weediness or plant pest potential in W8 for any of the agronomic characteristics, yield and grading characteristics, or ecological interactions such as plant-insect interactions, plant-disease interactions, and plant interactions with abiotic stressors. It can be concluded Russet Burbank Event W8 is no different from the control in terms of agronomy, tuber yield and grade, and susceptibility to insects or diseases other than late blight.

11.9 References

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12.0 Environmental Safety Assessment

The environmental safety of Russet Burbank W8 is supported by extensive testing including agronomic performance, trait efficacy and specificity, genetic characterization, compositional assessment, VNT1 protein evaluation, and bioinformatics screening for toxins and allergens. Throughout these studies, the concept of familiarity of potato was considered. Familiarity has been widely used to describe the interactions that a plant may have in nature by considering the biology of the crop, the introduced trait, the receiving environment, and the interactions with all of these factors (Hokanson et al. 1999). This concept allows regulatory decision-makers to draw upon past experience with introduction of plants into the environment and compare genetically modified plants to their non-modified counterparts.

Information on W8 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.

12.1 Potential to become a weed of agriculture or to be invasive of natural habitats

Weediness is a term used to describe the ability of a plant to become a weed (survive and thrive) outside of cultivation. Multiple field trials with W8 did not provide any evidence for altered growth characteristics such as accelerated tuber sprouting, increased plant vigor, increased tuber set, delayed senescence, or other key agronomic characteristics associated with weediness or survival outside of cultivation. Additionally, all field sites were monitored for 2 years after harvest for volunteer activity as required by USDA-BRS compliance. Through two years of field studies and up-to-date volunteer monitoring of every site, no differences have been observed that would lead us to believe that W8 has properties that would increase the survivability compared to conventional potato.

Potato is a poor competitor and does not thrive in a non-cultivated environment (Love 1994). Due to modern agricultural practices, it is highly 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, in the northern U.S. and Canada, most production areas experience deep frost penetration in the soil, minimizing the likelihood of over-winter survival (CFIA 1996). Results of the agronomic assessment show no differences between W8 and its conventional control that would alter survivability, and thus it is unlikely that W8 possesses increased potential to become a weed of agriculture or to be invasive of natural habitats.

12.2 Potential for gene flow to sexually compatible plants

Gene flow from Russet Burbank Event W8 is nonexistent because the variety is sterile. 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 tends to be limited to about 20 meters 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 W8 and untransformed plants in the field, the impact would be negligible to future plantings since tubers rather than seed are removed from the field. 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.

The tetraploid species *S. fendleri* and diploid *S. jamesii* are the only wild species that grow within the borders of the United States (Bamberg et al. 2003; Spooner et al. 2004*).* These species mostly occur outside of the major potato growing regions in Texas, New Mexico, Utah, Colorado, and Arizona. These species reside in dry forests, scrub desert, and sandy areas at altitudes of 5,000 to 10,000 feet, well isolated from most commercial production areas (Bamberg and del Rio 2011). Therefore, pollen flow from commercial events to wild species is extremely unlikely simply because of the geographic location. In addition, the Atlas of Wild Potatoes, (Hijmans et al. 2002) makes the statement that no wild potato species occur in Canada.

Based on conversations with Bamberg, Love reported that no one has ever reported finding hybrids between native and cultivated potatoes in the U.S., although gene transfer has been accomplished using special laboratory techniques (Love 1994). Love concluded that, based on the barriers that exist, including geographic isolation as described above, endosperm imbalances, and multiple ploidy levels, natural hybridization is highly unlikely, and introgression by cross hybridization over multiple generations, is impossible or at least highly improbable (Love 1994).

The US EPA has concluded that, based on its review of the scientific literature, successful gene introgression between native and cultivated potatoes in the U.S. is virtually excluded due to constraints of geographical isolation and other barriers to natural hybridization. These barriers include incompatible, meaning unequal, endosperm balance numbers that lead to endosperm failure and embryo abortion, multiple ploidy levels and incompatible mechanisms that do not express reciprocal genes to allow fertilization to proceed. No natural hybrids have been observed between these species and cultivated potatoes in the U.S. (US EPA 2011).

In conclusion, the Russet Burbank potato variety and W8 produce few flowers and are male sterile. Other factors limiting outcrossing include the tendency for most fertile varieties to be self-pollinated, an inability to attract honey bees because they lack nectar, a limited pollen transfer range of about 20 meters, and the fact that true seeds would be unlikely to grow into mature potatoes since potato seeds are not saved and propagated in a 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. In addition, potatoes are not known to escape from commercial fields or show weediness potential. Wild potato varieties are rare in North America and for the most part geographically isolated from commercial production areas, further reducing concerns about cross-pollination with wild species (CFIA 1996). No biologically relevant differences were identified in W8 which could contribute to increased potential for gene flow.

12.3 Potential to become a plant pest

Data collected and reported in the agronomic performance section show that in multi-year and multi-site field trials, no specific differences leading to increased weed or plant pest potential were present. Weediness and invasiveness are already considered above and the data on abiotic and biotic stressors included in the agronomic performance section indicate that there are no meaningful differences in susceptibility to pests and diseases common to potatoes except for the intended resistance to late blight. The agronomic data supplied in this submission show no evidence of an altered response to a wide range of pests and diseases. Thus, no increased potential to become a plant pest would likely result from cultivation of W8.

12.4 Potential impact on non-target species including humans

The modifications to Russet Burbank that resulted in W8 were intended to enhance both the late blight resistance and the quality of potatoes. In the agronomic performance section, the data show that W8 is effective against late blight but responds like Russet Burbank control to a panel of other diseases. Also, no differences were observed for insects or other arthropods interacting within the potato ecosystem indicating a lack of altered impact on non-target organisms during cultivation. The methodology used to develop W8 does not result in the expression of new polypeptides in the plants other than the VNT1 protein responsible for late blight resistance. A bioinformatics assessment showed no new toxins or allergens that could affect humans or other non-target species. The compositional analysis did not identify any altered nutritional components that would be biologically significant and there were no changes in glycoalkaloids. Thus there is no indication that the impact on humans or other non-target organisms would be altered by cultivation of W8.

In conclusion, no altered potential for a negative impact on non-target organisms, including humans, from the cultivation of Russet Burbank Event W8 is expected. In fact, the reduction of acrylamide levels in food products made with these potatoes could be beneficial to humans considering the potential negative health effects associated with ingestion of acrylamide (NTP 2012).

12.5 Potential impact on biodiversity

The modifications to Russet Burbank resulting in W8 were intended to enhance late blight resistance and quality, not agronomic characteristics, of potatoes. Planting, cultivation, management and harvesting techniques were not affected by the incorporated traits, with the exception that late blight resistant potatoes should require less fungicide use. No other natural defense mechanisms have been activated; therefore, the modified potatoes have no new properties to enhance their weediness, invasiveness, or pest resistance and therefore display no traits which would have a direct impact on endangered species or biodiversity. The changes in potato quality intentionally introduced into these events have no more impact on biodiversity than any other commercial potato variety as shown by our multi-site and multi-year field trials. Furthermore, the W8 event is likely to be planted in areas that are already growing potatoes and would not result in a significant expansion of planted acres. For these reasons, it is unlikely that the cultivation of W8 would have any greater impact on biodiversity than conventional potatoes.

12.6 Summary of the Environmental Safety Assessment

The environmental safety of W8, for which detailed information is included in this submission, was assessed by comparing W8 with the Russet Burbank control variety. Familiarity of potato was considered during evaluation of the data, including agronomic performance, trait efficacy, specificity, compositional assessment, toxicology, and allergenicity.

Information on W8 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.

Regulated field trials over multiple years with W8 did not provide any evidence for increased plant pest characteristics such as accelerated tuber sprouting, increased plant vigor, increased tuber set, or delayed senescence. Due to modern agricultural practices it is highly 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. Wild potato varieties are rare in North America and for the most part geographically isolated from commercial production areas, reducing concerns about cross-pollination with wild species. Field studies confirmed there were no differences in ecological interactions between W8 and the Russet Burbank control. Planting, cultivation, management and harvesting techniques were not affected by the incorporated traits, with the exception that late blight resistant potatoes should require reduced fungicide. The introduction of W8 would not result in a significant expansion of planted acres or a change in the areas where potatoes would normally be grown. Therefore, the potential to impact insects and other nontarget organisms, weed or disease susceptibility, endangered species, or biodiversity is negligible from the cultivation of W8.

The data presented here demonstrate that introduction of Russet Burbank Event W8 will have a similar environmental impact when compared to untransformed 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 or biological impacts.

12.7 References

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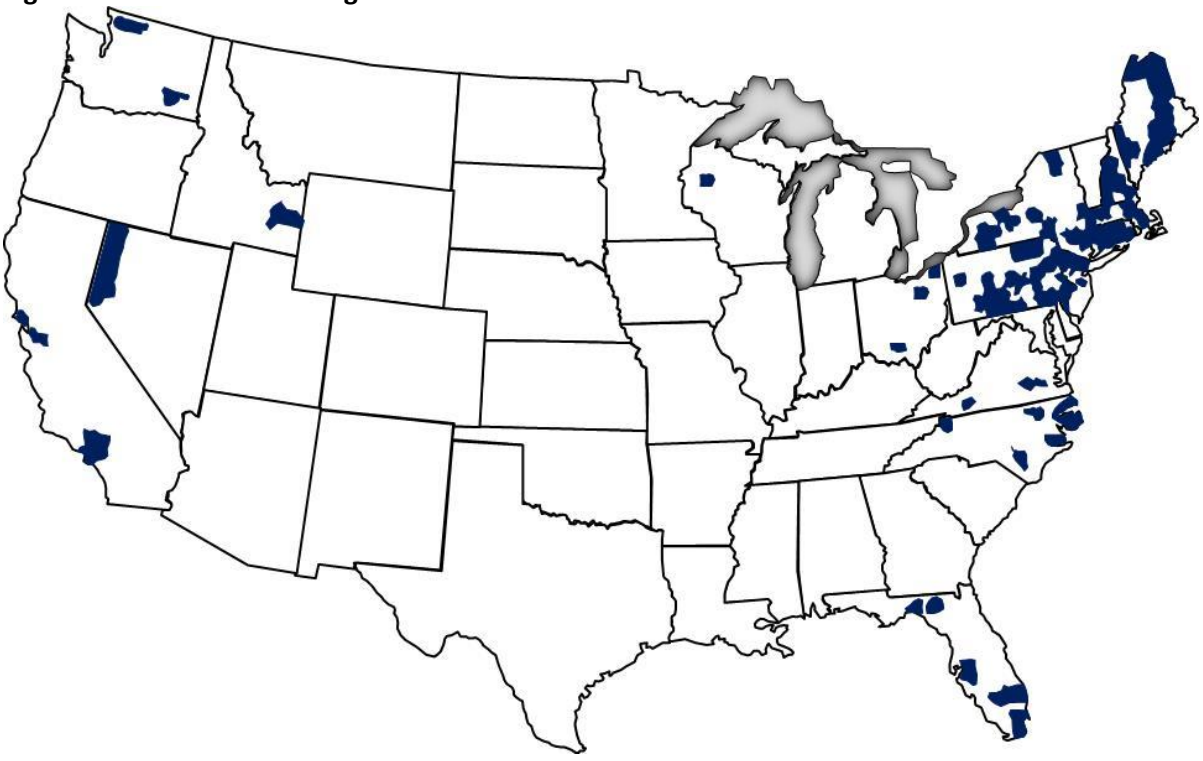
13.0 Crop Production and the Potato Industry

Russet Burbank Event W8 has multiple traits that provide benefits to potato processors, growers and consumers including late blight resistance, low acrylamide potential, reduced black spot, and lowered reducing sugars. These benefits were added to Russet Burbank potatoes through tools of modern biotechnology such as gene silencing using potato genomic DNA and in the case of late blight resistance, genes were obtained from a wild potato species. Integrating these traits simultaneously through conventional breeding would not be possible because potatoes are tetraploid, highly heterozygous, and are sensitive to inbreeding depression. Additionally, in the case of Russet Burbank, the variety is sterile. Commercially grown potato varieties such as the Russet Burbank are vegetatively propagated to maintain desirable traits developed over many years.

13.1 Benefits of Potatoes with Late Blight Resistance

Historically, late blight has long been a problem in potatoes, and was the cause of the Irish potato famine in the 1840s. It remains one of the most devastating diseases in modern potato production. The potato late blight oomycete overwinters in tubers infected from previous seasons which can give rise to infected potato seedlings. In infected plants, 1 potato leaf can hold 10,000 late blight spores, which can be spread through wind or animal movement. Late blight is prevalent in many areas of the United States and is now found in nearly every leading potato state (Figure 13-1).

Figure 13-1. Areas of late blight infection in 2012 are shaded.



The late blight pathogen spreads quickly through infected fields, causing widespread foliar necrosis resulting in plant death and significant yield reduction. Infected tubers can cause rot that destroys entire lots in storage. If late blight is not detected early or before pressure is high, susceptible varieties may require fungicide treatment every 3 to 5 days (for a total of 5 to 15 total applications before harvest)

and still incur yield losses of 20% to 70%. In terms of pesticide use, fall harvested potatoes require 23% of the total pesticides used for major crops including corn, soybean, cotton and wheat, which is second only to corn at 40% of the pesticide use (O'Donahue et al. 2011). Considering that potatoes are planted on a little over 1 million acres and corn is planted on about 90 million acres in the US, pesticide use per acre is much higher for potatoes than for corn. The average cost to control late blight in 2000 was estimated to be $205/ acre for a total cost of $77.1 million in the US. In addition, lost revenues related to poor yield from potato plants decimated by late blight resulted in an additional $210.7 million in 2000 (Guenthner et al. 2001).

A more recent study (Context 2014) suggests that annual fungicide use on potatoes costs about $90 million in the US, with about $60 million specifically for late blight management. This same study recommends that late blight resistant potatoes like W8 may require some fungicide applications, but could result in savings of $29 million per year with reductions of 290 million lbs of fungicide active ingredient. Other savings would be realized from labor, water, fuel, equipment maintenance, and the benefit of continual plant manufactured resistance that would not be subject to loss of effectiveness from rain or other environmental factors.

As one of the most important potato pathogens, resistance to late blight could have significant benefits with respect to reduced use of pesticides, but also could effectively improve yield compared with diseased potatoes. One result could be less chemical residue in food, land, and waste water. Even with pesticide application, some disease tends to persist in potato fields so effective resistance could result in healthier plants and better yield. Late blight affects both foliage and tubers. Evidence suggests that tuber damage is far more likely to occur if the plant foliage is infected than by picking up the disease agent only through soil contact. Tuber infection with late blight results in lesions, rot, and ultimately economic loss in storage. The loss of entire crops due to late blight is not uncommon in fields and storages in developed countries as well as in developing countries.

13.2 Benefits of Potatoes with Reduced Acrylamide Potential

Based on a Federal Register notice published in 2013, FDA has proposed guidance for industry on the reduction of acrylamide levels in food products (FDA 2013). 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." An extensive list of potential mitigation techniques were summarized in the guidance document including the reduction of sugar levels in potatoes (FDA 2013). The list also includes variety development and selection, focusing on varieties that are more resistant to cold-induced sweetening, and lower levels of reducing sugars or asparagine. Many of the methods in FDA's Guidance document are consistent with those reported in the Acrylamide Toolbox published by Food Drink Europe (2011).

The introduction of Innate™ potatoes with low acrylamide potential would provide potatoes that are largely indistinguishable from existing varieties. The reduction in asparagine and sugars using Innate™ technologies and the resulting reduction in acrylamide upon heating will address food industry needs with respect to the FDA's Draft Guidance (2013). As such, this biotechnology approach to lowering acrylamide was mentioned in the guidance document as a promising method to develop potato varieties with reduced acrylamide potential.

At the time of harvest, french fries made with W8 tubers contained 85% less acrylamide than the control. When potatoes were stored throughout nine months at 46˚F, acrylamide levels in W8 were 78

to 84% lower than control Russet Burbank (Tables 10-4 and 10-5 in Chapter 10: Trait Efficacy). The low acrylamide potatoes will provide an option that addresses this potential health issue for all sectors of the potato processing market and thus should increase the demand for these Innate™ W8 potatoes.

13.3 Benefits of Potatoes with Reduced Black Spot

The blackening that occurs after potatoes are bruised affects quality and recovery in processing french fries and chips. Potatoes that have been damaged and show black spot must be trimmed or could be rejected before processing, resulting in quality challenges or economic loss or both. In many instances potato growers have contracts that provide incentives for delivering "bruise free" potatoes. A significant reduction in black spot could result in higher profit for the farmer as a result of decreased visible damage. Also, these black spots are considered defects in potato chip and french fry processing, causing economic loss from trimming or culling potatoes with black spot. Potatoes may develop black spots from pressure bruising which results from the weight of potatoes in deep piles during storage. The weight of the potatoes causes damage to the potato tissue resulting in dark colors that must be removed through trimming.

Bruising and discoloration are also problems in the market for fresh whole potatoes. Potato lots that don't meet USDA bruise tolerance standards are diverted to other uses at lower prices. Another possible advantage of silencing black spot could be the enabling of new markets for "freshly cut" potatoes, without pre-cooking or using sulfites or other preservatives to maintain color and flavor. The convenience of fresh-cut potatoes could boost demand for Innate™ W8 potatoes. Focus group market research indicates that consumers will readily accept packaged fresh-cut potatoes in the refrigerated produce section of supermarkets.

13.4 Benefits of Potatoes with Lowered Reducing Sugars

In breeding programs for processing potatoes, one of the most desired characteristics is low levels of the reducing sugars glucose and fructose. High levels of reducing sugars lead to undesirable dark colors and bitter flavors in fries and chips. High sugar levels can result from any stress to the plants during growing or harvesting such as drought or heat stress (Bethke et al 2009), but develop rapidly with low temperature storage (Driskill et al. 2007). Typical storage temperatures for potatoes for the frozen fry market are 46 - 48˚F (Driskill et al. 2007) to maintain sugar levels while decreasing potato storage disease potential. If potatoes could be stored at a lower temperature without adversely affecting sugar level, the result would be less disease damage and less weight-loss from shrinkage due to lower respiration rates.

With introduction of pSIM1278 into Russet Burbank potatoes (Collinge and Clark 2013), we found silencing of the promoters for *R1* and *PhL* to result in slight reductions in reducing sugars at the time of harvest or after one month of storage. The silencing cassette contained in pSIM1678 results in silencing of *VInv* in W8, which results in lower levels of reducing sugars throughout the storage period. *VInv* silencing may also allow for lower temperature storage which will reduce yield loss from respiration and disease. Many potatoes must be stored for as long as 3 to 12 months before processing, and the ideal storage potato maintains a low level of reducing sugar throughout storage. A consistent focus of breeders for processing potatoes would be to have low reducing sugars at the time of harvest that remain low throughout their storage life (Driskill et al. 2007). There is also evidence that *VInv* silencing reduces the incidence of high sugar potatoes and sugar ends (see Chapter 11: Agronomic Performance, Table 11-4), both quality attributes that result in economic loss by causing potatoes to be rejected by

french fry and chip processors, resulting in lower prices as they are only suitable for making dehydrated potatoes or cattle feed. Additionally, significantly lower levels of acrylamide were found after frying (see Chapter 10: Trait Efficacy, Table 10-4).

13.5 Potato uses and exports

Potatoes are grown commercially in 36 of the 50 states in the US. Total US production in 2012 was 467 million hundred weight (cwt), with total value of $3.91 billion, planted on 1.15 million acres (USDA ERS 2013). The commercial uses include 36% as frozen, 26% sold fresh, 15% chips and shoestrings (julienne cut crispy snacks), 11% dehydrated, 6% seed potatoes, 4% other frozen products, and 1% canned (NPC 2013). Annual per capita potato use in the US in 2012 was 117 lbs per person (USDA ERS 2013).

The states with the largest volume and sales value in 2012 were Idaho, Washington, Wisconsin, North Dakota, Colorado, Oregon, Minnesota, Michigan, California, and Maine (NPC 2013). These data represent the combined markets including all processing and fresh varieties.

US exports of all potatoes and potato products for the 2013 fiscal year (July 2012-June 2013) were 1,561,896 metric tons (MT) valued at $1.64 billion, which are both 4 % increases over fiscal year 2012 (USPB 2013). US exports of frozen potato products for fiscal year 2013 increased by 2 % for volume and 6 % by value over fiscal year 2012, with 926,553 MT and a value of just over $1 billion, respectively. US exports of fresh potatoes increased by 13 % in fiscal year 2013 to 456,366 MT, but value was reduced by 4 % at $198 million compared to fiscal year 2012. The decline of value was mainly due to a reduction in value of exports to Canada. Finally, US exports of dehydrated potatoes decreased by 3 percent in fiscal year 2013 to 114,408 MT, but the value stayed about the same at $192 million compared to fiscal year 2012 (USPB 2013). Overall, 2013 was the most valuable year to date with over $1 billion in value for the first time.

A majority of the potato exports were frozen fries at 62% of the export market, fresh potatoes made up 13% of the market, chips and dehydrated potatoes each had 12% of the export market, and seed made up 1% of the export market (NPC 2013). Finally, the top three export markets for US potatoes in 2012 were Japan, Canada, and Mexico with values of $404 million, $342 million, and $185 million, respectively.

13.6 Submissions to Other Regulatory Agencies

An Experimental Use Permit (EUP) application was submitted to EPA on December 16, 2013, for field testing of Innate™ late blight resistant potatoes on more than 10 acres across the United States. An EUP, also for late blight resistant potatoes, with a Petition for Temporary Tolerance Exemption was submitted February 20, 2014. A Section 3 Registration will be filed after experiments are completed under the EUPs. Simplot has initiated and will complete a consultation process for food safety and nutrition with the Food and Drug Administration (FDA) prior to commercial distribution of potatoes from Russet Burbank W8.

An assessment of the safety of W8 will also be submitted to Health Canada and the Canadian Food Inspection Agency (CFIA). Furthermore, submissions in support of approval to import products from the events will be made to the Japan Ministry of Health, Labor and Welfare (MHLW) and the Ministry of Agriculture, Forestry, and Fisheries (MAFF). Submissions are anticipated for other key international markets including Mexico.

13.7 Impact on the Organic Market

Organic potatoes in 2011 represented less than 1.0% (0.7%) of total potato plantings with 8,273 acres of certified organic potatoes (USDA ERS 2014). Of the 13,258 organic acres, California, Colorado, and Oregon were the top three organic potato producers with 6,520 acres, 2,138 acres, and 1,654 acres, respectively. Potato varietal selection and breeding are similar between organic and conventional potato production as commercial potatoes are predominantly planted from tuber seed. Risks to organic growers would be most likely to occur with accidental mixing of planting material or of potatoes in farming, transportation, or processing channels. These risks are the same as those that organic growers already experience when keeping their organically grown potatoes separate from conventionally grown potatoes. Because potatoes are clonally propagated and the Russet Burbank variety is sterile, there is no risk of contaminating seed supplies through cross-pollination. Organic farmers routinely plant organic tuber seed material and any incidence of cross-pollination in production fields will not affect the harvested potatoes. Producers of organic true potato seed (TPS) will be able to protect their seed from Innate™ material by ensuring that the seed production fields are sufficiently isolated from Innate™ potato fields. Potential outcrossing can be prevented by separating fields planted for organic TPS production by at least 20 meters from fields planted with Innate™ potatoes (Conner and Dale 1996). However, outcrossing would not be relevant for Russet Burbank W8 since the variety is sterile.

13.8 History of Biotech Potatoes

A review of the history of the introduction of Monsanto's genetically modified potatoes in 1995 in the U.S. (Thornton 2008) indicates the importance of a careful assessment of the performance of W8, synchronizing approvals in North American and trading countries, and weathering public acceptance issues that may result from activism against food industry members that adopt the new technology. This history points to the importance of consumer acceptance when introducing any Innate™ crops and products to the market. Since the time when biotech potatoes were on the market briefly, the adoption of biotechnology in the food supply has increased consistently. Herbicide tolerant (HT) soybeans went from 17 percent of U.S. soybean acreage in 1997 to 68 percent in 2001 and 93 percent in 2013. The adoption of HT corn went from 4.3 percent in 1997 to 8 percent in 2001 and then accelerated to 85 percent of U.S. corn acreage in 2013. Plantings of insect resistant Bt corn grew from about 8 percent of U.S. corn acreage in 1997 to 76 percent in 2013 (USDA ERS http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/recent-trends-in-ge-adoption.aspx#.UnGtr_ns_cA). These two crops provide significant quantities of biotechnology-derived ingredients in widely marketed processed food and feed products, such as protein, oils, starches, and sweeteners. Many of the conventional processed potato products already contain one or more of these biotech ingredients. With widespread adoption of food ingredients from biotech crops, today's food industry should be far more likely to embrace biotech potatoes such as Russet Burbank W8, particularly in light of the positive consumer attributes of the W8 potatoes.

13.9 Stewardship of Innate™ W8 Potatoes

Stewardship of Potatoes

Throughout development of the Innate™ W8 potatoes, the Biotechnology Quality Management System (USDA-BRS Compliance assistance program) has served as a stewardship program to conduct field trials, monitor movement, and complete testing while regulated. As a part of BQMS, qualified and experienced potato growers were identified, and contractual agreements were established to ensure compliance and conformance with requirements for growing Innate™ potatoes. Processes were developed that

included Standard Operating Procedures (SOPs) and forms for all critical control points to guide internal and external collaborators to conduct activities using Innate™ potatoes.

The following management procedures were included for effective implementation of BQMS:

1. Document and record control
2. Resource management
3. Training external and internal associates
4. Planning and process realization
5. Conducting internal audits
6. Implement corrective / preventive actions
7. Continuous improvement of the system
8. Resolution of compliance issues

Targeted training programs were used to educate and bring awareness on handling Innate™ W8 potatoes in storage, transport, planting, harvest, post-harvest, processing, and final disposition to ensure that no Innate™ potato material entered food or feed channels prior to de-regulation. Both internal and external audits were used to verify that planned activities occurred and personnel were adhering to the protocols recommended by BQMS. This stewardship program includes corrective and preventive actions to avoid compliance and conformance incidents. While BQMS served as a quality management system during product development, with commercial introduction we anticipate a full extension of such methods as outlined in the 'Excellence Through Stewardship®' (BIO 2007) program.

Identity Preservation

To help prevent trade disruptions, international approvals will be sought from key trading partner countries before the Innate™ W8 potatoes are launched commercially. The initial introduction will build up slowly as seed becomes available and will be controlled within existing processing channels to ensure that W8 potatoes enter only the intended markets. This will provide an extended period of time to assess consumer acceptance and to address grower and industry awareness. A limited introduction in a vertically integrated supply chain will be well controlled by grower and processor agreements. In this situation, conventional products will be considered "identity preserved" with respect to the well-controlled stewardship of the Innate™ crop and its products. As Innate™ potato adoption increases, programs for identity preservation will be implemented as needed. It's expected that development and implementation of identity preservation systems will add some cost to the supply chain. The total costs will depend upon the type and extent of market penetration.

Synchronized Regulatory Approvals

We intend to follow the recommended stewardship policy statement released by the Biotechnology Industry Organization in May 2007.

"To help ensure the continued adoption of agricultural biotechnology globally and to continue to have products of agricultural biotechnology bring value to the marketplace, BIO's Food and Agriculture Section supports actions that facilitate the flow of goods in commerce and minimize trade disruptions. BIO's Food and Agriculture Section believes that henceforth individual member companies should, prior to commercialization meet applicable regulatory requirements in key countries identified in a market and trade assessment that have functioning regulatory systems and are likely to import the new biotechnology-derived plant products."

This voluntary guideline was adopted because, according to BIO: “asynchronous authorizations combined with importing countries maintaining ‘zero tolerance’ for recombinant-DNA products not yet authorized results in the potential for major trade disruptions. The potential occurrences of trade disruptions will only increase given the substantial amount of research that will bring many new products and combinations of products to market.”

13.10 Conclusions: Crop Introduction and the Potato Industry

Russet Burbank W8 potatoes provide the potato industry the opportunity to keep the highly desired characteristics of the leading french fry and fresh potato variety with multiple enhancements that could not be added through traditional breeding. Overall improvement in yield and lowered fungicide use with late blight resistance should benefit all members of the food value chain by reducing environmental impacts associated with fungicide use, and potentially reducing acreage devoted to potato production. The combination of low asparagine and reducing sugars results in greater than 70% reductions in acrylamide even after extended cold storage, addressing the potential health risk for consumers and the food industry. Quality improvements related to lower levels of reducing sugars and black spot provide benefits to processors and consumers, and invertase silencing could positively impact quality and yield from potato storage. Finally, reduced invertase could enable storage of processing potatoes at significantly lower temperatures, decreasing loss from disease and yield losses from higher respiration rates related to typical storage at 46 – 48 ˚F. Lastly, the combination of low asparagine and reducing sugars results in greater than 70% reductions in acrylamide even after extended cold storage, addressing this potential health risk.

Knowledge about potato biology, especially the fact that Russet Burbank is a sterile variety, should alleviate potential questions regarding outcrossing to other varieties or organic potatoes. It is anticipated that the combination of the planned stewardship program along with approval in key international markets will prevent trade disruptions through careful management of the supply chain.

13.11 References

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14.0 Conclusions: Determination of Nonregulated Status for Russet Burbank W8 Potatoes

The potato industry and customers will benefit from introduction of late blight resistant Russet Burbank potatoes, with lower reducing sugars, black spot, and acrylamide potential. Considering that the Russet Burbank variety is sterile, tools of modern biotechnology such as Innate™ technologies, are ideally suited for simultaneously incorporating multiple traits. The propagation of commercial potatoes through cloning mitigates concerns about increased plant pest potential such as seed dispersal, survival outside of cultivation, or outcrossing. Based on multi-year field trials evaluating composition, agronomic performance, and disease stressors, as well as the basic understanding and familiarity of potato biology, W8 poses no significant risk of persistence in the environment or altered environmental interactions as a result of weediness or increased plant pest potential.

Results of efficacy studies with Russet Burbank Event W8 confirm the expected late blight resistance, along with reductions in black spot, reducing sugars, and acrylamide. Significant reductions in reducing sugars and acrylamide were observed throughout long term storage.

Collectively, the ubiquitous nature of resistance genes like *Rpi-vnt1* and their encoded resistance proteins such as VNT1 in *Solanum* species, including potatoes, lack of toxins or allergens, mode of action not based upon inducing pest toxicity, and extremely low expression levels provides confidence in the safe use of potatoes created to express VNT1.

The data presented here demonstrate that introduction of W8 will have a similar environmental impact as control Russet Burbank 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 or biological impacts.

We now seek nonregulated status for Russet Burbank event W8 based on the weight of evidence demonstrating that the W8 potatoes are unlikely to pose a plant pest risk and respectfully submit that these plants should not be classified as “regulated articles” as defined under 7 CFR Part 340.

15.0 Acknowledgements

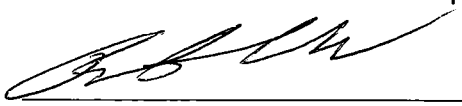
The J.R. Simplot Company would like to acknowledge the efforts of the following Regulatory Scientists for their contributions in this submission: Matt Pence, Ph.D., Sathya Adimulam, Stephanie McInerney, Jeff Hein, Janet Layne, Erika Roach, Juan Pablo Burzaco, and Eric Rosenbaum. Additionally, we would like to acknowledge the R&D and Commercial Teams led by Craig Richael, Ph.D. and Kerwin Bradley, respectively.

16.0 Statement of Grounds Unfavorable

JR Simplot is not aware of any information indicating that Innate™ potatoes may pose a greater plant pest risk than conventional potatoes. There are no adverse environmental consequences anticipated with its introduction based on the available data and information. The benefits of introduction of commercial events are provided in this submitted petition.

17.0 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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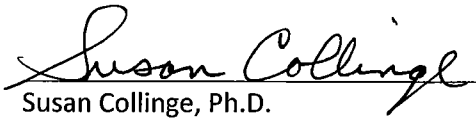

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