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PETITION FOR DETERMINATION OF NON-REGULATED STATUS

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

(Signature) JBurton

Statement of Grounds

As with W8, Simplot's Z6 potatoes have late blight protection, reduced black spot, and lower reducing sugars and free asparagine, which together contribute to low acrylamide potential. Considering that the Snowden variety, like other potatoes, is difficult to breed, biotechnology applications are ideally suited for simultaneously incorporating multiple traits. The vegetative propagation of commercial potatoes helps mitigate concerns about seed dispersal, survival outside of cultivation, and outcrossing, that could contribute to increased plant pest potential. Field trials evaluating phenotypic performance and insect and disease interactions demonstrate that Z6 poses no significant risk of persistence in the environment as a result of weediness or increased plant pest potential.

The data presented in this submission demonstrate that introduction of Z6 potatoes will have a similar environmental impact as W8 and conventional potatoes and pose no increased risk to the environment. The introduction and cultivation of these potatoes is not expected to cause any adverse environmental or biological impacts, or detrimental effects on plant health.

Simplot seeks an extension of the deregulation of the W8 event in 14-093-01p and requests non-regulated status for Z6 based on the weight of evidence demonstrating that these potatoes are unlikely to pose a plant pest risk. We respectfully submit that Z6, and its progeny, should not be classified as "regulated articles" as defined under 7 CFR Part 340.

Petition for Extension of Non-Regulated Status for Z6 Potatoes with Late Blight Protection, Low Acrylamide Potential, Lowered Reducing Sugars, and Reduced Black Spot

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

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J.R. Simplot Company

OECD unique identifier: SPS-ØØØZ6-5 (Z6)

May 31, 2019

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Certification

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

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Table of Contents

Table of Figures

List of Tables

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

Abbreviation	Definition
Agp	ADP-glucose pyrophosphorylase gene
A. tumefaciens	*Agrobacterium tumefaciens*
ASN	Probe used to detect *Asn1* sequence
ASN1	*Asn1* gene-derived probe used in DNA gel blot hybridization
Asn1	Asparagine synthetase-1 gene
CFR	Code of Federal Regulations
CLR	Combined literature range
CVR	Conventional variety range
cwt/A	Unit of measure equal to 100 lbs/acre or weight (lbs) of tubers harvested/acre divided by 100
DIGII	Molecular weight markers
DIGVII	Molecular weight markers
EPA	Environmental Protection Agency
ETS	Excellence Through Stewardship
FDA	Food and Drug Administration
FG, FG1, FG2	Field grown year
GBS, GBS1	Probe used to detect one region of the *Gbs* promoter sequence
GBS2	Probe used to detect second region of the *Gbs* promoter sequence
Gbss	Granule-bound starch synthase gene
IB	Internal band
ILSI	International Life Sciences Institute
Innate®	A branded biotechnology approach that uses plant genes to enhance desired traits
INV	Probe used to detect *VInv* sequence
JB	Junction band
kb	Kilobase
LB	Left border (25-base pair) is similar to *A. tumefaciens* T-DNA border
ORF	Open reading frame
pAGP	Truncated sequence that is not a functional promoter
pAgp	Promoter of the ADP glucose pyrophosphorylase gene
PCR	Polymerase chain reaction
pGbss	Promoter of the granule-bound starch synthase gene
PhL	Phosphorylase L gene
PPO	Polyphenol oxidase enzyme
Ppo5	Polyphenol oxidase-5 gene
RT-qPCR	Real time-quantitative PCR
R-gene	Resistance gene
R-protein	Resistance protein
R1	Southern blot probe used to detect the R1 cassette
R1	Water dikinase R1 gene
RB	Right border
Rpi-vnt1 gene	Resistance against *P. infestans* from *S. venturii*

Somaclonal variation	Genetic and/or phenotypic variation among clonally propagated plants of a single donor clone; generated by tissue culture and other forms of vegetative propagation
SPS-∅∅∅Z6-5	OECD identifier for Z6
T-DNA	Transfer DNA
USDA-APHIS	United States Department of Agriculture-Animal and Plant Health Inspection
VInv	Vacuolar invertase
VNT	Probe used to detect *Rpi-vnt1* sequence
VNT1	VNT1 protein
WT	Wild-type

Summary

The J.R. Simplot Company developed the SPS-ØØØZ6-5 (Z6) potato event as a retransformation of the previously deregulated event V11 (15-140-01p). The retransformation introduces late blight protection and invertase down regulation to the quality traits of reduced black spot, lower reducing sugars, and the benefit of lower acrylamide potential. The recipient variety, Snowden, is an important commercial variety for the chipping industry.

Z6 was developed using the same constructs and transformation method as W8, a previously deregulated event (14-093-01p). Simplot is submitting data for Z6 as an extension of W8, the antecedent organism, demonstrating similarity for trait mechanism-of-action in the same crop. Simplot requests a determination from the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) that event SPS-ØØØZ6-5 (Z6) and its progeny no longer be considered regulated articles under 7 CFR 340.

Molecular, agronomic, phenotypic, and compositional assessments demonstrate that Z6 is similar to W8, has low plant pest potential, and provides valuable traits to both the potato industry and consumers.

Z6 was obtained by *Agrobacterium*-mediated retransformation of V11 with pSIM1678. V11 had been generated by transforming Snowden with pSIM1278. The T-DNA from pSIM1278 down regulates four potato gene transcripts through RNA interference (RNAi):

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

These traits were reviewed when V11 was deregulated.

The pSIM1678 T-DNA was designed to down regulate the potato vacuolar invertase gene (*VInv*) transcript through RNAi and express the *Rpi-vnt1* gene:

- *VInv* (vacuolar invertase) for lower reducing sugars, contributing to low acrylamide potential; and
- *Rpi-vnt1* (protection against *Phytophthora infestans* from *Solanum venturii*) for foliar late blight protection.

The VNT1 protein is expressed at levels of less than 500 ppb in tuber and leaf. Human exposure to VNT1 is expected to be negligible due to the low expression level of the protein, which also limits exposure to non-target organisms and the environment. The VNT1 protein has a non-toxic mode of action and signals the programmed death of pathogen-infected plant cells limiting spread of the disease.

Molecular analyses demonstrate that Z6 maintains the insert from pSIM1278 as shown with V11 and contains a single copy of the insert from pSIM1678. In addition, backbone sequences were not introduced, and the inserts are stable through multiple cycles of potato vegetative propagation.

Agronomic and phenotypic analyses demonstrate that Z6 is equivalent to Snowden and other conventional potatoes. Agronomic and phenotypic data support the conclusion that an increase in plant pest or volunteer potential for Z6 is unlikely.

Compositional analysis of Z6 was conducted to evaluate key nutrient (proximates, vitamins, amino acids, minerals) and glycoalkaloid levels compared to controls. In addition, concentrations of free amino acids, and sugars were measured to evaluate trait efficacy.

The traits introduced into Z6 enhance disease protection and potato quality without enhancing weediness, or impacting on agronomic characteristics, insects and other non-target organisms, weed or disease susceptibility, endangered species, or biodiversity. Planting, cultivation, management, and harvest processes are not affected other than a potential reduction in fungicide use. Based on these data and analyses, Simplot requests that USDA-APHIS grant an extension of non-regulated status for the Z6 events and their progeny.

1.0 Rationale for Z6 Potato Varieties

SPS-ØØØZ6-5 (Z6) is a potato event developed by the J.R. Simplot Company. The rationale for developing Z6 potatoes is the same as for developing the antecedent organism, W8 Russet Burbank, deregulated on September 2, 2015 (14-093-01p). It provides an additional potato variety with late blight protection, low acrylamide potential, lowered reducing sugars, and reduced black spot. The introduced traits provide increased value by potentially lowering costs associated with disease management, processing, and storage.

The development of these traits in potato is accomplished by introducing partial or full-length potato gene sequences through transformation. A summary of the target genes and their intended functions is provided in Table 1-1. The addition of multiple traits into potato is difficult to achieve through traditional breeding because potato is tetraploid, highly heterozygous, and sensitive to inbreeding depression. Incorporating these traits is accomplished through transformation of each potato variety of interest.

Table 1-1. Summary of Z6 Genes and Intended Traits

Construct	Gene Target	Mechanism	Intended Trait	Intended Benefit
pSIM1278	*Asn1*: asparagine synthetase-1[1,2]	RNAi down regulation	Reduces free asparagine	Contributes to low acrylamide potential[3]
	R1: water dikinase[1,2]	RNAi down regulation	Lowers reducing sugars	Contributes to low acrylamide potential[3]
	PhL: phosphorylase L[1,2]	RNAi down regulation	Lowers reducing sugars	Contributes to low acrylamide potential[3]
	Ppo5: polyphenol oxidase-5[1,2]	RNAi down regulation	Reduces enzymatic darkening	Reduced black spot
pSIM1678	*VInv*: vacuolar invertase[2]	RNAi down regulation	Lowers reducing sugars	Prevents excess darkening during frying and contributes to low acrylamide potential[3]
	Rpi-vnt1: R-gene[2]	Protein overexpression	Confers protection to *P. infestans*	Late blight protection

[1] Previously deregulated trait in Simplot petition 13-022-01p, 15-140-01p, 14-093-01p, and 16-064-01p.
[2] Previously deregulated trait in Simplot petition 14-093-01p and 16-064-01p.
[3] Acrylamide is formed primarily from asparagine and reducing sugars heated at temperatures above 120 °C, as occurs during frying.

Simplot has transformed the Snowden variety because it is the second most important public variety for chipping after Atlantic (Figure 1-1). The Snowden variety stores well, offers generally good yields, and although is used in fresh and dehydrated markets, predominantly goes to the making of chips.

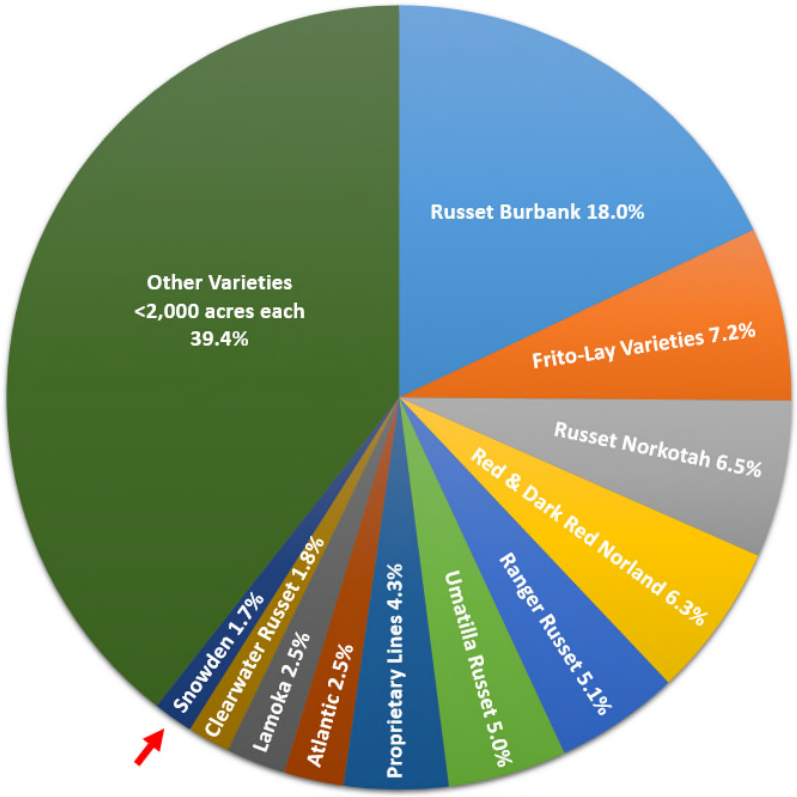

Figure 1-1. 2017 Certified Seed Potato Acres, By Variety
The top potato varieties grown for seed in the U.S. during 2017. Forty-one different varieties were grown on more than 300 acres. Potatoes grown on less than 2000 acres are combined and account for 39.4% of all potatoes planted. Source: NPC, 2018

1.1 Basis for Determination of Non-Regulated Status under 7 CFR §340.6

The Animal and Plant Health Inspection Service (APHIS) and the U.S. Department of Agriculture (USDA) have responsibility under the Plant Protection Act (7 U.S.C. 7701-7772) to prevent the introduction or dissemination of plant pests into or within the U.S. Part 340 regulates the introduction of organisms produced or altered through genetic engineering that are or may be considered plant pests.

The USDA-APHIS regulation 7 CFR §340.6(e) provides for an applicant to petition APHIS for evaluation of data submitted for a determination, based on similarity to an antecedent organism, that a regulated article, the genetically modified crop, does not pose a potential for plant pest risk and therefore, should not be regulated as such. A finding for deregulation would be made based on the similarity of the additional regulated article to an antecedent organism, i.e., an organism that has already been the subject of a determination of non-regulated status by USDA-APHIS under 7 CFR 340.6. The antecedent organism is used as a reference for comparison to the regulated article under consideration, as provided by the regulations.

Simplot is submitting data for Z6 as an extension of W8, the antecedent organism, demonstrating similarity for trait mechanism-of-action in the same crop, and requests a determination from USDA-

APHIS that events SPS-ØØØZ6-5 (Z6), and its progeny, be no longer considered regulated articles under 7 CFR 340.

1.2 Rationale for Using the Extension Process

Z6 was derived with the same transformations as W8, and contains the same traits as said antecedent event. Z6 is a retransformation of the V11 event that was deregulated on January 13, 2016 in petition 15-140-01p (Table 1-2).

Table 1-2. Regulatory Status of Simplot Events

Variety	Construct used for transformation:		Construct used for transformation:	
	pSIM1278		pSIM1678	
	Event	Status	Event	Status
Russet Burbank	E56	Non-regulated 17-122-01[1]	W8	Deregulated 9/2/15 14-093-01p
Ranger Russet	F10	Deregulated 11/10/14 13-022-01p	X17	Deregulated 10/28/16 16-064-01p
Atlantic	J3	Deregulated 11/10/14 13-022-01p	Y9	Deregulated 10/28/16 16-064-01p
Snowden	V11	Deregulated 01/13/2016 15-140-01p	Z6	Subject of this extension

[1] USDA Am I Regulated response letter confirmed non-regulated status of E56.

Z6 is a new variety of the same species (*Solanum tuberosum*) with the same incorporated T-DNA as W8 (Table 1-3). The observed phenotype, introduced traits, conclusions from molecular, agronomic, phenotypic, and compositional assessments are the same for Z6 and W8. Z6 does not exhibit additional traits beyond what was expressed in W8. Two additional events, X17 and Y9 (16-064-01p), were previously deregulated as an extension of W8 (14-093-01p).

Table 1-3. Comparison between Z6 and Previously Deregulated W8, X17, Y9

	W8	X17	Y9	Z6
USDA Number	14-093-01p	16-064-01p		Not yet issued
Date of Deregulation	9/1/2015	10/28/2016		Not yet deregulated
Date of Environmental Assessment	7/1/2015	7/1/2015*		Not yet assessed
Recipient Organism	Potato (*Solanum tuberosum* subsp. *tuberosum*)	Same	Same	Same
Variety	Russet Burbank	Ranger Russet	Atlantic	Snowden
Fertility	Male Sterile	Male Fertile	Male Fertile	Male Fertile
Transformation Method	*A. tumefaciens*	Same	Same	Same
Constructs	pSIM1278 and pSIM1678	Same	Same	Same
Benefits	Late blight protection, low acrylamide potential, lowered reducing sugars, and reduced black spot	Same	Same	Same
Traits and Gene Functions	*Rpi-vnt1*: late blight protection	Same	Same	Same
	Asn1: down regulated, reduced free asparagine			
	R1: down regulated, lower reducing sugars			
	PhL: down regulated, lower reducing sugars			
	VInv: down regulated, lower reducing sugars			
	Ppo5: down regulated, reduced enzymatic darkening			
Molecular	Well characterized and contains stable inserts	Same	Same	Same
Agronomic	Agronomically and phenotypically similar to conventional parent variety, other than the intended late blight protection	Same	Same	Same
Composition	Compositionally similar to conventional parent variety, other than intended changes to free asparagine and reducing sugars	Same	Same	Same
Plant Pest	Similar to parent variety in terms of reproductive fitness, gene flow, and other plant pest characteristics	Same	Same	Same

*FONSI for X17 and Y9 was based on the Environmental Assessment for antecedent potatoes, W8.

1.3 Submissions to Other U.S. Regulatory Agencies

As a new transformation event, Z6 is regulated by USDA-APHIS Biotechnology Regulatory Services.

As a food product, Z6 is within the scope of the FDA policy statement concerning regulation of food products derived from new plant varieties, including those developed by recombinant DNA techniques. A voluntary safety and nutritional assessment of Z6 has been submitted to the FDA Center for Food Safety and Applied Nutrition.

Z6 contains a plant-incorporated protectant and is subject to registration by the EPA under the Federal Insecticide, Fungicide and Rodenticide Act. A Section 3 registration application for Z6 has been submitted to the EPA. Similar events W8, X17, and Y9 were registered by EPA on January 19, 2017 (8917-1, 8917-2, and 8917-3, respectively).

EPA is the U.S. regulatory agency primarily responsible for evaluating safety of the VNT1 protein under the Federal Food, Drug, and Cosmetic Act. The EPA has issued a permanent tolerance exemption for the VNT1 protein in potato (40 CFR, §174.534).

1.4 Prior Environment Release of Z6

Z6 has been field tested in the U.S. since 2016. Z6 has been grown in six states, as authorized by the USDA-APHIS notifications listed in Appendix A.

2.0 Development of Z6 Potato Variety

SPS-ØØØZ6-5 (Z6) is a new potato event developed by the J.R. Simplot Company. Z6 was developed from the Snowden variety. Snowden was transformed with pSIM1278 to create V11, which was deregulated by the USDA. V11 was transformed with pSIM1678 to create Z6, which is the subject of this extension request. The transformation, selection and testing protocols were that same as those used for W8, X17, and Y9.

2.1 Characterization of the Recipient Variety

The Snowden variety was developed by the University of Wisconsin and released in 1990 as a public variety (PAA, 2017). Plants are tall, semi-erect, and have lightly pigmented stems with slight pubescence. The leaves are open with an olive-green color and slightly pubescent. They produce few flowers that are medium sized and white with yellow anthers. The tubers are round to oval with buff-colored skin and white flesh. Snowden is a high-yielding variety that produces a high tuber set. It responds well to nitrogen and needs high irrigation. This variety has high specific gravity, good storability, and short dormancy. Snowden is an excellent chipping variety from the field as well as from storage. Snowden potatoes store for three to five months at 10 °C with low sugar levels (UNL, 2014).

Although Snowden is male fertile, the probability of outcrossing remains low due to the lack of pollinators and geographic overlap between cultivated and wild potato species. Pollination of fertile potato varieties is mostly by bumblebees (*Bombus impatiens*), which has a relatively short flight range (less than three kilometers) (OECD, 1997). Pollen dispersal by nectar seeking pollinators is limited because potato flowers lack nectar limiting their attractiveness to honeybees (*Apis mellifera*) and *Bombus fervidus* species. Wind plays an insignificant role in pollen dispersal (OECD, 1997).

For fertile potato varieties, 80-100% of true seed is derived from self-pollination (Plaisted, 1980). A potential source of volunteers is true potato seed, produced by pollination. Seed develops inside small fruiting bodies formed on the potato vine. True potato seed is highly heterozygous, leading to longer establishment times and tuber sets, resulting in lower yield than what is typical with vegetatively propagated potato tubers (Pallais et al., 1988). Plants produced from true seed are no weedier than volunteer plants produced from overwintered tubers and are relatively easy to control in rotational crops.

While there appears to be minimal, if any, overlap geographically between cultivated and wild potatoes in the U.S., there is a possibility that a few wild potato plants may be growing near potato fields (Love, 1994). However, the potential for hybridization between wild and domesticated potatoes is extremely unlikely. Although approximately 200 species of wild potatoes are known, only two species grow within the U.S. borders, including the tetraploid species *Solanum fendleri* (recently reclassified as *Solanum stoloniferum*) and the diploid species *Solanum jamesii* (Bamberg and Rio, 2011; Bamberg et al., 2003; Hijmans and Spooner, 2001). In addition to geographic separation, other biological barriers to gene transfer include different ploidy levels and endosperm imbalances. Hybridization between native and cultivated potatoes have not been reported in the U.S., although gene transfer has been accomplished using special laboratory techniques (Love, 1994).

Potatoes are vegetatively propagated and differ from genetically propagated or seed grown crops. Maize and soybean, for example, produce seed through sexual recombination (meiosis) resulting in progeny that are genetically and phenotypically unique. Potato seed tubers are not true seed but are vegetative propagated tissue. Potato plants arising from vegetative propagation are genetically identical

having not undergone meiosis, recombination, or segregation. Vegetative propagation is not sexual reproduction, and the propagated material is considered genetically and phenotypically stable. Nomenclature used for potato cycles of vegetative propagation is shown in Figure 2-1.

Somaclonal variation occurs when genetically dissimilar individuals are derived from vegetative propagation. It is especially common during tissue culture that includes a callus stage. *Solanum tuberosum* varieties are prone to somaclonal variation during tissue culture and may exhibit a degree of heterogeneity (OECD, 1997). Several steps were taken during event selection to mitigate somaclonal variation. Initially, a number of events were produced from retransformation of V11. A late blight assay was used to screen for plants expressing the VNT1 protein. Plants exhibiting partial resistance to late blight were discarded. Asymptomatic plants were selected and advanced for field testing. Anomalous phenotypes were identified and removed in replicated field trials grown in several geographic regions that were carefully monitored for undesirable or off-types. Any plants with off-types were removed. The selection process was conducted by experienced agronomists observing the growth characteristics of the transformed events compared to Snowden and V11. Further studies confirming that somaclonal variation did not occur in Z6, including phenotypic evaluations, are discussed in Section 7.

Figure 2-1. Vegetative Cycle Nomenclature
Plantlets, propagated from cuttings of G0, are grown typically in soil, by Nutrient Film Technique, or using hydroponics. Tubers from G0 plantlets are mini-tubers. An entire mini-tuber is planted in the greenhouse (G = greenhouse) or field (FG = field) to produce a G1/FG1 plant. Tubers from G1/FG1 plants may be cut (2-4 oz. pieces) or used whole as “seed pieces” to produce G2/FG2 plants. This process is repeated for increasing vegetative plants and “seed”.

3.0 Genetic Characterization of Z6

Z6 was successfully transformed with pSIM1278 and pSIM1678. DNA used for transformation, plasmid maps, and genetic elements tables can be found on pp. 43-49 (14-093-01p). Molecular analyses demonstrated that the pSIM1678 insert in Z6 was integrated at a single locus in the Snowden genome (Section 3.1). The characterization of the inserts in Z6 show that both inserts from pSIM1278 and pSIM1678 consist of nearly full-length T-DNA (Section 3.2). Further characterization demonstrated that Z6 contained no backbone sequences.

The molecular characterization of Z6 was performed using:

- Southern analysis showing pSIM1278 and pSIM1678 inserts integrate into single genomic loci (see Section 3.1);
- Southern, PCR, and sequencing analyses showing the structure of the inserts (see Section 3.2); and
- Southern analysis showing the absence of backbone sequences in Z6 (see Section 3.3).

Southern blot analysis was optimized to reduce the background in all blots. Because the pSIM1278 and pSIM1678 inserts contain potato elements, the probes used in most cases bind to elements in the inserts and also in the potato genome, creating many endogenous and some interfering bands. Reducing background reduces the appearance of endogenous bands. This optimization also leads to bands of interest which are fainter than they would be without reduced background interference. The internal bands are corroborated by sequencing of the insert and sequencing data are provided to support the Southern blot data.

Additionally, a control (pSIM1278 plasmid DNA spiked into Snowden genome) was added to the blots. The concentration of pSIM1278 plasmid DNA is targeted at one copy per genome equivalent. This low concentration of spiked plasmid served as a positive control for sensitivity of the pSIM1278 probes and as a size marker for bands internal to the pSIM1278 insert. As a positive control for the sensitivity of the probes, only faint bands were expected when the spiked DNA had homology to a probe. The band associated with spiking pSIM1278 plasmid DNA in each blot will likely be faint as a result of optimization to reduced background.

3.1 Single Genomic Insertion Locus in Z6

Genomic DNA samples from Z6, Snowden, and V11 were digested with the MfeI restriction enzyme and hybridized using a series of probes that span the length of each T-DNA. A Snowden sample spiked with plasmid DNA (pSIM1278 or pSIM1678) served as a positive control and ensured that probes were sensitive enough to detect a single copy in the genome. The V11 sample served as a control to distinguish between pSIM1278 and pSIM1678 bands in blots where probes recognized both inserts.

MfeI cuts frequently within the potato genome, but not within the T-DNA of pSIM1278. This results in a distinct band for each insert from pSIM1278. Only a single band is expected for any transformant containing a single insert from pSIM1278. For pSIM1678, MfeI cuts once within the T-DNA. This means that one or two distinct bands are expected for each insert from pSIM1678, depending on the probe used.

The pSIM1278 and pSIM1678 T-DNA have common elements, including the left and right border regions (LB and RB) and the Agp and Gbss promoters. Probes hybridizing to these regions were expected to

detect inserts from both pSIM1278 and pSIM1678 and result in two bands, one for the pSIM1278 insert and one for the pSIM1678 insert.

3.1.1 Single Insertion Locus in Z6 Associated with pSIM1278

The pSIM1278 transformation resulted in a single insert in the Snowden genome. Only one fragment was identified unique to V11 and Z6 by any of the seven probes (red arrows, Figure 3-1). This band (about 11.2 kb) corresponded to the pSIM1278 insert (10.1 kb) plus about 1.1 kb of flanking DNA. The size of the pSIM1278 insert was the same in each blot and distinct from the pSIM1278 plasmid control digestion product (Snowden p1278, black arrows). Detection of the plasmid control indicated that the probes had sufficient sensitivity to detect a single insert in the genome.

The pSIM1278 insert was distinguished from the pSIM1678 insert by comparing Z6 samples with Snowden and V11. Bands present in both V11 and Z6, but not Snowden, were associated with the pSIM1278 insert. Bands unique to Z6 were associated with the pSIM1678 insert. Because all seven of the pSIM1278 probes had complementarity with either pAGP, pGbss, or border sequences, and because these elements exist in pSIM1678, the pSIM1278 probe set also detected the pSIM1678 insert (Figure 3-2). All seven probes identified a 7.6 kb pSIM1678 digestion fragment unique to Z6 (blue arrow, Figure 3-1). The 7.6 kb pSIM1678 digestion fragment unique to Z6 for probe 1278.5 is faint as a result of very little sequence homology between 1278.5 and the pSIM1678 insert (Figure 3-2). As a result, the blot probed with 1278.5 showed clear bands for the insert from pSIM1278 and only a faint, but visible, band for the insert from pSIM1678 (Figure 3-1). This was expected. Furthermore, probe 1278.1 showed complementarity to an additional sequence in pSIM1678, as it identified a 2.4 kb band in Z6 (blue arrow, Figure 3-1). These data were consistent with the characterization of the pSIM1678 insert.

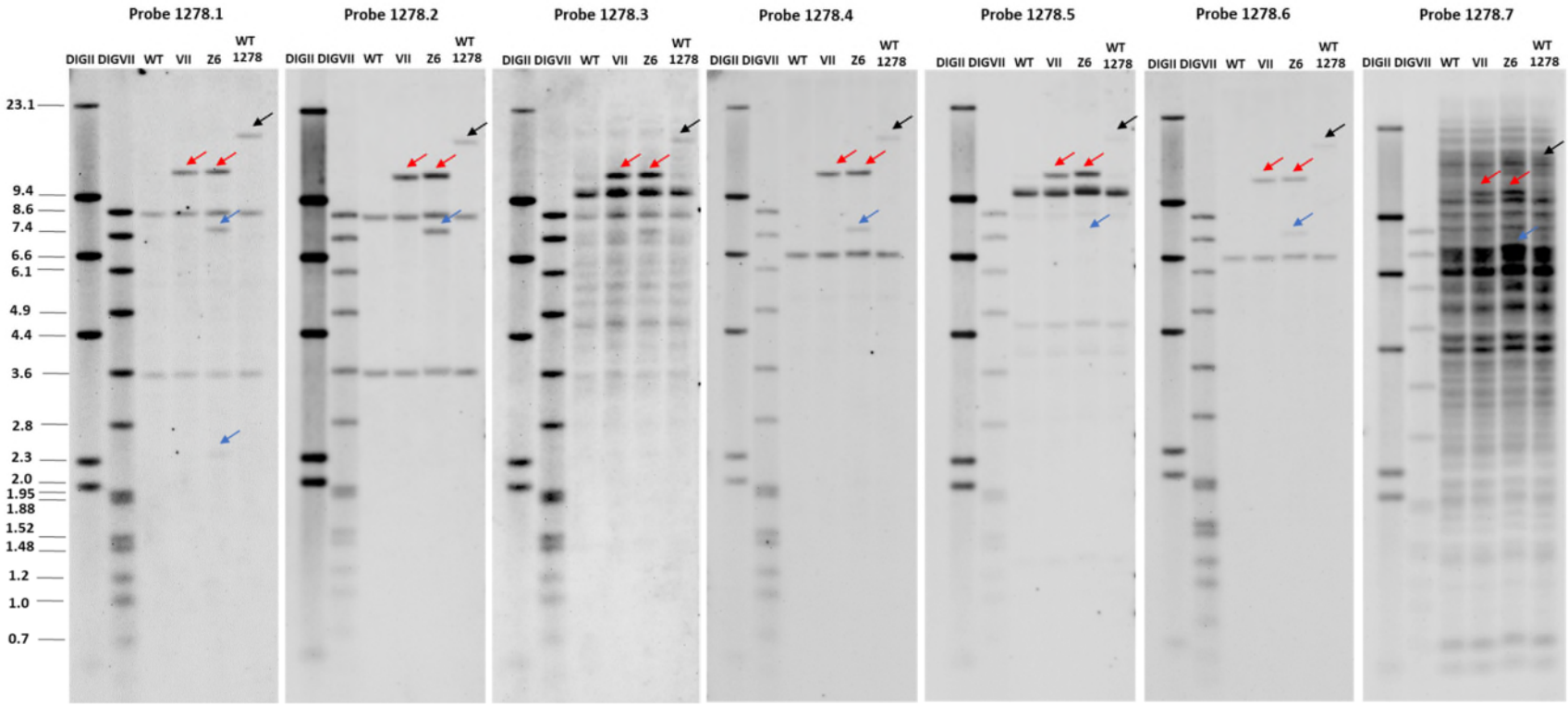

Figure 3-1. pSIM1278 and pSIM1678 Copy Number in Z6 Using pSIM1278 Probes

Southern blots of MfeI digested genomic DNA isolated from Snowden (WT), V11, Z6, and WT spiked with pSIM1278 plasmid DNA (WT p1278). Blots were hybridized with 7 unique pSIM1278 probes (1278.1–1278.7). In addition to endogenous bands common to all samples, three other types of bands were observed: bands unique to V11 and Z6 corresponding to the pSIM1278 insert (red arrow), bands unique to Z6 and corresponding to the pSIM1678 insert (blue arrow), and bands associated with spiking pSIM1278 plasmid DNA (black arrow) into the WT sample. The molecular weight markers, DIGII and DIGVII, were included in each gel and labeled in kb at the left of the first gel.

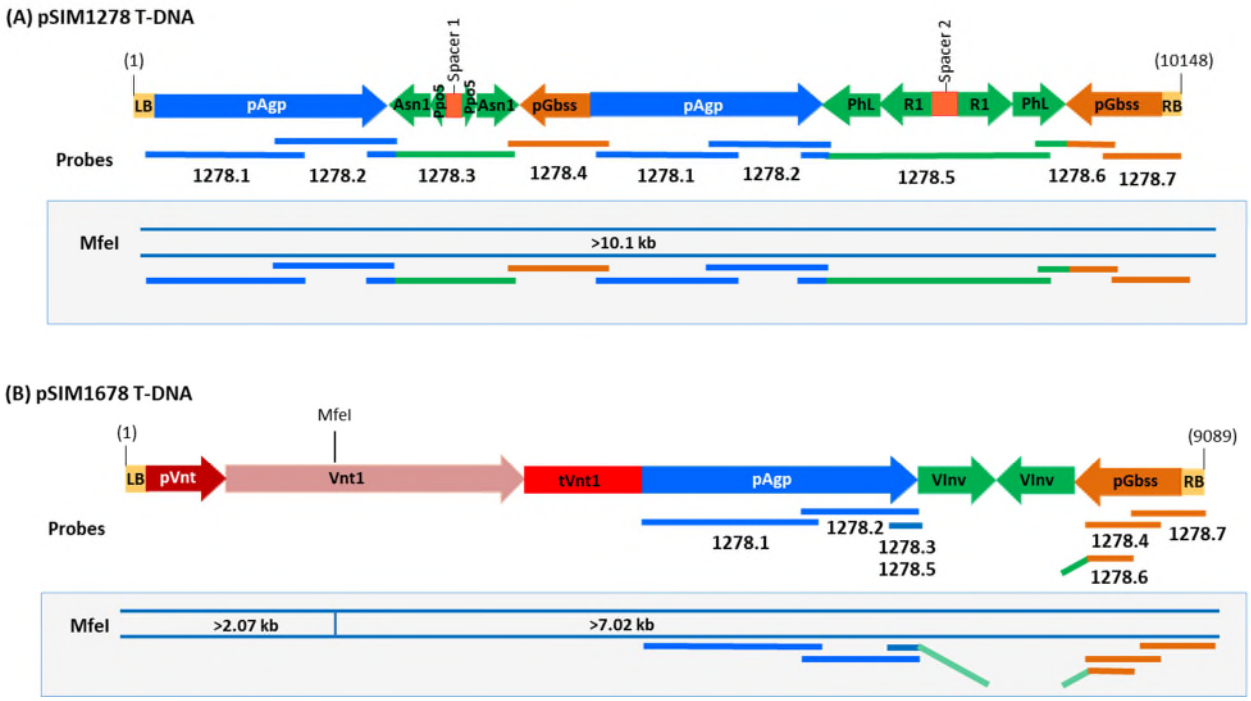

Figure 3-2. pSIM1278 Insert Number Characterization

(A) Seven probes hybridized across the pSIM1278 T-DNA. Many hybridized to more than one site within the T-DNA as there were two copies of Agp and Gbss promoter elements. The 1278.3, 1278.5 and 1278.6 probes are indicated using two colors, as they overlap multiple elements. The overlap of these probes with the pAgp or pGbss elements allowed these probes to detect the pSIM1678 insert. (B) Hybridization sites for the 1278 probe set on the pSIM1678 T-DNA. All seven 1278 probes could detect the pSIM1678 insert. Expected digestion products and probe binding sites are shown in boxes below the T-DNA maps.

Probes for the insert from pSIM1278 showed that a single insert from this construct was present in Z6. This is consistent with the data for V11.

3.1.2 Single Insertion Locus in Z6 Associated with pSIM1678

Transformation with pSIM1678 resulted in a single insert in the Snowden genome. The MfeI digested fragments from Z6, V11, and Snowden samples were hybridized with nine probes designed to detect pSIM1678 T-DNA. The sequences for pAGP, pGbss, and border regions are shared between the pSIM1278 and pSIM1678 inserts. Several of the pSIM1678 probes (1678.1, 1678.5, 1678.6, and 1678.9) are complementary to these regions in the pSIM1278 T-DNA (Figure 3-3). Hybridization with these four probes was expected to result in two bands in Z6 samples, one from the pSIM1678 insert, and another from the pSIM1278 insert. Hybridization with the remaining five pSIM1678 probes, which lack complementarity to pSIM1278 T-DNA, was expected to result in one band specific to Z6, and no bands in V11. The expected banding patterns were observed with each probe indicating a single pSIM1678 insert in Z6 (Figure 3-4).

No bands specific to pSIM1278 or pSIM1678 were identified in the Snowden samples. Detection of the plasmid control (black arrows) indicated that the probes had sufficient sensitivity to detect a single insert in the genome. Red arrows were used to indicate fragments corresponding to the pSIM1278 insert, and blue arrows denoted the pSIM1678 insert (Figure 3-4).

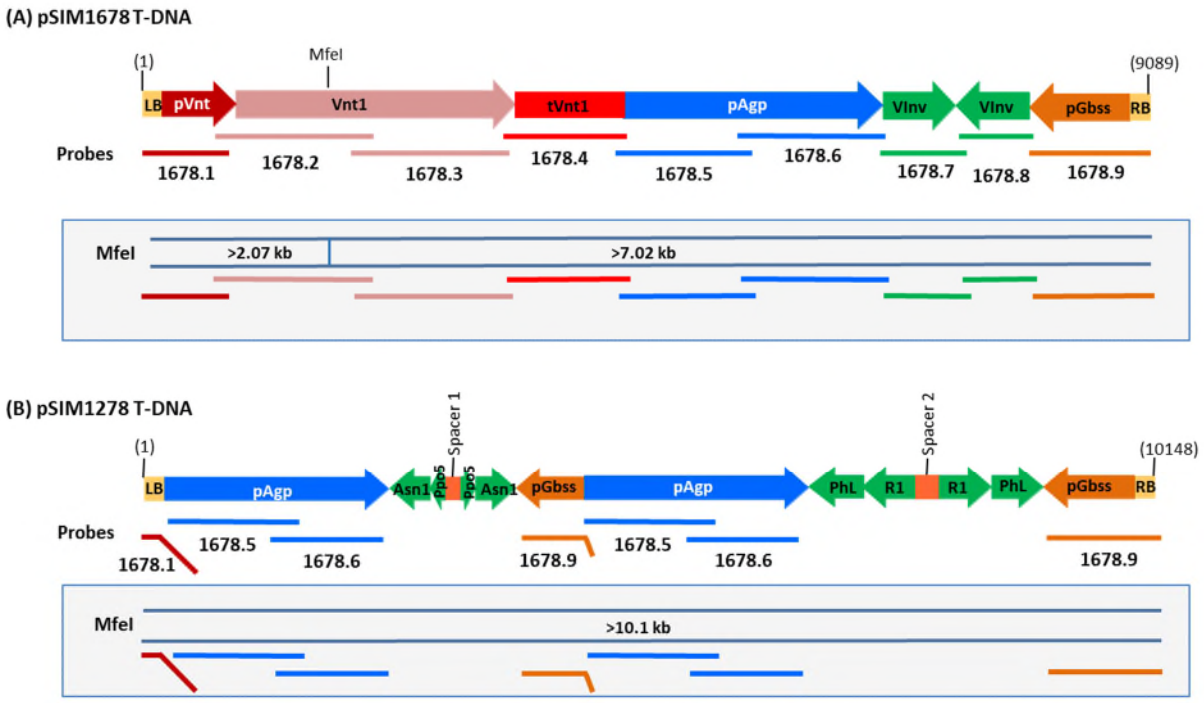

Figure 3-3. Binding Sites for 1678 Probe Set on pSIM1278 and pSIM1678 T-DNA

A) Nine probes hybridized across the pSIM1678 T-DNA. (B) Hybridization sites for the 1678 probe set on the pSIM1278 T-DNA. Probes 1678.1, 1678.5, 1678.6 and 1678.9 also detected elements in the pSIM1278 insert. Expected digestion products and probe binding sites are shown in boxes below the T-DNA maps.

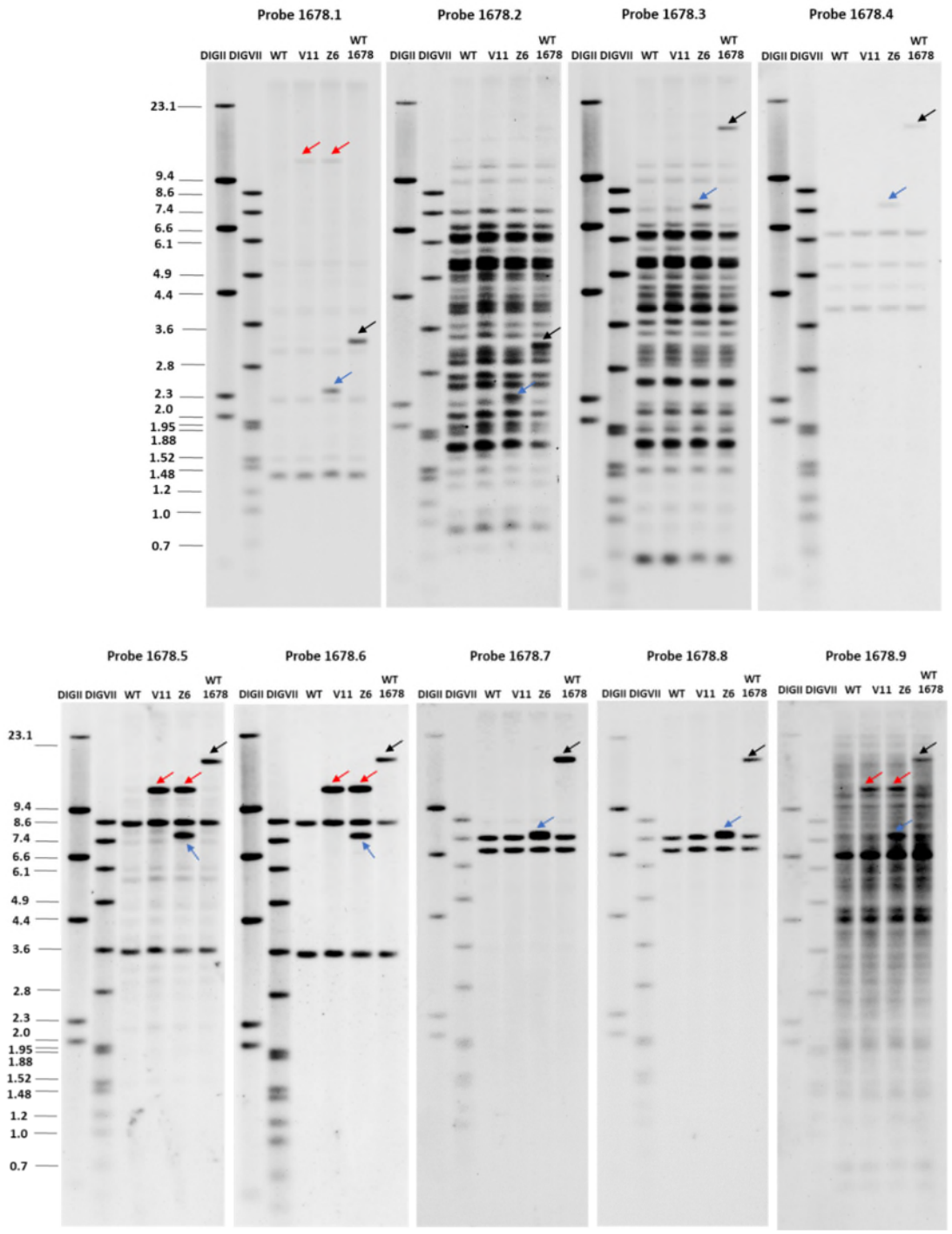

Figure 3-4. pSIM1278 and pSIM1678 Copy Number in Z6 Using pSIM1678 Probes
Southern blots of MfeI digested genomic DNA isolated from Z6, V11, Snowden (WT), and Snowden spiked with pSIM1678 plasmid DNA (WT p1678). Blots were hybridized with the indicated probe (1678.1–1678.9). In addition to endogenous bands common to all samples, three other types of bands were observed: 1) same in Z6 and V11—corresponding to pSIM1278 (red arrow), 2) unique to Z6—corresponding to pSIM1678 (blue arrow) and, 3) associated with spiked pSIM1678 plasmid DNA (black arrow) observed in WT sample. The molecular weight markers, DIGII and DIGVII, were included in each gel and labeled in kb.

Probes for the insert from pSIM1678 showed that a single insert from this construct was present in Z6.

3.2 Structures of the pSIM1278 and pSIM1678 Inserts in Z6

The structures of the pSIM1278 and pSIM1678 inserts in Z6 are shown in Figure 3-5 and Figure 3-12. Both pSIM1278 and pSIM1678 inserts consist of a nearly full-length T-DNA.

Southern blot analyses supporting the insert structures in Z6 are provided in Section 3.2.1 and Section 3.2.2.

3.2.1 pSIM1278 Insert Structure in Z6

The Southern analysis, shown above in Figure 3-1 was used to determine that the pSIM1278 insert in Z6 consists of a single copy of the T-DNA. The junctions between the pSIM1278 insert and the plant genome were determined using DNA sequencing. A series of additional Southern blots, shown below, were used to confirm the structure of the pSIM1278 insert. AGP, ASN, GBS, and R1 probes were hybridized to genomic DNA following digestion with EcoRV, HindIII, PacI/XbaI and a double digest with EcoRI and ScaI. The restriction sites, digestion products and sizes, and probe binding sites are shown in Figure 3-5. The Snowden (WT) sample was analyzed with and without spiked pSIM1278 (p1278) or pSIM1678 plasmid (p1678). The spiked plasmid was at a targeted concentration of a single copy per genome equivalent, prior to digestion. The spiked plasmids served as positive controls for sensitivity of the probes and as size markers for bands internal to the pSIM1278 or pSIM1678 insert and T-DNA.

Internal bands were detected in Z6 and V11 samples indicating that the pSIM1278 T-DNA sequence between the EcoRV (2339) and ScaI (8233) cut sites was intact (Figure 3-10 and Figure 3-11). Internal fragments with expected sizes resulted from the restriction digest with EcoRV, HindIII, EcoRI/ScaI, or PacI/XbaI. The 2.3 kb EcoRV, 4.2 kb HindIII and 3.8 kb EcoRI/ScaI fragments were detected by the AGP and GBS probes (Figure 3-6 and Figure 3-7). A 5.3 kb PacI/XbaI fragment that hybridized to AGP and GBS probes, and a 1.3 kb PacI/XbaI fragment that hybridized to the GBS probe, were both detected (Figure 3-8, Figure 3-9). The 0.7 and 2.3 kb EcoRV fragments and a 4.2 kb HindIII fragment were observed when hybridized with the ASN probe (Figure 3-10). The 1.3 kb HindIII band and 3.8 kb and 0.8 kb EcoRI/ScaI bands were detected by the R1 probe (Figure 3-11). Both ASN and R1 probes are specific to pSIM1278 and did not hybridize to pSIM1678 sequences. Many of the blots have faint bands due to optimization for background reduction. Although faint, the background is minimal and there are no bands interfering with bands of interest. The internal bands are corroborated by sequencing of the insert (Section 3.2.3.; Appendix C).

The analysis of junction fragments provided a means to confirm the presence of a single insert. Multiple inserts would result in an unexpected number of junction bands. The presence of MfeI sites in both left and right flanking regions made it possible to predict the sizes of the junction bands resulting from MfeI and XbaI digests (3.3 kb and 7.4 kb, respectively). Both fragments were detected in blots hybridized with the AGP probe (Figure 3-8). The 7.4 kb MfeI/XbaI junction band was also detected by the GBS probe (Figure 3-9). The junction bands provide support for the structure of the pSIM1278 insert as they overlap with internal fragments (Figure 3-5).

The flanking regions contain 1 kb of DNA that was confirmed by sequencing. However, the EcoRV, HindIII, EcoRI, and ScaI sites in the flanking regions were not identified, so the exact size of the junction bands resulting from restriction digestions using EcoRV, HindIII, EcoRI, and ScaI enzymes could not be predicted. The minimum sizes of the junction bands based upon known sequence and enzyme digestion

sites were determined, as shown in Figure 3-5. In each case a corresponding band was identified in the appropriate Southern blot. The three left junction bands associated with EcoRV, HindIII and EcoRI/ScaI digests were identified exclusively by the AGP probe. The size of each band (5.3 kb EcoRV, 18 kb HindIII and 9 kb EcoRI/ScaI) was determined from the gel and was consistent with expectations. Similarly, three fragments associated with the right junction (5.2 kb EcoRV, 8.6 kb HindIII, and 4.4 kb EcoRI/ScaI) were detected by the GBS and R1 probes (Figure 3-7, Figure 3-11) and all were consistent with the expected sizes (Figure 3-5). There were no unexpected bands that would suggest additional inserts of pSIM1278 T- DNA.

These data confirmed that Z6 contains a single, nearly full-length T-DNA from pSIM1278.

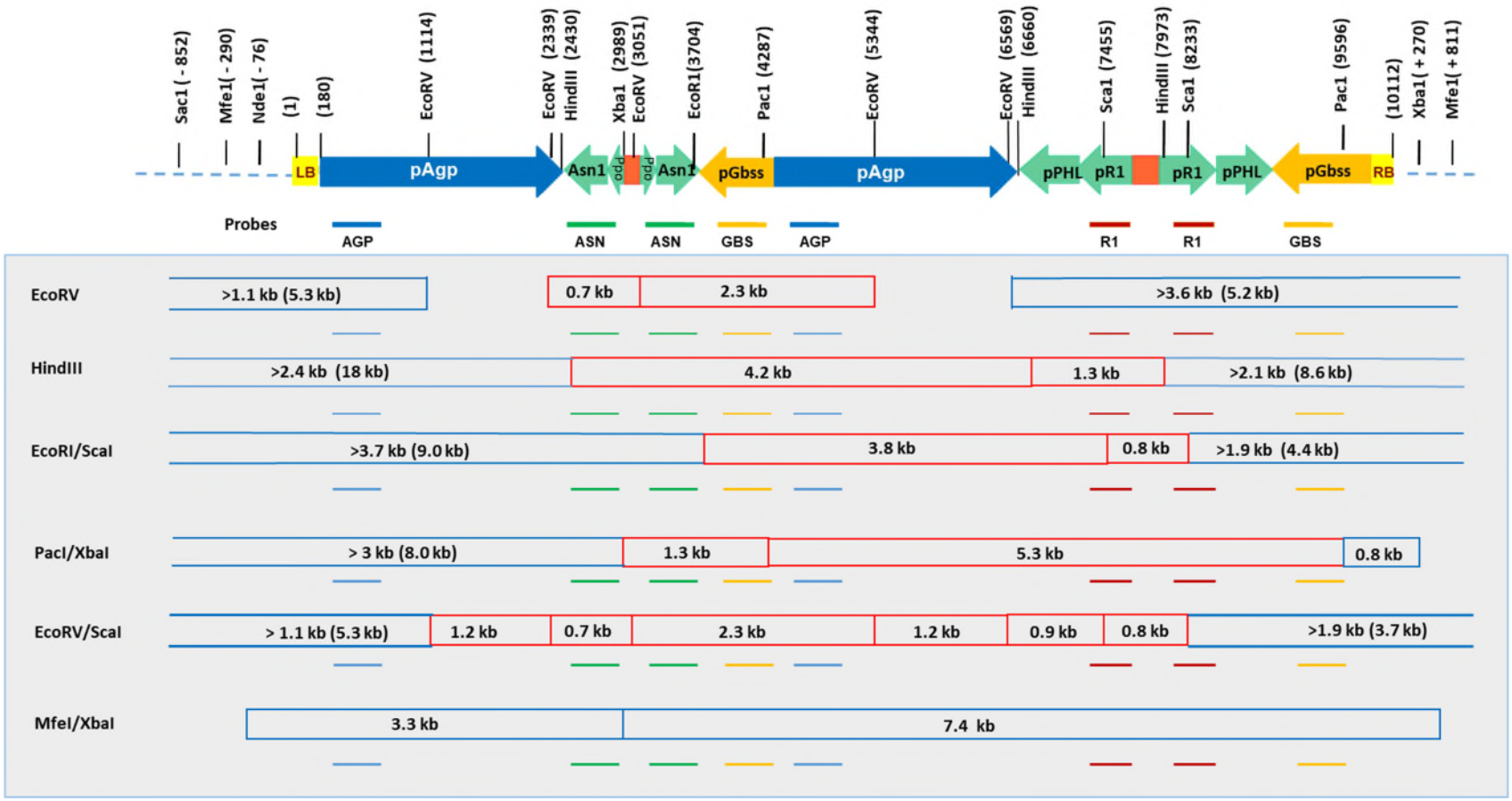

Figure 3-5. Structure of the pSIM1278 Insert in Z6 with Digestion Patterns and Probe Binding Sites

The figure represents the structure of the pSIM1278 insert in Z6, including restriction sites. The digestion pattern for selected enzymes is shown as colored boxes with the digest and fragment size indicated. The probes that hybridize to each digestion product are indicated below the fragment with colored lines. Red boxes denote internal bands and blue closed boxes indicate bands of known sizes due to identification of restriction sites within flanking DNA. Open-ended blue boxes indicate junction bands where the exact location of the second restriction site in the flanking region is unknown. The expected size for each these bands is indicated (i.e. distance to the end of the insert plus 1 kb to account for empirically-verified flanking region) along with the measured size based upon Southern blots (parentheses).

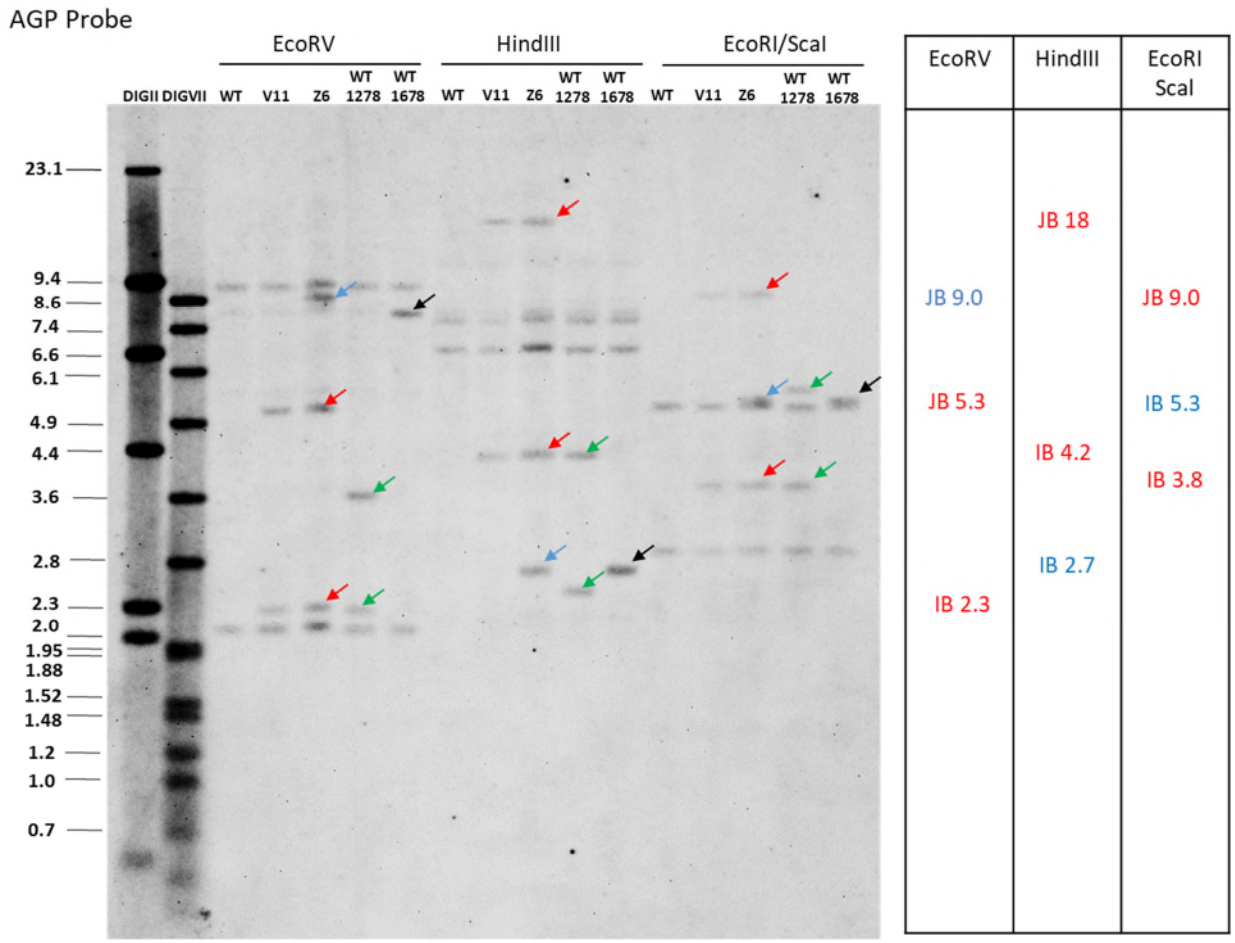

Figure 3-6. Southern Hybridization of Genomic DNA with AGP Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue), the spiked pSIM1278 plasmid (green) and pSIM1678 plasmid (**black**). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

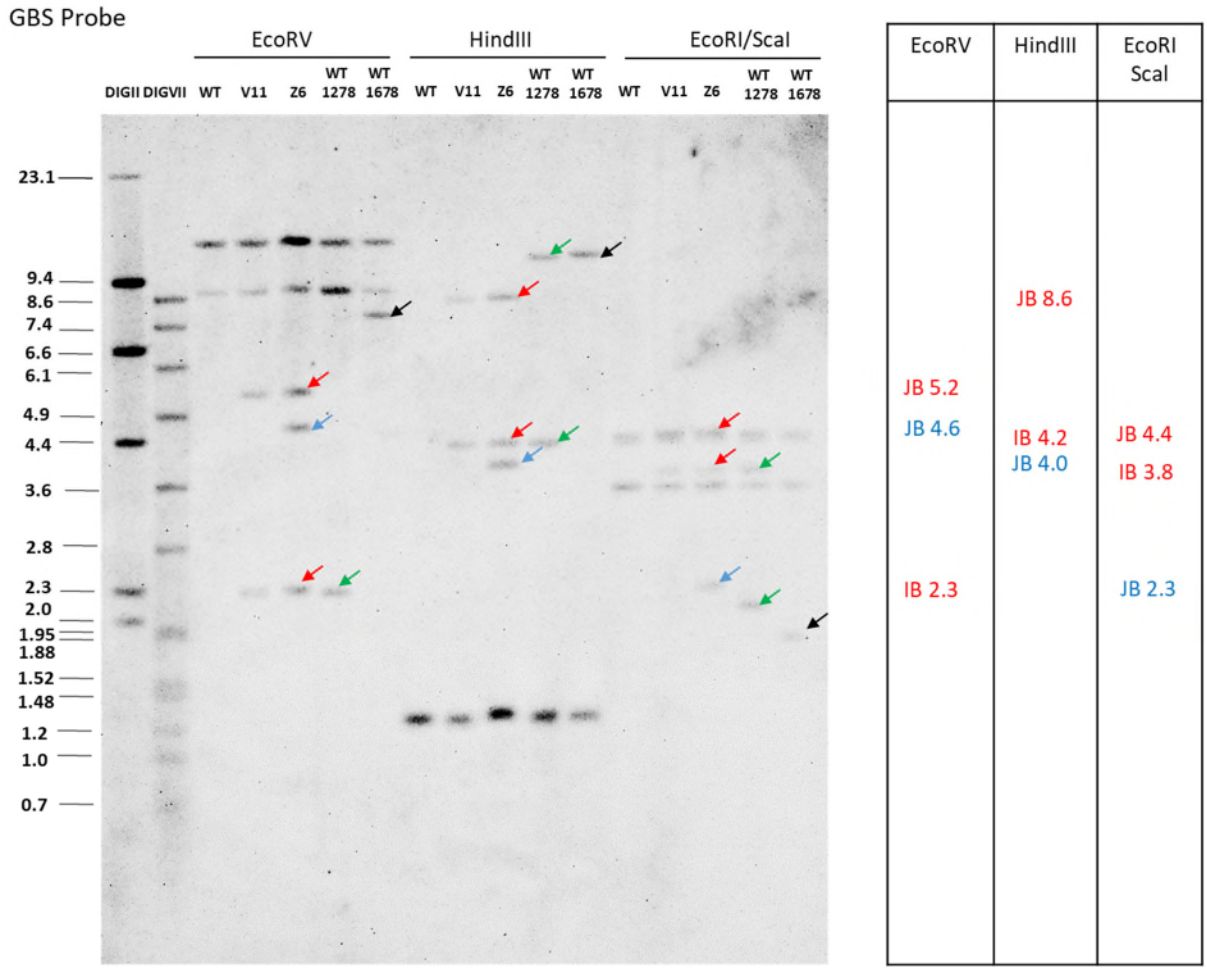

Figure 3-7. Southern Hybridization of Genomic DNA with GBS Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the GBS probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue), the spiked pSIM1278 plasmid (green) and pSIM1678 plasmid (**black**). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

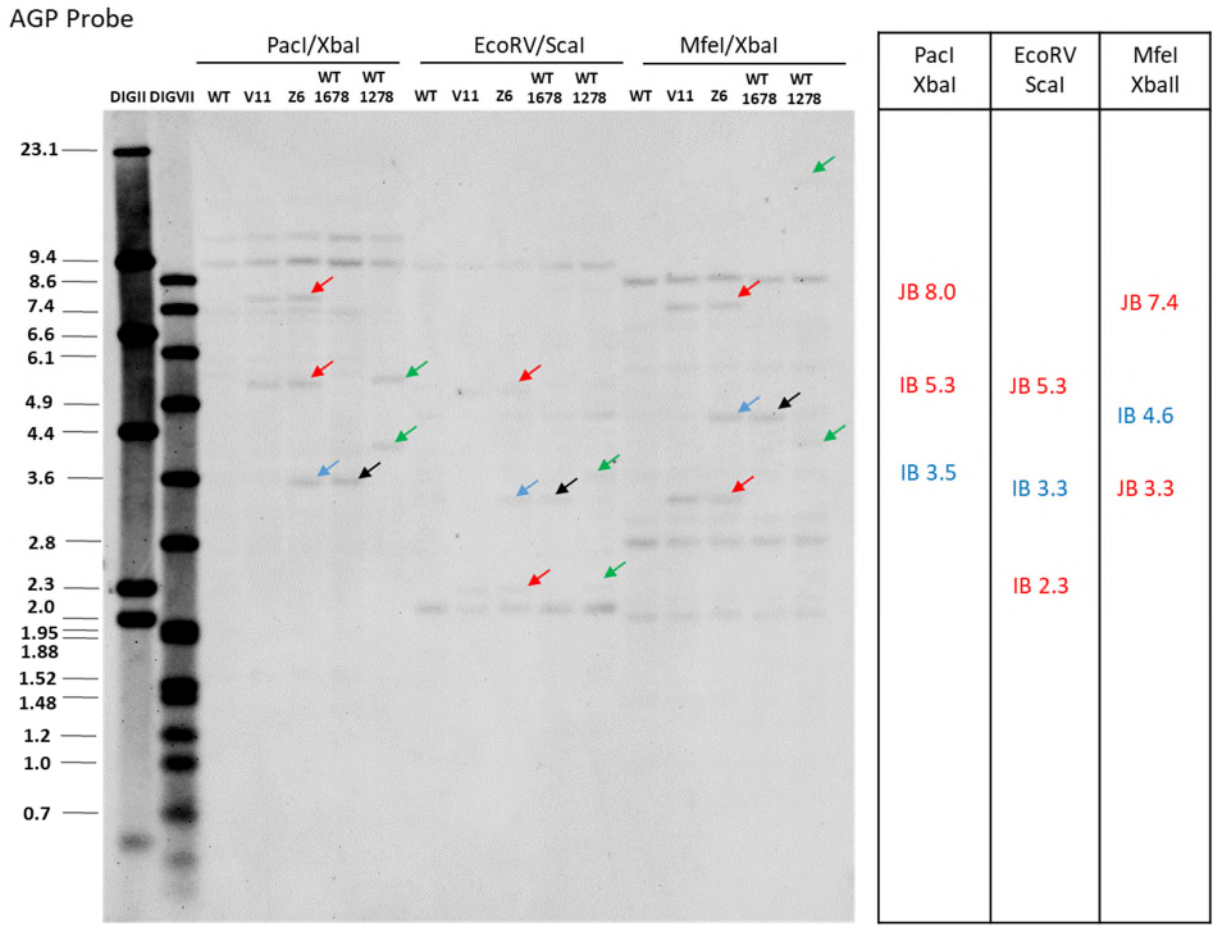

Figure 3-8. Southern Hybridization of Genomic DNA with AGP Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with PacI/XbaI, EcoRV/ScaI, and MfeI/XbaI and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue), the spiked pSIM1278 plasmid (green) and pSIM1678 plasmid (**black**). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

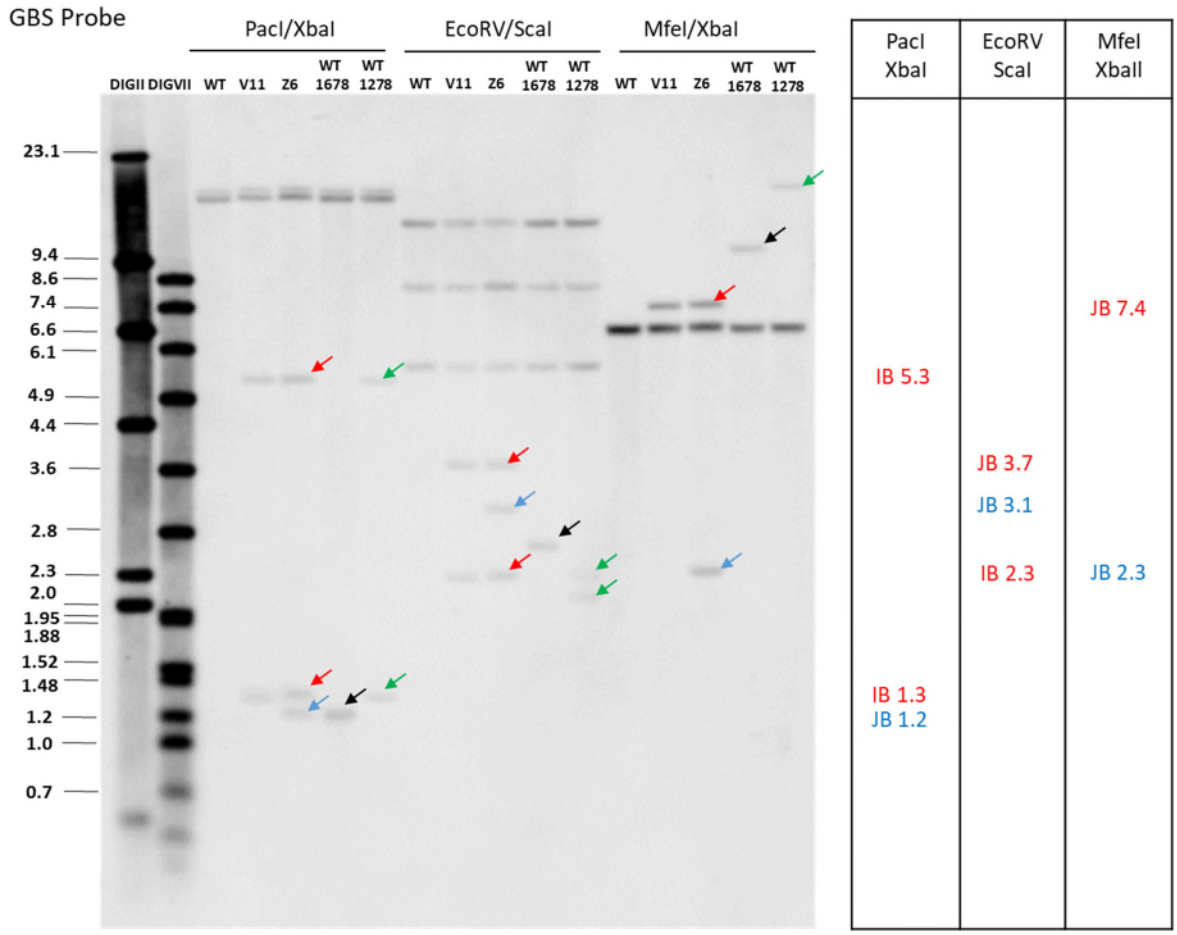

Figure 3-9. Southern Hybridization of Genomic DNA with GBS Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with PacI/XbaI, EcoRV/ScaI, and MfeI/XbaI and hybridized with the GBS probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue), the spiked pSIM1278 plasmid (green) and pSIM1678 plasmid (**black**). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

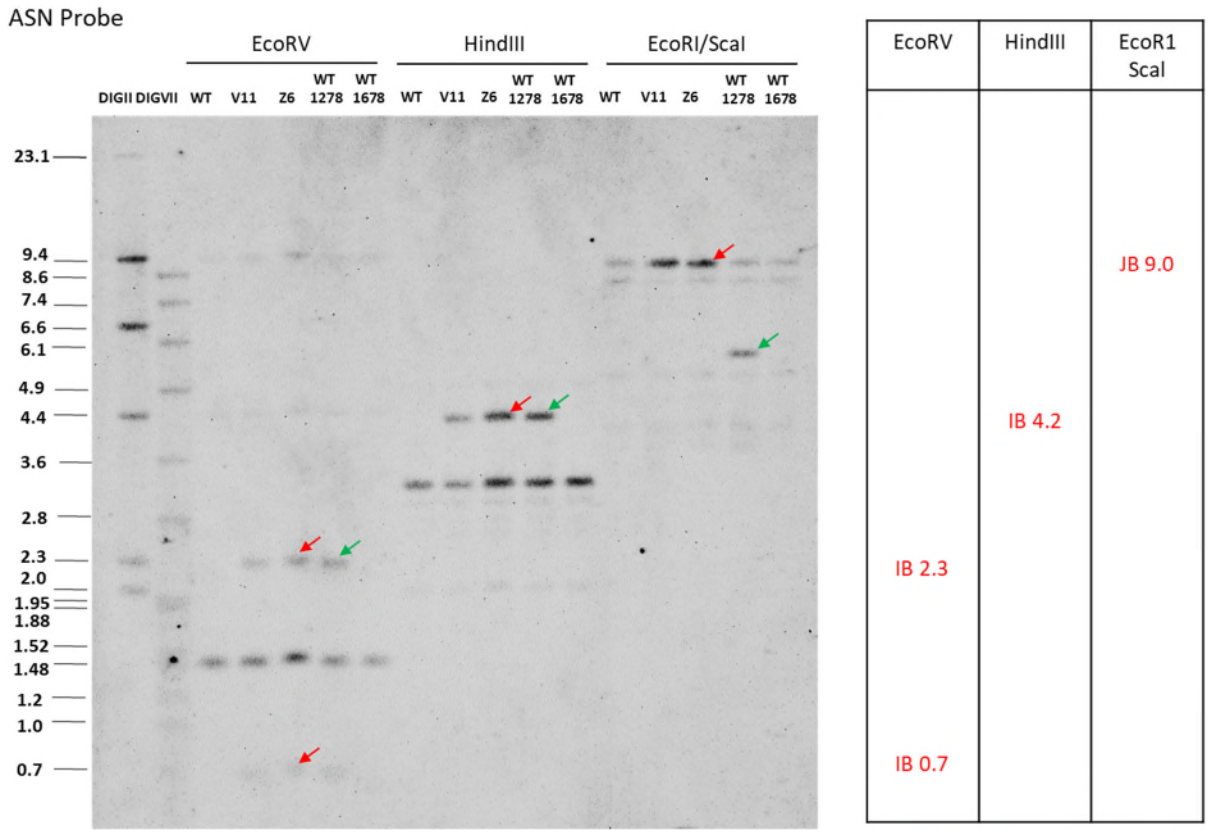

Figure 3-10. Southern Hybridization of Genomic DNA with ASN Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was 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. Colored arrows were used to distinguish between bands associated with the pSIM1278 insert (red) and the spiked pSIM1278 plasmid (green). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

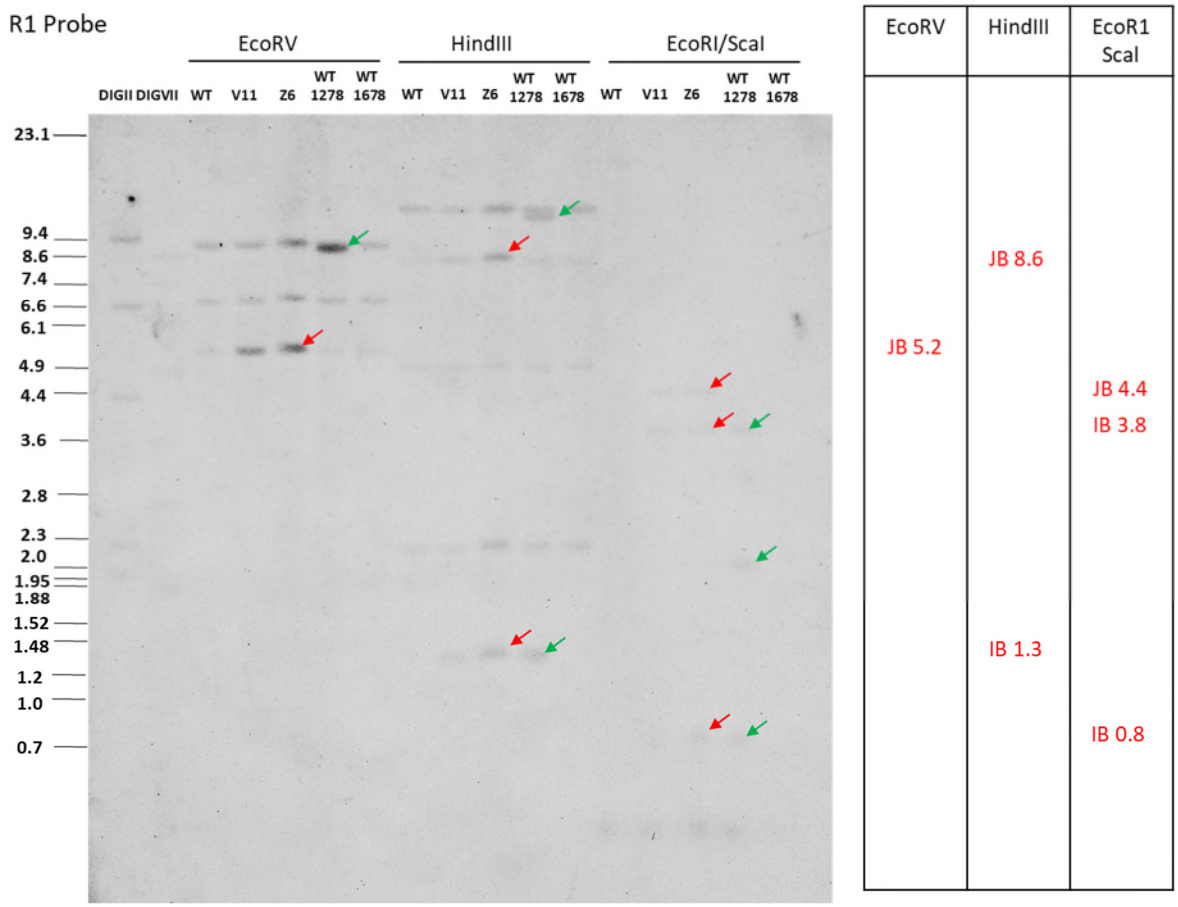

Figure 3-11. Southern Hybridization of Genomic DNA with R1 Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was 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. Colored arrows were used to distinguish between bands associated with the pSIM1278 insert (red) and the spiked pSIM1278 plasmid (green). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

3.2.2 pSIM1678 Insert Structure in Z6

The Southern analysis using MfeI digestion (Figure 3-4) determined the pSIM1678 insert in Z6 consists of a single, full-length copy. The junctions between the pSIM1678 insert and the plant genome were determined using DNA sequencing. Additional Southern blot analysis was conducted to confirm the pSIM1678 insert structure. The pSIM1678 insert structure was characterized like the pSIM1278 insert with additional probes (VNT and INV). The structure of the insert, restriction sites, digestion products, and probe binding sites are summarized in Figure 3-12. Like the pSIM1278 characterization, the internal bands (IB) are shown in red and junction bands (JB) in blue with open and closed boxes. The expected size of each band was provided for cross-reference with the Southern blot analysis. All the internal bands and junction bands detected in Z6, but not in Snowden or V11, are associated with the pSIM1678 insert.

To determine pSIM1678 insert structure, internal bands were analyzed first because their size and copy number are predictable for a simple insert (Figure 3-12). The Southern blots probed with AGP and GBS (Figure 3-6-Figure 3-8) were evaluated as the pSIM1278 and pSIM1678 T-DNA both contain Agp and Gbss promoters. Three internal pSIM1678 T-DNA fragments (3.5 kb PacI/XbaI, 4.6 kb XbaI, and 5.3 kb EcoRI/ScaI) were detected by both AGP and INV probes (Figure 3-6, Figure 3-8, and Figure 3-13). The 2.7 kb HindIII and 3.3 kb EcoRV/ScaI fragments were detected by the AGP probe (Figure 3-6 and Figure 3-8). The 1.2 kb PacI/XbaI fragment was also detected using the INV probe (Figure 3-13), which hybridized to pSIM1678, but not pSIM1278. The internal bands are corroborated by sequencing of the insert (Appendix D).

Similar to the pSIM1278 insert, the plant genome junction regions for the pSIM1678 insert adjoin the left and right border regions (LB and RB) of the insert. The junction sequence analysis revealed a 9-bp deletion of the pSIM1678 T-DNA left border region and a 35-bp deletion of the right border region. Restriction sites were also identified on each flanking region (Figure 3-12).

The VNT probe was designed to hybridize to the promoter region of *Rpi-vnt1* and binds near the left border of pSIM1678 T-DNA, which makes it ideal for detecting the left junction fragments. Three restriction sites (XbaI, ScaI, and MfeI) were identified on the flanking region near left border of pSIM1678 T-DNA. The 3.9 kb XbaI, 1.1 kb ScaI and 2.4 MfeI junction fragments detected by the VNT probe (Figure 3-14) connect the left side of the pSIM1678 insert with the flanking region as depicted in Figure 3-12.

The right junction DNA sequencing revealed restriction sites for MfeI, ScaI, and PacI, which can be used to characterize the right side of the insert using the INV and GBS probes. Unlike the GBS probe, the INV probe is specific to the pSIM1678 insert. The 3.1 kb EcoRV/ScaI, 2.3 kb XbaI/MfeI and 2.3 kb EcoRI/ScaI junction bands were detected in Z6, not Snowden and V11 samples, by INV probe as expected (Figure 3-13, Figure 3-15). Southern with XbaI/PacI digested DNA of Z6 resulted in two internal fragments (3.5 and 1.2 kb) and both were observed using the INV probe (Figure 3-13). The XbaI/MfeI and EcoRI/ScaI digests bisect the VInv cassette resulting in an internal band and a junction band for each digest. The XbaI/MfeI (4.6 kb IB and 2.3 kb JB) and EcoRI/ScaI (5.3 kb IB and 2.3 kb JB) bands were detected by the INV probe (Figure 3-13, Figure 3-15) confirming the presence of partial Vnt1 cassette and a complete copy of the VInv inverted repeat cassette on the right side.

These data indicate that the insert from pSIM1678 in Z6 consists of a single, nearly full-length T-DNA from pSIM1678.

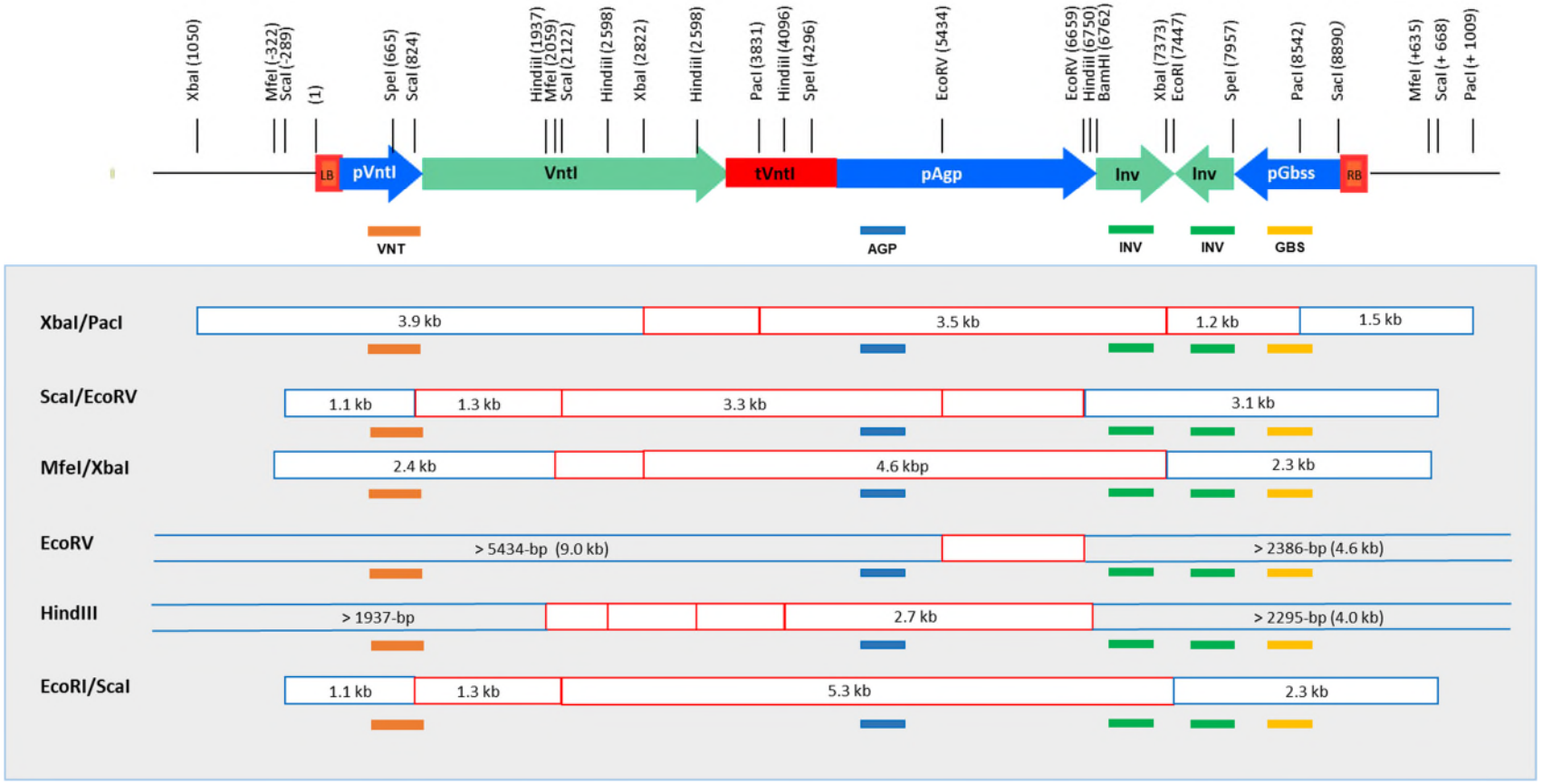

Figure 3-12. Structure of the pSIM1678 Insert in Z6 with Digestion Patterns and Probe Binding Sites

The structure of the insert from the pSIM1678, 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 colored lines. All expected probe binding sites are indicated, but only the digest/probe combinations necessary to support the model are provided. Red boxes denote internal bands (IB) associated with the pSIM1678 DNA construct. Blue closed boxes indicate junction bands of known sizes due to identification of restriction sites within flanking DNA. Open-ended blue boxes indicate junction bands where the exact location of the second restriction site on flanking region is unknown. The expected size for each these bands is indicated (i.e. distance to the end of the insert plus 1 kb to account for empirically-verified flanking region) along with the measured size based upon Southern blots (parentheses).

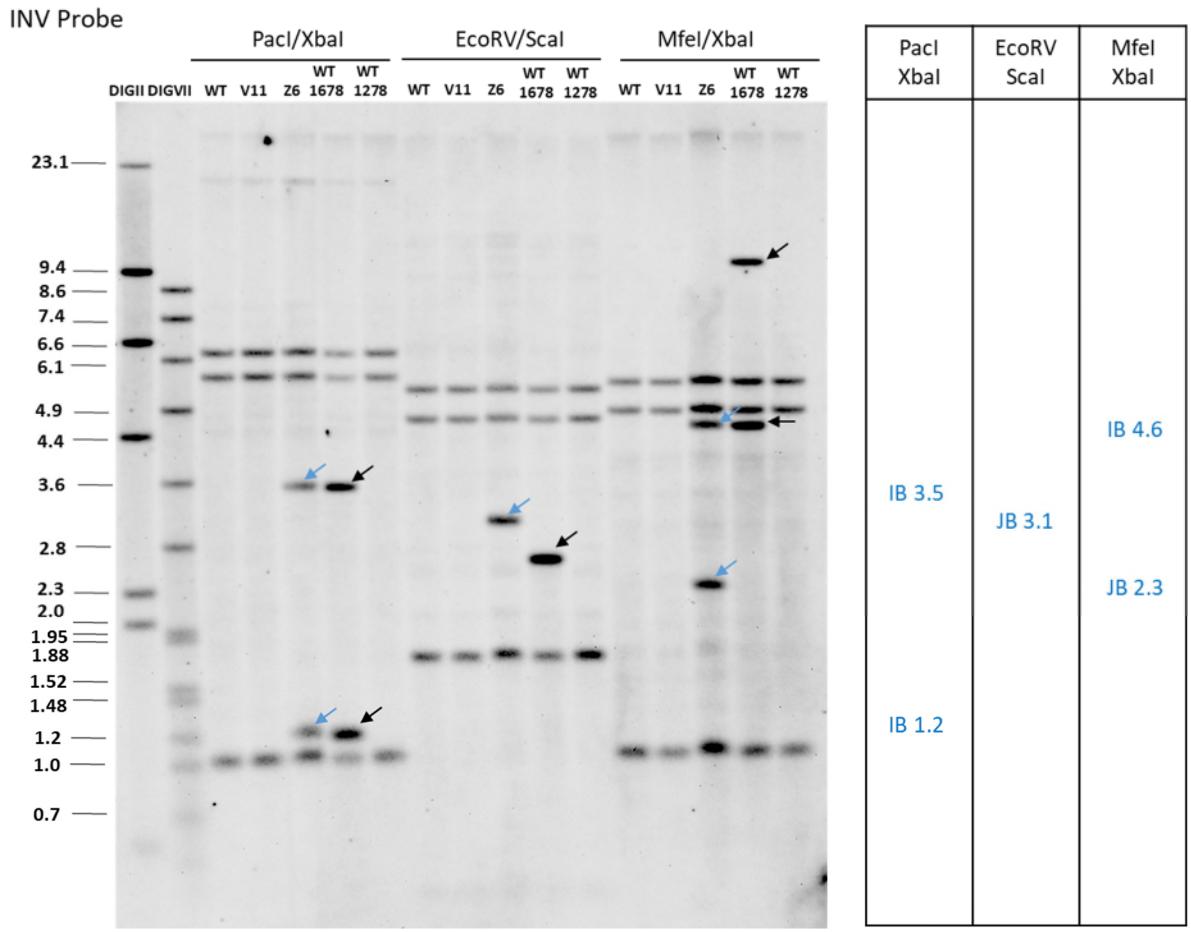

Figure 3-13. Southern Hybridization of Genomic DNA with INV Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278 plasmid DNA was digested with PacI/XbaI, EcoRV/ScaI, and MfeI/XbaI and hybridized with the INV probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between bands associated with the pSIM1678 insert (blue) and the spiked pSIM1678 plasmid (black). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

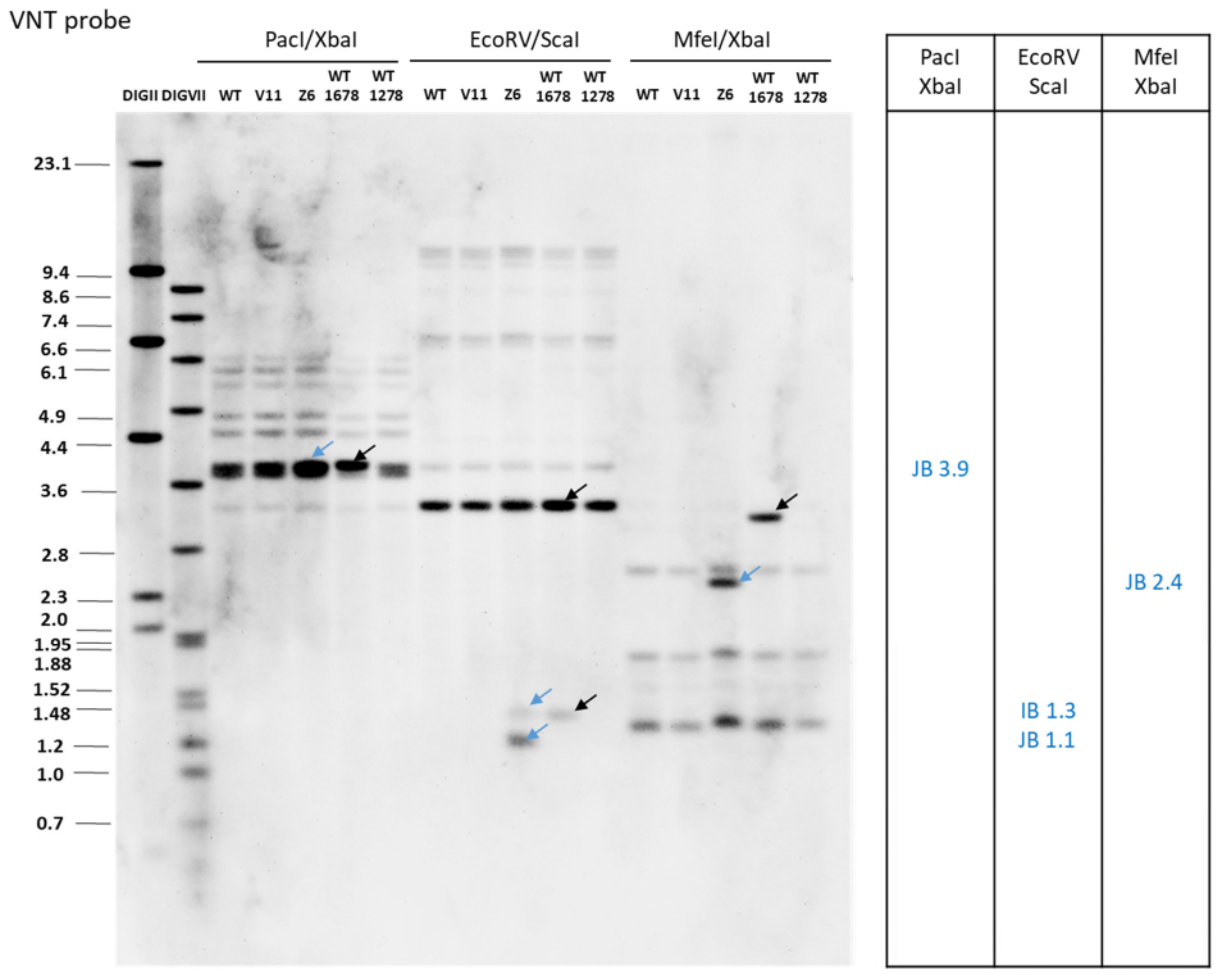

Figure 3-14. Southern Hybridization of Genomic DNA with VNT Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278 plasmid DNA was digested with PacI/XbaI, EcoRV/ScaI, and MfeI/XbaI and hybridized with the VNT probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between bands associated with the pSIM1678 insert (blue) and the spiked pSIM1678 plasmid (black). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

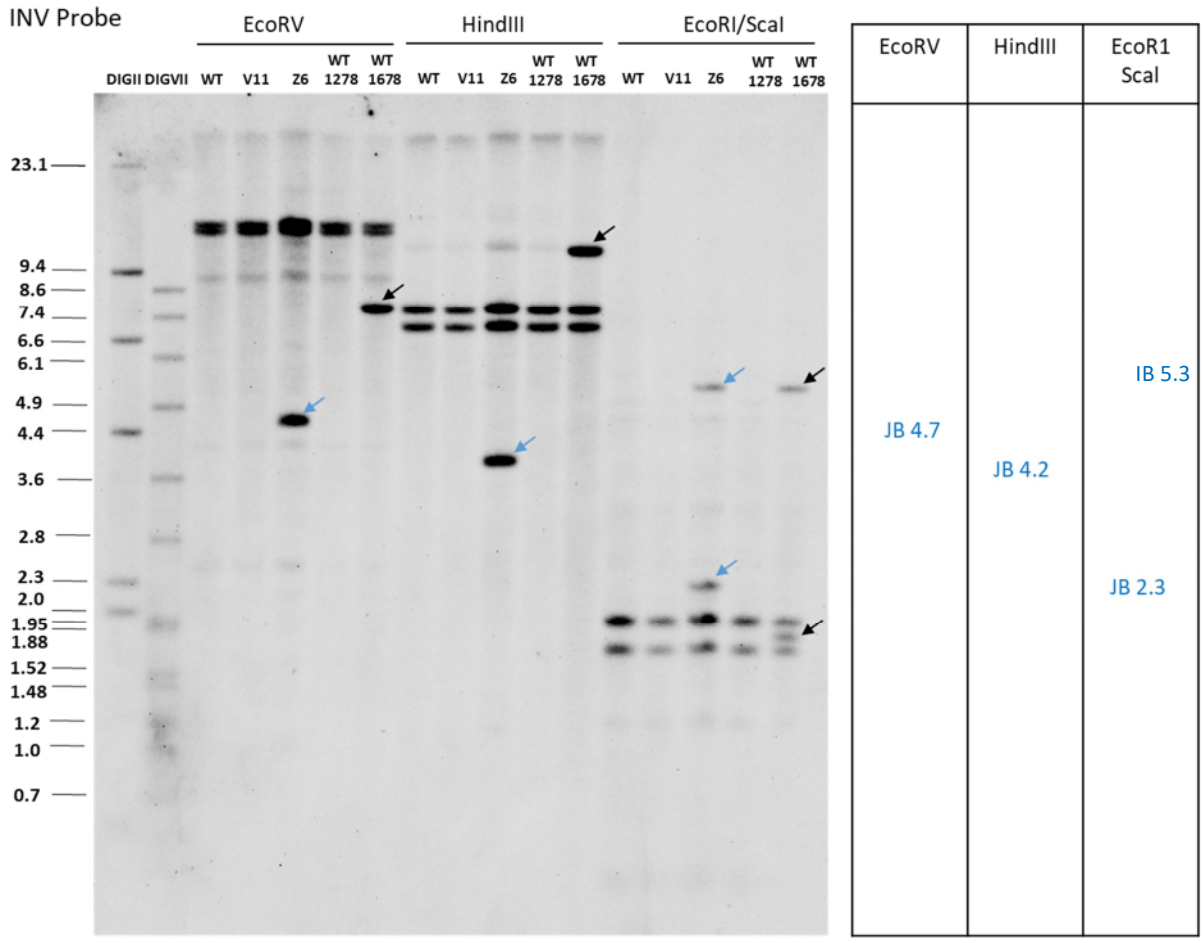

Figure 3-15.Southern Hybridization of Genomic DNA with INV Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278plasmid DNA was digested with EcoRV, 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. Colored arrows were used to distinguish between bands associated with the pSIM1678 insert (blue) and the spiked pSIM1678 plasmid (black). The estimated sizes of insert-specific bands are summarized in the associated table using the same color convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kb.

3.2.3 Sequence Characterization of pSIM1278 and pSIM1678 Insert Structures in Z6

Mate pair libraries and multiplexed targeted sequencing were applied to the challenges of identifying inserts containing native, potato sequences and inverted repeats. Insertion locus identification with bioinformatic scripts indicated one insert each from pSIM1278 and pSIM1678 in the Z6 genome. Insert flanking sequences and insert sequence validation was characterized and confirmed by next generation sequencing analysis and Southern blotting. Methods for sequencing are provided in Appendix B.

3.3 Insert Sequence Validation

Illumina data were used to validate the insert structure that was determined by Southern blotting. The Z6 Illumina library was generated by sequencing mate pair DNA fragments sharing sequence identity to either pSIM1278 or pSIM1678 after targeted capture. The reference pSIM1278 insert (event V11) and

the reference pSIM1678 insert were assembled from Sanger sequenced flanking genomic junctions and the T-DNA sequence. Illumina mate pair sequencing reads were aligned to both references as well as the genome. Targeted capture and resulting enrichment yielded thorough depth of coverage of read pairs across the entire pSIM1278 insert, including the junctions and adjacent flanking genomic DNA (Figure 3-16A) and the entire pSIM1678 insert, including the junctions and adjacent flanking genomic DNA (Figure 3-16B). The alignments were inspected to confirm accuracy by depth of coverage and sequence quality scores across the entire pSIM1278 insert and pSIM1678 insert.

Sequence data (Appendix C) showed that the pSIM1278 insert contains a nearly full-length T-DNA with a 14 bp deletion from the left border and a 23 bp deletion from the right border. Sequence data (Appendix D) showed that the pSIM1678 insert contains a nearly full-length T-DNA with a 9 bp deletion from the left border and a 36 bp deletion from the right border.

The sequences described are consistent with the structures of the pSIM1278 and pSIM1678 inserts determined by Southern blot analyses.

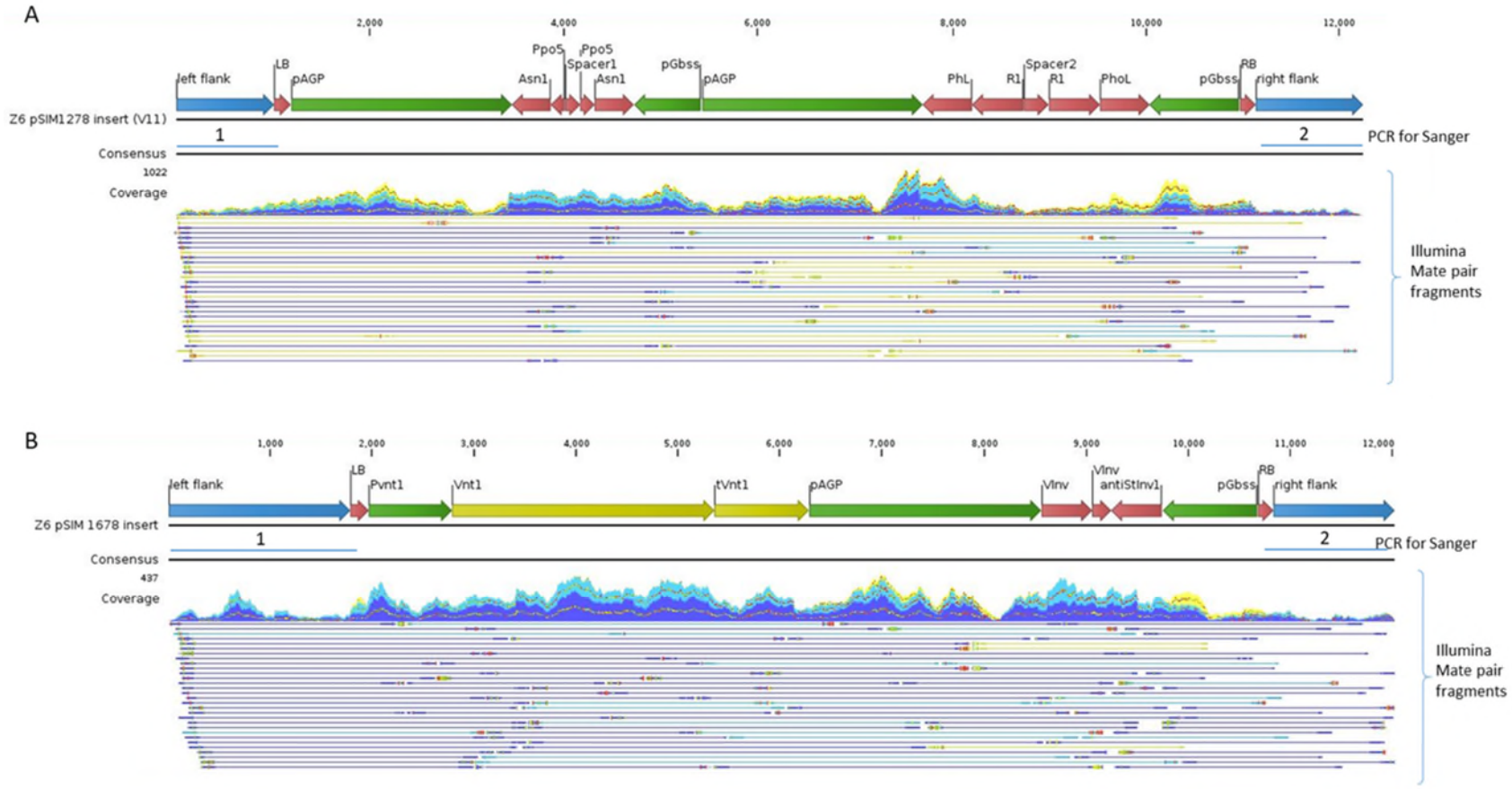

Figure 3-16. Summary of the Sequence Characterization of the pSIM1278 and pSIM1678 Inserts in Z6

Maps of the (A) pSIM1278 insert (V11) and (B) pSIM1678 insert are shown with blue lines used to represent regions Sanger sequenced, where 1 is the left junction and 2 is the right junction. Illumina sequence coverage depth of paired reads is displayed, indicating that there are more reads than shown. Yellow corresponds to reads that align to multiple places on the inserts (i.e. inverted repeats or duplicated promoters).

3.4 Absence of Backbone

Southern blot analysis was used to screen for the presence of backbone sequences in Z6. The pSIM1278 and pSIM1678 transformation plasmids had identical backbone sequences. Eight probes were developed that span the full sequence of the backbone DNA in pSIM1278 and pSIM1678. The probes were hybridized to restriction-digested genomic DNA from Z6 and Snowden variety. The pSIM1278 plasmid was used as a positive control.

Digestion of pSIM1278 with EcoRI produced three bands (12.5 kb, 1.57 kb, and 5.6 kb) detectable by the probe set (Figure 3-17A). A linear representation of the plasmid backbone shows the expected fragment sizes following digestion and probe binding sites (Figure 3-17A). Samples were digested with EcoRI for the Southern blots hybridized with probes BB1-BB6. A double digest (EcoRI/ScaI) was used for Southern blots hybridized with probes BB7 and BB8. Because the sequence of the spacer 2 element in the pSIM1278 T-DNA was derived from the Ubi7 promoter and is also present in the backbone, probe BB8 detected both the pSIM1278 plasmid control and the Z6 insert from pSIM1278 (Figure 3-17B).

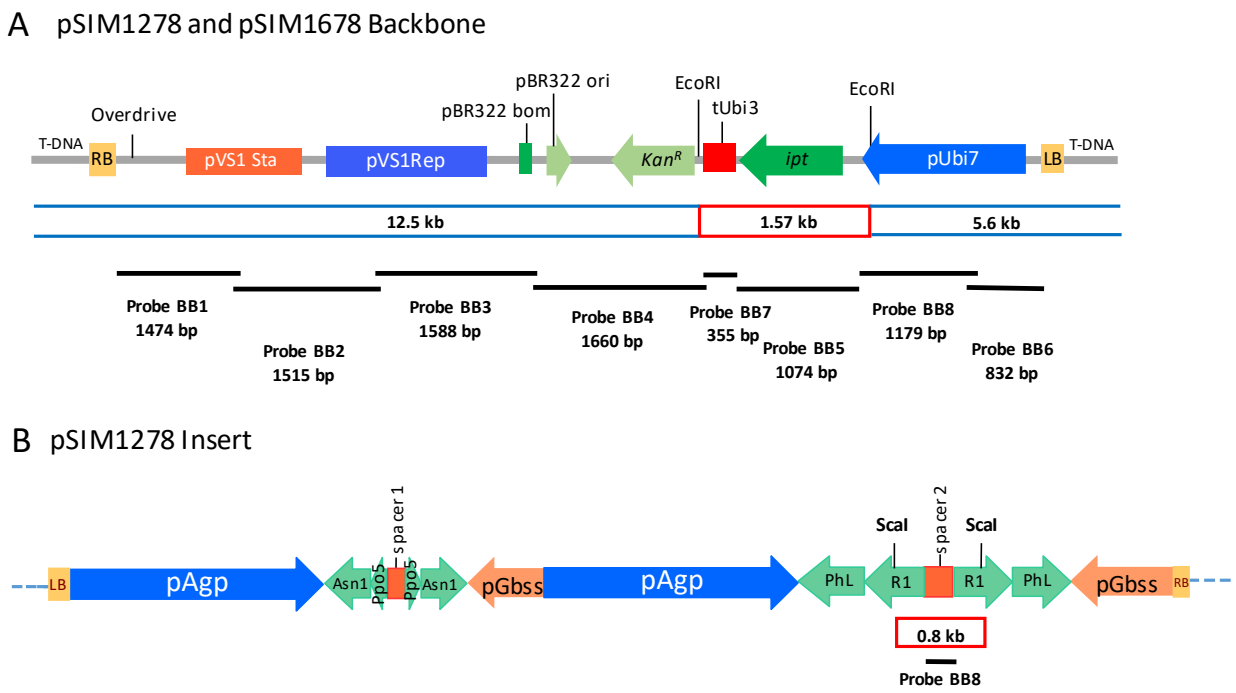

Figure 3-17. Probe Alignment for the pSIM1278/pSIM1678 Backbone and pSIM1278 T-DNA
(A) Linear map of backbone for pSIM1278 and pSIM1678. Eight probes (BB1-BB8) to detect backbone are indicated with the size of probes in base pairs (bp). EcoRI sites and expected band sizes for the pSIM1278 control are indicated as colored boxes. (B) Structure of the pSIM1278 T-DNA shows probe BB8 detects the spacer 2 region and produces a 0.8 kb band when digested by ScaI.

No hybridization signal corresponding to backbone DNA in the Z6 genome was detected in Southern blots hybridized with probes BB1-BB4 (Figure 3-18). The expected 12.5 kb EcoRI fragment was detected for the positive control sample (pSIM1278) in each of these blots. Endogenous bands not related to the transformation were detected in all samples hybridized with probe BB3 (Figure 3-18).

There were no backbone fragments detected in the genome of Z6 when hybridized with probes BB5-BB8 (Figure 3-19). The 1.57 kb EcoRI plasmid fragment described in Figure 3-17A was detected in the positive control sample (pSIM1278) on blots hybridized with probes BB5, BB7, and BB8. The 5.6 kb EcoRI/ScaI plasmid fragment (Figure 3-17A) was detected in positive control samples by probes BB6 and BB8 (Figure 3-19). The 0.8 kb band (Figure 3-17B) was detected in the Z6 and pSIM1278 control sample in the blot probed with BB8 (Figure 3-19). This band corresponded to the spacer 2 element of the pSIM1278 insert (Figure 3-17B). Endogenous bands not related to the transformations were observed in all samples in the blots hybridized with probes BB6–BB8 (Figure 3-19).

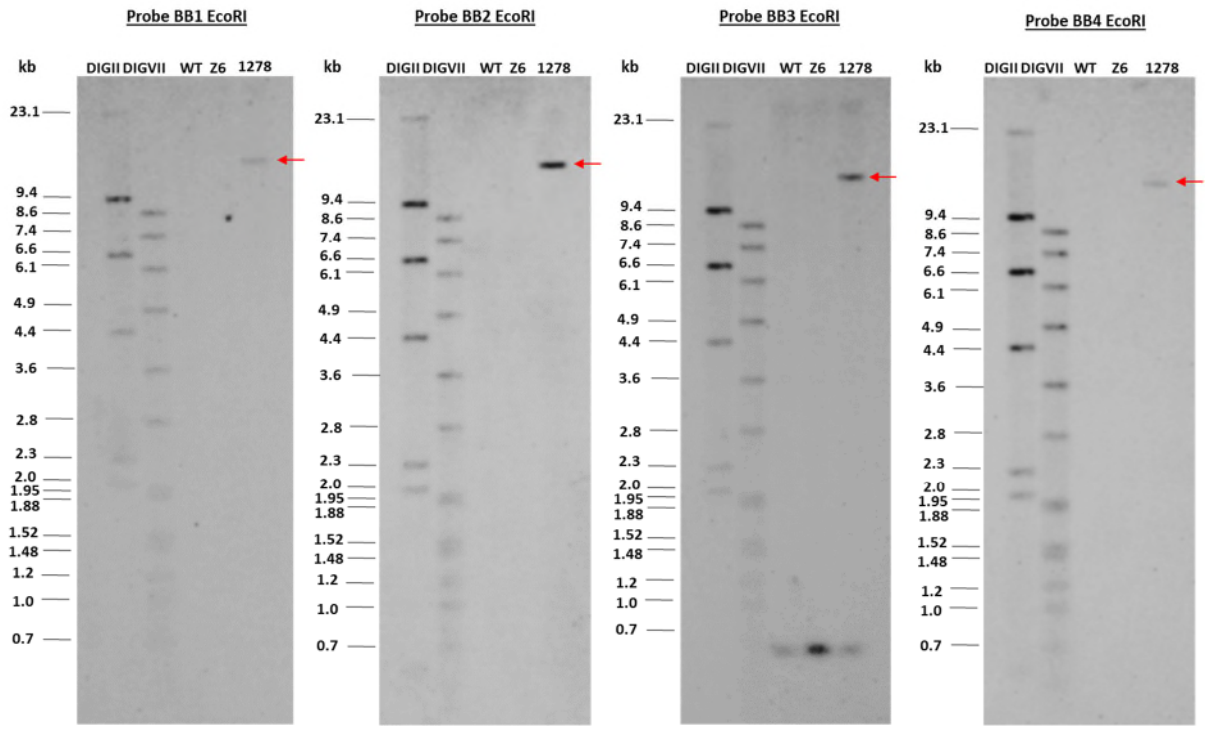

Figure 3-18. Southern Blots Probed with BB1-BB4 Show No Evidence of Backbone DNA in Z6
Southern blots of genomic DNA isolated from Snowden (WT), Z6, and WT spiked with pSIM1278 (1278). Molecular weight mark markers, DIGII and DIGVII, are labeled in kb. Red arrows indicate expected 12.5 kb bands unique to the plasmid control (1278).

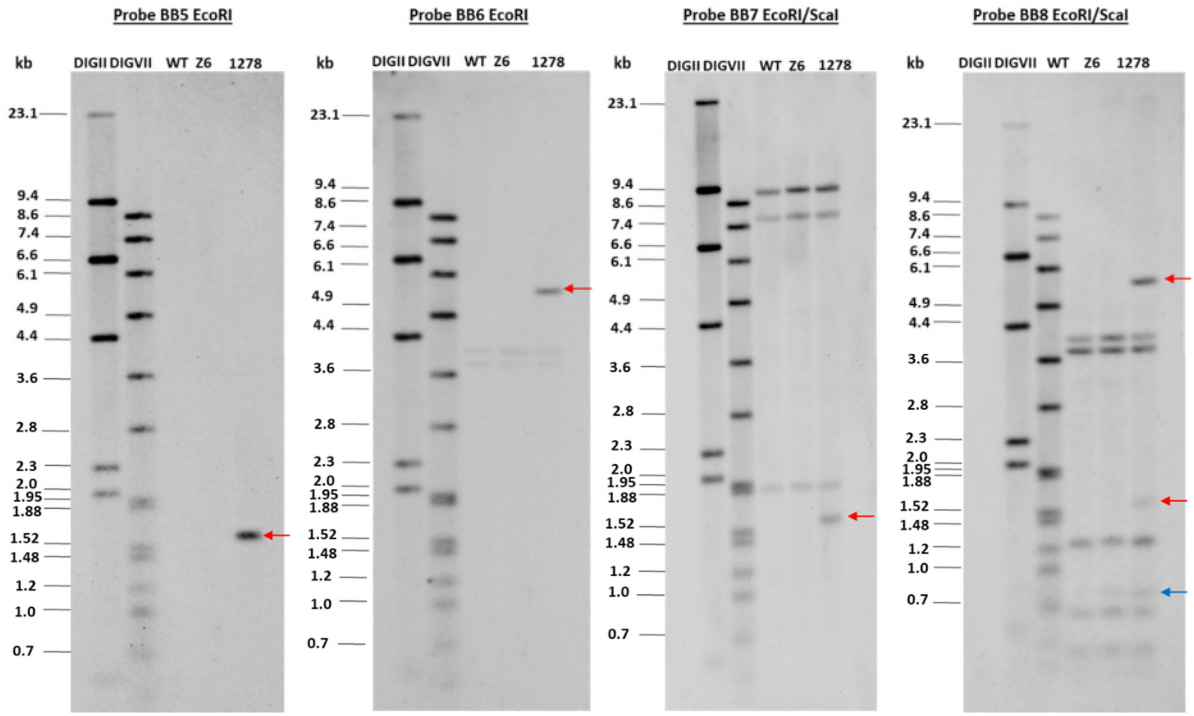

Figure 3-19. No Evidence of Backbone DNA in Z6 Using Probes BB5-BB8
Southern blots of genomic DNA isolated from Snowden (WT), Z6, and WT spiked with pSIM1278 (1278). Molecular weight markers, DIGII and DIGVII, are labeled in kb. Red arrows indicate expected bands (1.57 kb and 5.6 kb) unique to the plasmid control (1278). Blue arrow indicates 0.8 kb band associated with the spacer 2 element of the pSIM1278 insert in Z6.

There were no hybridization signals observed with Z6 genomic DNA that corresponded to backbone sequences from pSIM1278 and pSIM1678. Fragments from the pSIM1278 positive control samples were correctly identified using the eight probes. The results confirmed the absence of pSIM1278 and pSIM1678 plasmid backbone DNA in Z6.

3.5 Summary of the Genetic Characterization of Z6

Molecular characterization showed that Z6 contains a single insertion for each of the pSIM1278 and pSIM1678 inserts. For Z6, the structure of the pSIM1278 and pSIM1678 inserts consist of a nearly full- length T-DNA. No backbone sequences were introduced into Z6 during transformation. Because potato plants arising from vegetative propagation are genetically identical having not undergone meiosis, recombination, or segregation, the inserts are expected to be stable. The insertions in Z6 have been confirmed to be stable over multiple cycles of vegetative propagation (data not provided).

4.0 Gene Down Regulation in Z6 Tuber and Leaf

Z6 was developed by transforming Snowden variety with pSIM1278 and then retransforming with pSIM1678. The pSIM1278 construct introduced two cassettes intended to down regulate expression of *Asn1, Ppo5, PhL*, and *R1* transcripts. The retransformation with pSIM1678 introduced a cassette to down regulate *VInv* transcripts.

Each cassette is driven by promoters primarily active in tubers to facilitate tuber-preferred down regulation. The effectiveness and specificity of target gene down regulation were evaluated by northern blot analysis in potato tuber and leaf.

RNA was isolated from field-grown Z6 and Snowden (WT) and analyzed by northern blot to determine the extent of down regulation of the target genes. The results showed that *Asn1, Ppo5*, and *VInv* transcripts were down regulated in tubers (Figure 4-1). Partial down regulation of *PhL* and *R1* transcripts was observed in tubers. The 18S rRNA and total RNA levels were consistent across samples allowing direct comparison of *Asn1, Ppo5, PhL, R1*, and *VInv* transcripts between samples in tubers or leaves. No changes in target transcript expression were observed in Z6 leaves (Figure 4-2).

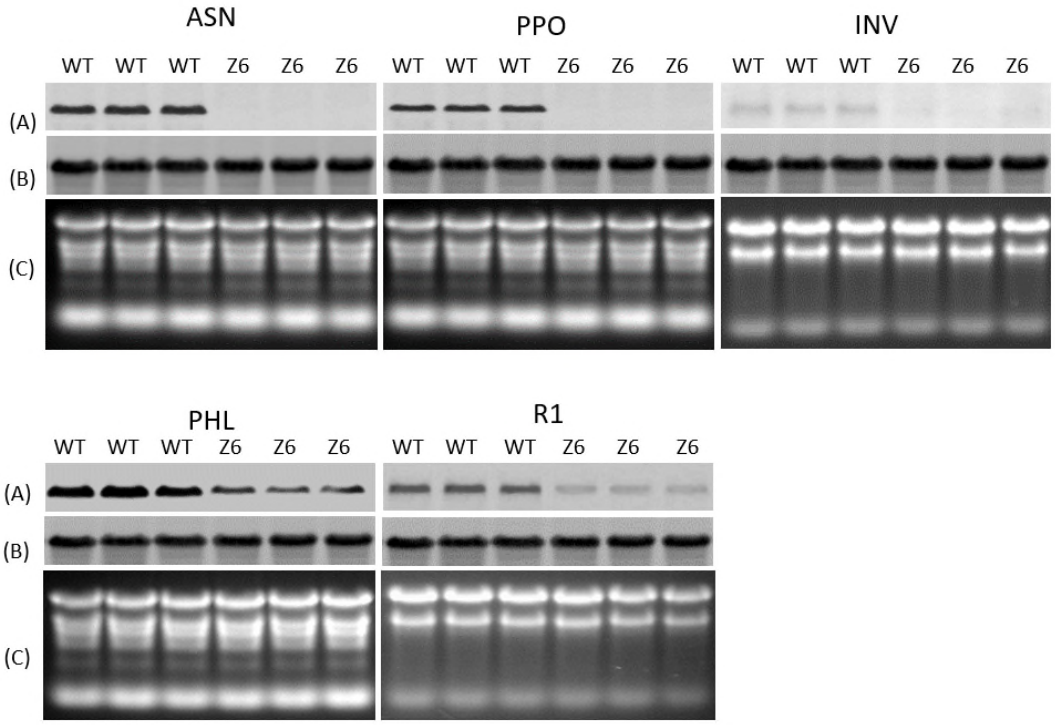

Figure 4-1. Reduced Expression of Target Transcripts in Z6 Tubers
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, INV, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide. WT = Snowden.

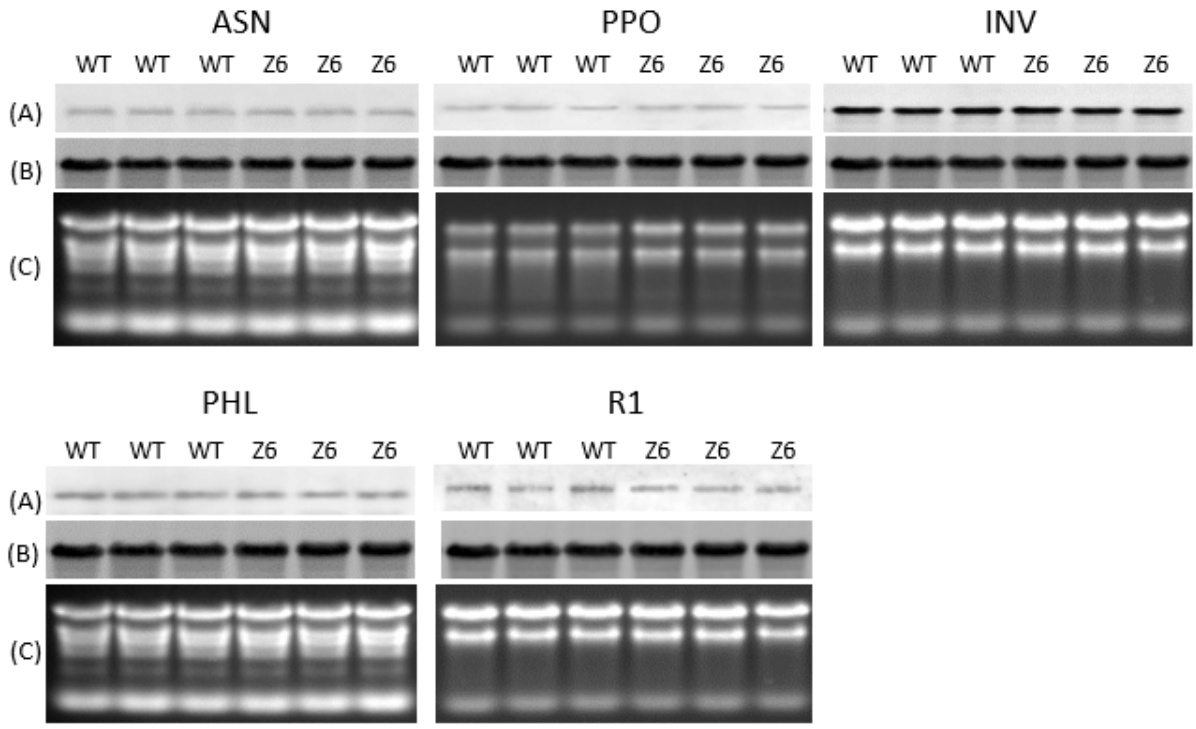

Figure 4-2. No Changes in Target Transcript Expression in Z6 Leaves
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, INV, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

5.0 The VNT1 Protein in Z6

VNT1 is the only introduced protein in Z6. Information about the levels of VNT1 protein and *Rpi-vnt1* transcript in Z6 leaf, stem, root, flower, and tuber are summarized below. These data will be reviewed by the EPA as part of a Section 3 registration application for Z6. The VNT1 protein has the permanent tolerance exemption necessary for its production in potatoes (40 CFR, §174.534).

5.1 Levels of the VNT1 Protein in Z6

Expression of the *Rpi-vnt1* gene in Z6 is driven by the native VNT1 promoter. In general, R-proteins are expressed at low levels in plants—in some cases estimated to be as low as 18 ppt (Bushey et al., 2014). Numerous homologs of *Rpi-vnt1* are present in potato varieties and other wild *Solanum* species (Jupe et al., 2012). In order to distinguish the VNT1 protein in Z6 from endogenous homologs in potato, a western immunoblot assay was developed. A VNT1 polyclonal antibody was generated using a VNT1-specific peptide, SQKGYQHVTFPKK, which is part of the LRR (Leucine-rich repeat) domain. R-protein homologs made development of a VNT1-specific antibody challenging. Despite the high sensitivity of the antibody, cross-reactivity was high in tuber and leaf samples.

VNT1 protein levels were assessed in field-grown Z6 leaves and tubers. The limit of quantitation (LOQ) for VNT1 protein was conservatively established at 500 ppb in potato tubers and leaves. Collectively, the data demonstrated that expression levels of the VNT1 protein were not above background levels. The methods used for these assessments are equivalent to those previously used and presented in petitions 14-093-01p (for event W8) and 16–064–01p (for events X17 and Y9).

5.2 Levels of Rpi-vnt1 Transcript in Z6

Quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) was used to verify expression of the *Rpi-vnt1* transcript in Z6 leaf, stem, root, flower, and tubers. Total RNA was isolated from these plant parts of Z6 and Snowden and subjected to RT-qPCR using *Rpi-vnt1*-specific primers. The methods used for these assessments are equivalent to those previously used and presented to USDA related to petitions 14-093-01p (for event W8) and 16–064–01p (for events X17 and Y9).

Rpi-vnt1 mRNA transcript levels in Z6 and Snowden leaves were compared to levels in the native *S. venturii* leaf (Figure 5-1). Expression of the *Rpi-vnt1* gene was similar between Z6 and *S. venturii* leaf. As expected, expression of *Rpi-vnt1* in Snowden leaf was not detected. Reduced levels of *Rpi-vnt1* transcript were measured in Z6 tuber compared to Z6 leaf. High R-gene expression in leaves, but not in tubers, is consistent with other work on the expression levels of R-genes (Pel, 2010). Like W8, the data indicate that the *Rpi-vnt1* gene is transcribed in Z6 leaves and tubers, and transcription is consistent with low levels of gene expression and protein accumulation in the plants.

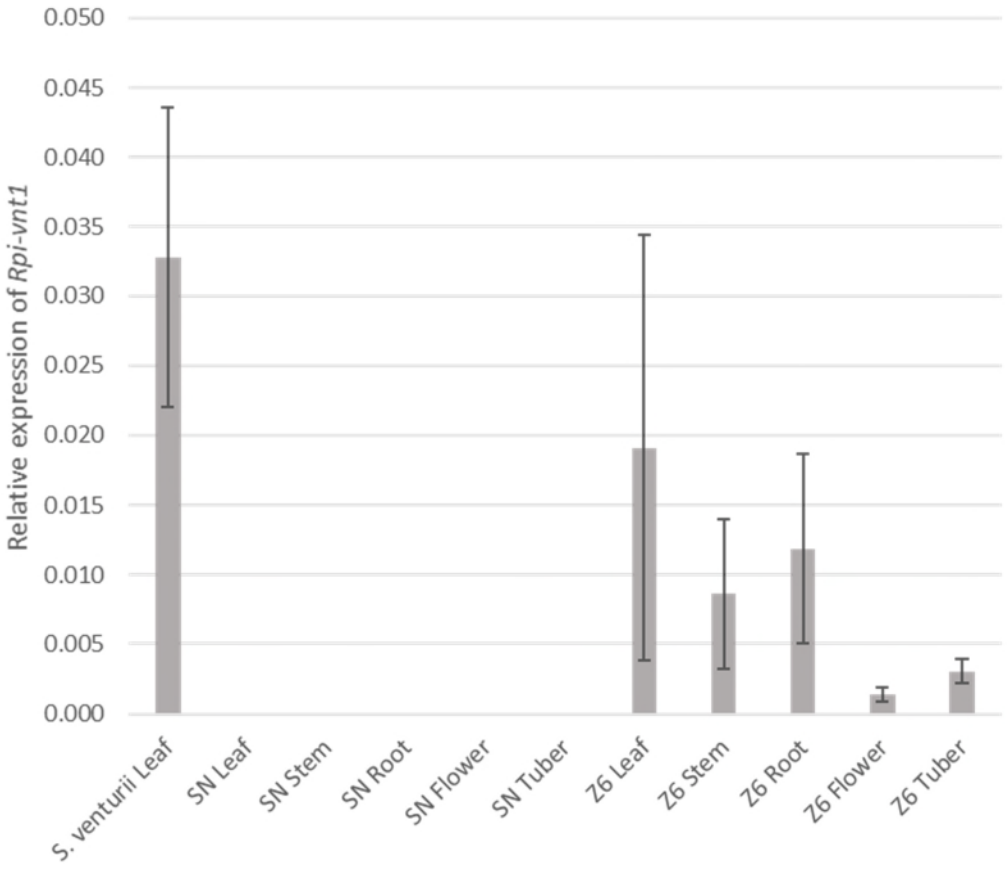

Figure 5-1. Rpi-vnt1 Transcript Levels in Z6 Measured by RT-qPCR

The *Rpi-vnt1* RT-qPCR assay was performed on three biological replicates of Z6 and Snowden (SN) leaf, stem, root, flower, and tubers, analyzed in triplicate. Expression of *Rpi-vnt1* was normalized to endogenous reference genes (*Elongation Factor 1α* and *APRT)* by calculating a ΔCq value. The ΔCq for each replicate was exponentially transformed to ΔCq-Expression by $2^{-\Delta Cq}$, showing relative expression of *Rpi-vnt1* for all samples analyzed. *S. venturii* leaves used as a positive control.

6.0 Comparative Assessment of Z6

As was done for W8, phenotypic and compositional comparative assessments were conducted to compare Z6 to conventional potatoes that have a long history of safe use in the environment and as food and feed.

For Z6, the most relevant comparator is the non-transformed parental variety, Snowden. The only difference between the transformed event and Snowden is that Z6 contains inserts from pSIM1278 and pSIM1678. Statistical analysis was used to determine whether Z6 was different from Snowden.

Other important comparators include additional varieties of conventional potatoes that are grown in typical potato growing regions. These represent potato varieties that are planted commercially and are used to assess the normal range of phenotypic and compositional variation. For agronomic and phenotypic studies, commercial potato varieties were used to generate a conventional variety range (CVR) for all parameters. A list of varieties and data points associated with each are presented in Table 6-1. These varieties were selected to represent a range of commercially planted potato varieties that are commonly on the market in the United States. For composition, the ILSI crop database (ILSI, 2019) and scientific literature were used to provide a combined range of analyte values for conventional potatoes, giving further context to results.

Table 6-1. Varieties and Number of Data Points used in the CVR

Variety	N Per Characteristic
Atlantic	28
Bintje	20-32
C0095051-7W	16
Gala	16
Golden Sunburst	15-30
Lamoka	8
Nicolet	24-32
Purple Majesty	16
Ranger Russet	20-40
Red Thumb	20-32
Russet Burbank	9-53
Russet Norkotah	16-24
Snowden	49-52
TX 278	8

Comparative assessment data were interpreted, after statistical analysis, as follows:

- When p-values were available and the p-value indicated no statistical significance, Z6 was considered to be equivalent to its control Snowden, and the assessment was considered complete; and
- If the p-value indicated statistical significance or if a p-value was not present, the mean value for the event was compared to a CVR, or combined literature range (CLR). Mean Z6 values within

these ranges were considered within the natural variation for potatoes, and Z6 was considered to be equivalent to conventional potatoes.

The phenotype and compositional comparative assessments for Z6 are provided in Section 7.0 and Section 8.0, respectively. The analyses indicate that Z6 is comparable to Snowden and other conventional potatoes with respect to the characteristics measured. Overall, these analyses indicate that Z6 is not materially different from conventional potatoes, poses no more risk than conventional potato varieties in food, feed, and the environment, and is as safe and nutritious as conventional potato varieties.

7.0 Phenotypic Performance and Field Observations for Z6

The purpose of the phenotypic performance and field observation studies was to assess Z6 phenotype compared to the Snowden variety, when grown in potato-producing regions of the U.S. Observations throughout the growing season were used to make a thorough assessment of the characteristics listed in Table 7-1.

Table 7-1. Characteristics Evaluated for Z6

Phenotypic Performance	Tuber Evaluation	Stressors
Early Emergence	Total Yield	Insect Stressors
Final Emergence	US#1 Yield	Disease Stressors
Stems Per Plant	Tubers Per Plant	Abiotic Stressors
Plant Vigor	Tuber Grading	
Plant Height	Specific Gravity	
Vine Senescence	Total Internal Defects	

The assessments, described in detail below, demonstrated that Z6 is agronomically and phenotypically comparable to its parental variety and other conventional potatoes. Introducing the pSIM1278 and pSIM1678 inserts into Snowden did not result in unintended effects associated with weediness or pest-like characteristics. The phenotypic comparability of Z6 with conventional controls supports the lack of somaclonal variation in the event.

7.1 Field Trial Details

Field trial years and locations for Z6 studies are shown in Table 7-2. The agronomic practices, including soil preparation, fertilizer application, irrigation, and pesticide-based control methods, were location-specific and typical for potato. Cultivation practices were recommended by regional potato extension specialists and agronomists.

The field trials were established in a randomized complete block (RCB) design. The treatments included Z6 and Snowden. Every block (replicate) included a plot (four rows) of each treatment. Rows were approximately 20 ft long and the typical seed spacing was one tuber every 10-12 in. The seed tubers were placed by hand or machine to a depth of approximately 6 in. Additional method details are equivalent to those described in petitions 14-093-01p (for event W8) and 16–064–01p (for events X17 and Y9).

Table 7-2. Z6 Field Trial Locations

Year	Site State	Site County	Rows x Tubers/Row	Material Planted	Planting Date, Harvest Date
2018	ID	Canyon	4x20	FG1	4/05/2018, 8/15/2018
	ID	Bonneville			5/08/2018, 9/18/2018
	MI	Montcalm			5/08/2018, 9/18/2018
	WI	Waushara			5/04/2018, 9/12/2018

Note: Additional varieties including other events, controls, and references were included in the experimental design at some sites.

7.2 Z6 Phenotypic and Tuber Assessment Results

Summaries of the phenotypic and tuber characteristics (yield and grading) are shown in Table 7-3. Z6 tubers were assessed using methods equivalent to those used for chipping potatoes, including Y9 in petition 16-064-01p. Total yield was determined after harvest by weighing all tubers from a single row of each plot and converting to cwt/acre. U.S. #1 yield was determined by subtracting the weight of tubers failing to meet the U.S. #1 standard prior to conversion. The average number of tubers per plant was determined by counting the total number of tubers in each yield sample and dividing by the total number of plants in the sample row. After harvest, all tubers from one row of each plot were transported to a facility equipped to conduct grading. The grading methods employed were identical to those used to grade commercial potatoes intended for the production of chips (USDA, 1997). Tuber grading size profiles were determined by weighing tubers from the sample sorted by size. The size

categories included: 2-3.25 inches in diameter (A); <2 inches in diameter (B); >3.25 inches in diameter (oversized); and unmarketable tubers based on physiological defects (pick-outs). Specific gravity was determined by using a weight in air/weight in water measurement. Sub-samples of the tubers used to determine yield 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). 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.

No statistical differences were detected between Z6 and Snowden for early emergence, final emergence, plant vigor, plant height, vine senescence, total yield, U.S. #1 yield, tubers per plant, tuber grading, and total internal defects. Z6 had more stems per plant and higher specific gravity, however, the means for these attributes were within the CVR. These changes do not indicate greater environmental impact of Z6 compared to conventional potatoes.

The data support the conclusion that Z6 is unlikely to have increased plant pest potential compared to conventional varieties.

Table 7-3. Phenotypic, Yield, and Grading Characteristics of Z6

Characteristic	Variety	N	Mean	P-Value[1]	SD	CVR Min[2]	CVR Max[2]
Phenotypic Performance							
Early Emergence (%)	Snowden	16	87.7	0.5153	9.9	0	100
	Z6	16	89.1		11.4		
Final Emergence (%)	Snowden	16	98.4	0.1471	2.7	10.6	100
	Z6	16	99.7		1.3		
Stems Per Plant (#)	Snowden	16	3.5	**0.0226**	0.6	1	6
	Z6	16	3.9		0.6		
Plant Vigor (1-5 Scale)	Snowden	12[3]	3.2	0.5571	0.4	1.3	5
	Z6	12	3.3		0.5		
Plant Height (cm)	Snowden	16	65.8	0.1153	12.5	16.4	108.7
	Z6	16	70.5		13.5		
Vine Senescence (%)	Snowden	16	0[4]	.	0	0	100
	Z6	16	0		0		
Tuber Evaluation							
Total Yield (cwt/a)	Snowden	16	572.1	0.7245	144.1	89.2	1410.6
	Z6	16	562.1		174.3		
U.S. #1 Yield (cwt/a)	Snowden	16	561.7	0.7868	140.3	118.9	693.5
	Z6	16	554		168		
Tubers Per Plant (#)	Snowden	16	11.4	0.4907	4.5	3.1	19.5
	Z6	16	11.8		4.3		
A (%)	Snowden	16	73.2	0.3907	8.7	28	83.3
	Z6	16	75.5		7.8		
B (%)	Snowden	16	5.4	0.0509	2.4	2	70.5
	Z6	16	7.4		3.3		
Oversized (%)	Snowden	16	19.7	0.1879	9.9	0	45.7
	Z6	16	15.8		9.8		
Pick-outs (%)	Snowden	16	1.7	0.4117	2.1	0	17.3
	Z6	16	1.3		1.2		
Specific Gravity	Snowden	16	1.060	**<.0001**	0.009	0.835	1.171
	Z6	16	1.066		0.011		
Total Internal Defects (%)	Snowden	16	0.1	0.469	0.3	0	93.8
	Z6	16	0		0		

[1] P-values indicating significant differences with controls are underlined and in bold. [2] The range of mean values of conventional varieties. [3]For vigor, no data were collected at WI-HANC due to vine killing. [4]No variability was observed in the senescence data – all zero.

7.3 Insect, Disease, and Abiotic Stressor Assessments

Once per month, any insects, diseases (other than late blight), and abiotic stressors observed in any of the plots were recorded. For each stressor, symptoms were rated as slight, moderate, or severe. For each stressor observation, observers indicated whether there were differences between Z6 and Snowden. Stressor evaluations were intended to be categorical and were not statistically analyzed.

Of 64 possible observations (4 locations x 4 repetitions x 4 months), no differences were observed between Z6 and Snowden (Table 7-4) in 7 cases where stressors were present.

The results support the conclusion that the ecological interactions of Z6 are the same as Snowden and conventional potatoes. Z6 is not expected to impact non-target organisms or differ with respect to insect, disease, or abiotic stressors, other than the intended late blight protection.

Table 7-4. Stressor Observations in Z6 and Snowden

Date	Site	Stressor	Z6 Rating	Snowden Rating
6/19/2018	Canyon, ID	Leafhopper (*Empoasca* sp.)	Slight	Slight
7/31/2018	Waushara, WI	Early Dying (*Verticillium* sp.)	Moderate	Moderate
8/2/2018	Canyon, ID	Dickeya (*Dickeya dianthicola*)	Slight	Slight
8/6/2018	Waushara, WI	Early Blight (*Alternaria solani*)	Moderate	Moderate
8/6/2018	Waushara, WI	Early Dying (*Verticillium* sp.)	Moderate	Moderate
8/14/2018	Waushara, WI	Early Blight (*Alternaria* solani)	Moderate	Moderate
8/14/2018	Waushara, WI	Early Dying (*Verticillium* sp.)	Moderate	Moderate

7.4 Conclusions for Agronomic Performance and Field Observations

Like W8, results of the agronomy trials confirmed that Z6 grown at locations representing major areas of potato production in the U.S. is phenotypically and agronomically similar to the control variety, Snowden. No phenotype that would indicate enhanced weediness, survivability, or plant pest potential was noted for Z6.

8.0 Compositional Assessment for Z6

As with W8, compositional analysis of Z6 was conducted to evaluate the levels of key nutrients (proximates, vitamins, minerals, amino acids) and glycoalkaloids. In addition, concentrations of free amino acids and sugars were analyzed to determine trait efficacy. The U.S. FDA will review the details of the compositional analyses as a component of the food and feed safety assessment of Z6 potatoes.

Tubers for the compositional assessment were generated from the field studies used for phenotypic testing (see Table 7-2). All tubers were grown in commercial potato-growing regions of the U.S. (Section 6.0).

Z6 and Snowden were assessed for analytes important to potato nutrition, and those related specifically to trait efficacy. Each sample (replication) consisted of six randomly selected, mid-sized tubers with skin. Samples were powdered in an industrial blender with liquid nitrogen and stored at -70 °C until analysis. Analytical testing was conducted by Covance Laboratories, Inc. Method details for compositional and statistical analyses are equivalent to those described in petitions 14-093-01p (for event W8) and 16-064-01p (for events X17 and Y9).

The nutritional assessment evaluated levels of proximates, vitamins, minerals, amino acids, and glycoalkaloids and demonstrated that Z6 is compositionally equivalent to conventional potatoes. Trait efficacy assessment evaluated free amino acids and reducing sugars, and demonstrated that, Z6 has lower levels of free asparagine and lower levels of reducing sugars compared to Snowden. The same conclusions were reached for W8, the antecedent event, compared to the Russet Burbank comparator.

8.1 Compositional Nutrient Analysis

Analyses were conducted to evaluate the nutritional composition of Z6 and assess the food quality, feed quality, and safety compared to Snowden and other conventional potatoes. The compositional assessments determined the concentrations of:

- Proximates, vitamins, and minerals (Table 8-1);
- Total amino acids (Table 8-2); and
- Glycoalkaloids (Table 8-3).

8.1.1 Proximates, Vitamins, and Minerals

Statistically significant differences between Z6 and Snowden were observed for carbohydrates, calories, moisture, Vitamin B3, and Vitamin C (Table 8-1). However, mean values for each of these analytes were within the CLR. These results indicated that Z6 was equivalent to conventional potatoes. The remainder of the analytes presented in Table 8-1 showed no statistically significant difference between Z6 and Snowden.

Table 8-1. Proximates, Vitamins, and Minerals in Z6 and Snowden

Variable (Fresh Weight Basis)	Variety	Mean	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Protein (%)	Z6	2.33	0.7562	0.174	16	2.11	2.73	0.7	4.6
	Snowden	2.31		0.149	16	2.08	2.61		
Total Fat (%)	Z6	0.158	0.1983	0.102	16	0.100	0.370	0.02	0.74
	Snowden	0.178		0.118	16	0.100	0.420		
Ash (%)	Z6	0.931	0.4871	0.0900	16	0.792	1.10	0.15	2.0
	Snowden	0.905		0.158	16	0.462	1.11		
Crude Fiber (%)	Z6	0.609	0.8192	0.0990	16	0.453	0.870	0.17	3.5
	Snowden	0.603		0.105	16	0.425	0.780		
Carbohydrates (%)	Z6	19.3	**0.0002**	2.41	16	16.2	23.3	3.68	29.4
	Snowden	18.1		2.56	16	14.5	24.5		
Calories (kcal/100 g)	Z6	87.4	**0.0006**	9.49	16	73.9	103	22.4	110
	Snowden	83.2		9.97	16	68.5	107		
Moisture (%)	Z6	77.4	**0.0003**	2.37	16	73.4	80.6	71.8	86.0
	Snowden	78.5		2.50	16	72.4	82.1		
Vitamin B3 (mg/100 g)	Z6	1.58	**0.0071**	0.243	16	1.28	2.05	0.88	3.43
	Snowden	1.46		0.275	16	1.15	2.00		
Vitamin B6 (mg/100 g)	Z6	0.142	0.0605	0.0130	16	0.121	0.160	0.065	0.204
	Snowden	0.133		0.0110	16	0.105	0.150		
Vitamin C (mg/100 g)	Z6	26.7	**0.0395**	3.03	16	21.5	31.2	6.97	51.4
	Snowden	24.8		3.01	16	19.7	30.1		
Copper (mg/100 g)	Z6	0.113	0.2273	0.128	16	0.0320	0.570	0.04	2.05
	Snowden	0.0831		0.0440	16	0.0250	0.160		
Magnesium (mg/100 g)	Z6	23.8	0.0809	2.10	16	20.1	28.2	14.6	40.6
	Snowden	22.6		2.20	16	20.0	29.4		
Potassium (mg/100 g)	Z6	479	0.1082	34.0	16	405	527	291	765
	Snowden	461		22.7	16	409	492		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges are from ILSI, 2019 and OECD, 2002.

8.1.2 Total Amino Acids

Levels of amino acids were measured for Z6 (Table 8-2). Although, differences were observed between most amino acid levels in Z6 and Snowden, such changes would have minimal impact on food and feed safety considering the low levels of protein (about 2% fresh weight) in potatoes.

Significantly lower aspartic acid + asparagine and significantly higher glutamic acid + glutamine were noted between Z6 and Snowden. These results were expected because of the down regulation of asparagine synthetase in Z6. The means for these analytes in Z6 were within the CLR.

A significant difference between Z6 and Snowden was also noted for all other total amino acids, with the exception of histidine and tryptophan (Table 8-2). In all cases, the mean for Z6 was within the CLR, so Z6 was considered equivalent to conventional potatoes.

Table 8-2. Total Amino Acids in Z6 and Snowden

Variable (Fresh Weight Basis)	Variety	Mean (mg/100g)	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2,3]	
						Min	Max	Min	Max
Alanine	Z6	74.8	**0.0007**	8.4	16	61.6	89.6	10.0	145
	Snowden	62.5		5.13	16	54.8	69.7		
Arginine	Z6	147	**0.0015**	17.4	16	124	192	46.2	234
	Snowden	127		11.4	16	114	153		
Aspartic Acid + Asparagine	Z6	308	**<.0001**	27.4	16	270	364	177	1548
	Snowden	502		50.0	16	408	594		
Cystine	Z6	33.8	**0.0003**	3.97	16	26.6	38.9	10.0	125
	Snowden	27.6		3.71	16	20.5	33.3		
Glutamic Acid + Glutamine	Z6	510	**<.0001**	42.4	16	426	584	152	956
	Snowden	369		27.8	16	326	429		
Glycine	Z6	79.2	**<.0001**	8.63	16	67.8	91.9	30.7	372
	Snowden	66.1		5.94	16	57.4	76.2		
Histidine	Z6	38.9	0.0658	4.93	16	32.8	50.0	10.0	105
	Snowden	35.2		4.33	16	29.1	43.7		
Isoleucine	Z6	86.0	**0.0047**	9.53	16	73.1	103	21.3	137
	Snowden	74.7		6.43	16	65.4	85.4		
Leucine	Z6	148	**0.0004**	18.9	16	124	181	53.0	224
	Snowden	120		11.2	16	104	140		
Lysine	Z6	124	**0.0033**	15.2	16	103	147	44.4	495
	Snowden	107		9.38	16	92.9	120		
Methionine	Z6	39.5	**0.0009**	3.47	16	34.2	46.7	10.0	83.6
	Snowden	34.7		2.55	16	30.3	39.4		
Phenylalanine	Z6	101	**0.0065**	11.2	16	85.7	121	41.4	131
	Snowden	90.3		7.51	16	78.4	104		
Proline	Z6	80.9	**0.0005**	8.55	16	70.4	98.7	31.9	232
	Snowden	67.8		6.79	16	58.2	79.2		
Serine	Z6	87.9	**0.0007**	10.5	16	74.1	104	10.0	140
	Snowden	75.7		7.00	16	66	86.9		
Threonine	Z6	94.2	**0.0028**	10.6	16	80.2	111	19.8	133
	Snowden	79.2		7.63	16	69	90.9		
Tryptophan	Z6	23.1	0.0582	1.97	16	19.7	25.9	10.0	32.1
	Snowden	22.0		1.72	16	18.7	24.8		
Tyrosine	Z6	89.4	**0.0008**	10.3	16	76	109	27.5	237
	Snowden	73.7		6.34	16	63.5	83.1		
Valine	Z6	109	**0.0166**	9.85	16	97.7	131	24.6	259
	Snowden	99.0		9.86	16	86.5	122		

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

[2]Negative values or values below the limit of detection, arising from variability measured in the samples, were adjusted to the limit of detection (10 mg/100 g).

[3]Combined literature ranges are from ILSI, 2019 and OECD, 2002.

8.1.3 Glycoalkaloids

Glycoalkaloids are toxins commonly found in Solanaceous crops, including potato. Ninety-five percent of glycoalkaloids in potato tubers are either α-solanine or α-chaconine (OECD, 2002). The widely accepted safety limit for total glycoalkaloids in tubers is 20 mg/100 g fresh weight (Smith et al., 1996).

The mean concentration of glycoalkaloids in Z6 was statistically lower than Snowden, was within the CLR, and was within the generally accepted safety limit (Table 8-3).

Table 8-3. Glycoalkaloids in Z6 and Snowden

Variable (Fresh Weight Basis)	Variety	Mean (mg/100g)	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Glycoalkaloids[3]	Z6	11.8	**0.0439**	3.16	16	7.26	19.0	3.20	210.4
	Snowden	13.9		3.70	16	9.08	23.5		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges from Kozukue et al., 2008.
[3]Total of α-solanine and α-chaconine.

8.2 Composition Efficacy Assessment of Z6

To evaluate trait efficacy, free amino acids (Table 8-4) and reducing sugars (Table 8-5) were assessed.

8.2.1 Free Amino Acids

The results show that Z6 tubers contained significantly less free asparagine and significantly more free glutamine than Snowden tubers (Table 8-4). However, the mean concentrations of free asparagine and free glutamine for Z6 were within the CLR and therefore considered within the normal range for potatoes. Free amino acid analysis demonstrated that, similar to W8, down regulation of asparagine synthetase was effective in reducing free asparagine in tubers.

Table 8-4. Free Amino Acids in Tubers at Harvest in Z6

Variable (Fresh Weight Basis)	Variety	Mean (mg/100 g)	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Asparagine	Z6	80.4	**<.0001**	14.8	16	55.4	104	31.4	456
	Snowden	309		44.1	16	237	397		
Aspartic Acid	Z6	45.1	0.5897	3.85	16	39	51.7	16.7	197
	Snowden	44.2		3.60	16	37.3	50.5		
Glutamic Acid	Z6	57.6	0.061	8.28	16	44.6	71.7	12.5	136
	Snowden	54.5		7.83	16	44.1	68.7		
Glutamine	Z6	259	**<.0001**	40.1	16	186	314	33.6	411
	Snowden	162		22.1	16	118	193		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined literature ranges are from ILSI, 2019.

8.2.2 Reducing Sugars

Z6 showed significantly lower levels of reducing sugars, fructose and glucose, at harvest (Table 8-5). The mean glucose and fructose levels in Z6 tubers are below the range for conventional potatoes, but this is not anticipated to affect potato nutrition, as the mean carbohydrates for Z6 are within the range for conventional potatoes (Table 8-1). These results can be attributed to partial down regulation of water dikinase and phosphorylase L, and down regulation of vacuolar invertase. Down regulation of water dikinase and phosphorylase L slows the breakdown of starch into sugars in the amyloplast. Down regulation of invertase slows the conversion of sucrose into fructose and glucose in the vacuole.

Sucrose levels were significantly higher in Z6 at harvest compared to Snowden (Table 8-5). However, the mean sucrose content for Z6 was within the combined literature range for potatoes at harvest.

Table 8-5. Sugars in Z6 and Snowden Tubers at Harvest

Variable (Fresh Weight Basis)	Variety	Mean	P-Value[1]	Standard Deviation	N	Range		Combined Literature Range[2]	
						Min	Max	Min	Max
Fructose and Glucose (mg/100 g)									
Fresh	Z6	6.76	**0.0517**	2.12	16	4.02	10.1	13	1208
	Snowden	17.7		12.7	16	7.52	55		
Sucrose (mg/100 g)									
Fresh	Z6	133	**0.0036**	16.7	16	109	161	39.7	1390
	Snowden	122		16.7	16	91.3	151		

[1]P-values indicating significant differences are underlined and in bold.
[2]Combined Literature Ranges are from Amrein et al., 2003 and Vivanti et al., 2006.

8.2.3. Acrylamide

At harvest, chips made with Z6 tubers contained significantly less acrylamide than chips made with Snowden (Table 8-6). The lower acrylamide levels were expected from down regulation of asparagine synthetase, water dikinase, phosphorylase L and vacuolar invertase, which reduced the free asparagine and reducing sugar reactants.

Table 8-6. Acrylamide in Chips from Z6 and Snowden at Harvest

Variable	Variety	Mean (ppb)	P-Value[1]	Standard Deviation	N	Percent Reduction	Range	
							Min	Max
Fresh	Z6	334	**0.0168**	94.7	8	77.8%	191	464
	Snowden	1,506		355	8		998	2,150

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

8.3 Compositional Assessment Conclusions

Like W8, a thorough compositional assessment was conducted for Z6 comprising of:

- Compositional nutritional assessment on analytes important to potato nutrition; and
- Compositional efficacy assessment on specific analytes related to the introduced traits and acrylamide.

The nutritional assessment evaluated levels of proximates, vitamins, minerals, amino acids, and glycoalkaloids, and demonstrated that Z6 is compositionally equivalent to conventional potato varieties and is as safe and nutritious for food and feed as conventional potatoes that have a long history of safe consumption. The efficacy assessment demonstrated that Z6 has lower levels of free asparagine, reducing sugars, and acrylamide compared to Snowden at harvest.

9.0 Environmental Safety Assessment for Z6

Like W8, the environmental safety of Z6 is supported by testing. Data from phenotypic performance, trait efficacy, genetic characterization, and compositional assessments were collected and analyzed to determine potential environmental risks associated with Z6. The following five criteria were considered:

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

The traits in Z6 are intended to enhance late blight protection and tuber quality of potatoes. Planting, cultivation, management, harvesting, and volunteer management are not expected to change, with the exception that, similar to W8, the cultivation of Z6 may reduce the amount of fungicide needed to control late blight. Conclusions from a previous Environmental Assessment of the W8 event (14-093-01p; Final Environmental Assessment) are applicable to Z6. Z6 is no more likely to impact the environment than W8.

10.0 Stewardship of Z6 Potatoes

Simplot is committed to the responsible introduction and stewardship of biotech potatoes, including the Z6 variety. The Simplot Stewardship Program for Innate® potatoes is based on industry best practices for managing the development, production, sale, distribution, utilization, and disposal of seed improved using biotechnology.

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

10.1 Stewardship for Commercial Products

After regulatory authorization, Innate® potatoes are managed by Simplot and its licensees in accordance with the Innate® Stewardship Program. This commercial stewardship program is designed to direct Innate® seed and harvested potatoes to Simplot-authorized growers, packers, processors, and marketers who agree to abide by crop stewardship requirements. Seed multiplication is contracted by Simplot with selected licensees who agree to transfer Innate® seed under the direction of Simplot to its licensed growers. The Simplot requirement for stewardship directs Innate® potatoes in the marketplace and facilitates separation between Innate® and non-biotech potatoes.

11.0 Conclusion: Extension of Non-Regulated Status for Z6

As with W8, Simplot's Z6 potatoes have late blight protection, reduced black spot, lower reducing sugars and free asparagine contributing to low acrylamide potential. Considering that the Snowden variety, like other potatoes, is difficult to breed, biotechnology applications are ideally suited for simultaneously incorporating multiple traits. Vegetative propagation of commercial potatoes helps mitigate concerns about seed dispersal, survival outside of cultivation, and outcrossing, that could contribute to increased plant pest potential. Field trials evaluating phenotypic performance and insect and disease interactions demonstrate that Z6 poses no significant risk of persistence in the environment as a result of weediness or increased plant pest potential.

The data presented in this submission demonstrate that introduction of Z6 potatoes will have a similar environmental impact as W8 and conventional potatoes and poses no increased risk to the environment. The introduction and cultivation of these potatoes is not expected to cause any adverse environmental or biological impacts, or detrimental effects on plant health.

Simplot seeks an extension of the deregulation of the W8 event in 14-093-01p and requests non-regulated status for Z6 based on the weight of evidence demonstrating that these potatoes are unlikely to pose a plant pest risk. We respectfully submit that Z6, and their progeny, should not be classified as "regulated articles" as defined under 7 CFR Part 340.

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13.0 Appendix A: Z6 USDA Notifications

Since 2016, field trials of Z6 have been conducted in several states in the continental U.S. (Table 13-1). The table shows plantings up until March 28, 2019.

Table 13-1. Z6 USDA Release Notifications and Planted Acreage Details

Year of Planting	Notification Number	Valid Date	State	Number of Counties Planted	Acreage (Whole Plot)
2016	16-082-105n	4/4/2017	MI	1	0.83
			ND	1	0.65
			PA	1	0.30
2017	17-045-104n	3/5/2018	ID	2	5.93
	17-066-102n	4/3/2018	MI	2	0.44
			WI	1	0.44
2018	18-066-101n	4/3/2019	ID	3	0.16
			UT	1	0.26
			MI	1	0.09
			WI	1	0.13
2019	19-052-102n	3/4/2020	AZ	1	0.65

14.0 Appendix B: Molecular Methods and Materials

The following methods were used to generate the molecular data presented for Z6.

Southern Blot Methods

Plant Material

Z6, Snowden, and V11 (G0) plants were grown for two months in Sunshine mix-1 (www.sungro.com) in two-gallon pots in a greenhouse with controlled temperatures (18 °C minimum/27 °C maximum) and light exposures (16-h photoperiod with an intensity of ~1500 µmol/m^2/s). After one to two months of growth, leaves were collected and used for genomic DNA isolation V11 samples were used to identify bands specific to the insert from pSIM1278.

DNA Isolation

A 1.0 g sample of young potato leaves was ground into a fine powder under liquid nitrogen using a mortar and pestle. The ground tissue was transferred to a pre-cooled 15 ml conical tube with a pre-cooled spatula and stored at -80 °C. Powdered tissue was mixed with 10 ml extraction buffer (0.35 M Sorbitol, 0.1 M Tris-HCl, pH 8.0, 0.05 M EDTA) and centrifuged at 3,000 g for 15 min at room temperature (RT). The pellet was suspended in 2 ml extraction buffer containing 200 µg RNase A. After incubating at 65 °C for 20 min with 2 ml nuclear lysis buffer (0.2 M Tris-HCl (pH 7.5), 0.005 M EDTA (pH 8.0) and 20 mg/ml CTAB Hexadecyl Trimethyl Ammonium Bromide) and 800 µl of 5% Sarcosyl, the sample was mixed with an equal volume of chloroform: isoamyl alcohol (24:1), vortexed for 1 min, and centrifuged at 3,000 rpm for 5 min at RT. The DNA was precipitated with an equal volume of isopropyl alcohol, washed with 70% ethanol, air dried, and dissolved in 400-700 µl 1X Tris/EDTA buffer (TE). DNA concentration was measured using Qubit Fluorometric Quantitation (Life Technologies) and quality was confirmed by electrophoresis on a 0.8% agarose gel in 1X Tris/Acetate/EDTA (TAE) for 30-40 min at 80 volts.

DNA Restriction Digestion and Electrophoresis

A 4.0 µg sample of plant DNA was digested overnight in 400 µl (final volume) reaction with at least 50 units restriction enzyme (Invitrogen) at 37 °C. Digested DNA was concentrated by ethanol precipitation (40 µl of 3 M NaOAc (pH 5.3) and 1 ml ethanol) at -80 °C for 10 min followed by a wash with 70% ethanol. The DNA pellet was dissolved in 20 µl 1X TE followed by the addition of 2 µl DNA gel loading buffer (40% sucrose and 0.35% Orange G (Sigma)).

Gel Preparation

Digested DNA was electrophoresed on a 0.7% agarose gel containing Tris-Acetate-EDTA (TAE) buffer for 24 h using 30 volts. The gel was depurinated by submersion in 0.25 N HCl for 2 x 10 min. After subsequent denaturation in 0.5 M NaOH/1.5 M NaCl for 2 x 15 min and neutralization in 1.5 M NaCl/0.5 M Tris-HCl (pH 7.5), for 2 x 15 min on a shaker at room temperature, the gel was equilibrated with 10X SSC for 10 min. The transfer of DNA to the nylon membrane was carried out with 10X SSC using capillary transfer.

DIG-Labeled Probe Preparation

Probes were labeled with DIG using PCR. The reaction mix contained Hotmaster Taq enzyme and buffer (Fisher BioReagents) according to Roche's DIG labeling protocol. A standard 50 µl reaction consisted of 5 µl of 10X Hotmaster Taq Buffer, 2-5 µl of 10 µM forward primer and reverse primer (Table 1), 5 µl of DIG labeled dNTPs (Roche), 10 ng plasmid template, and 0.75 µl Hotmaster Taq polymerase. The PCR amplification conditions were optimized for each DIG-labeled probe. PCR with unlabeled dNTPs was used as a positive control. Quality of the DIG labeled probes was assessed by analyzing a fraction of the product on a 1% agarose gel alongside the control. The probe was denatured before use by incubating at 95 °C for 5 min, and quenching on ice for 2 min.

Hybridization

Following transfer to a nylon membrane, the DNA was prehybridized in 40 ml pre-warmed DIG Easy Hybridization solution (Roche) at 42 °C for 1-4 h in a hybridization oven (Amerex Instruments Inc.) with rotating at 20-25 rpm. Hybridization was initiated by replacing the prehybridization buffer with a fresh amount of the same preheated solution containing 25-50 µl denatured DIG labeled probe and continuing the incubation with rotation (20-25 rpm) at 42 °C for about 16 h. The probe-containing hybridization solution was stored (-20 °C) and reused up to 3 times. The reused hybridization solution was heated to 68 °C for 10 min before use.

Detection

The hybridization solution was replaced by 100 ml washing solution I (2X SSC/0.1% SDS). The membrane was washed twice in washing solution I for 10 min at RT. The low stringency buffer was replaced immediately by preheated high stringency washing solution II (0.5X SSC/0.1% SDS, 60 °C). The membrane was washed twice in washing solution II at 65 °C for 20 min each at 25-30 rpm. This was followed by a rinse with 2X SSC to remove SDS. The membrane was rinsed with 150 ml of 1X DIG Washing Solution (Roche) in a tray for 2 min and incubated in 1X Blocking solution (Roche) for 0.5-3 h with shaking. The blocked membrane was incubated with DIG antibody solution (1:10,000 dilution of Anti-DIG-alkaline phosphate conjugate with 1X Blocking solution) for 30 min on a shaker. The membrane was washed twice (15 min each) with 1X DIG Washing Solution (Roche) and equilibrated with 1X detection buffer. The detection reaction was carried out with 2 ml CDP-Star solution (1:100 diluted stock of CDP-Star with 1X detection buffer) for 5 min. The membrane was wrapped in plastic film. Depending on the experiment multiple exposures were taken from 30 sec to 30 min. Images were developed with an Amersham Imager 600 (GE). Following detection, membranes were washed twice (15 min each) at 37 °C with stripping buffer (0.2 M NaOH and 0.1% SDS) and rinsed with 2x SSC for 5 min. Membranes were checked with Amersham Imager 600 to confirm the absence of signal prior to incubation with additional backbone probes.

Southern Blot Presentation

Southern blots are presented with an accompanying table to help with band identification. Two kinds of bands are observed on the blots, internal (IB) and junction (JB) bands. Internal bands are sequences within the insert that have predictable sizes. Junction bands extend from restriction enzyme sites in the insert to sites in the genomic DNA. Junction bands were expected to vary in size and were not always predictable. However, a minimal size could be estimated from distances between restriction sites within the insert and those identified during analysis of the flanking genomic sequences. The estimated junction band sizes were confirmed or modified based on migration during electrophoresis. Junction

bands were used to confirm the number of integration sites as there are never more than two junction bands per insert for each restriction digest and probe. The T-DNA is derived from potato DNA sequence so that some Southern blot probes targeting the insert also detect endogenous potato sequences. Bands with endogenous sequences are observed in both the control and event samples with the same size and intensity. These bands are not labeled to simplify the presentation.

Sequencing Methods:

Plant Material

Tissue culture plants were grown in Sunshine mix-1 (www.sungro.com) in two-gallon pots in a greenhouse controlled for temperature (18 °C minimum/27 °C maximum) and light (16 h photoperiod with an intensity of about 1,500 µmol/m^2/s). After one to two months of growth, leaf tissue was collected for genomic DNA isolation.

DNA Isolation

A 1.0 g sample of young potato leaves was ground into a fine powder using a mortar and pestle under liquid nitrogen. The ground tissue was transferred to a pre-cooled 15 mL conical tube with a pre-cooled spatula and stored at -80 °C. Powdered tissue was thoroughly mixed with 10 mL extraction buffer (0.35 M sorbitol, 0.1 M Tris-HCl, pH 8.0, 0.05 M EDTA) and the suspension was centrifuged at 3,000 rpm for 15 min at room temperature. The pellet was resuspended in 2 mL extraction buffer containing 200 µg RNase A. After incubating the suspended DNA at 65 °C for 20 min with 2 mL nuclear lysis buffer (0.2 M Tris-HCl pH 7.5, 0.005 M EDTA pH 8.0, 20 mg/mL CTAB, 800 µL 5% sarcosyl) it was mixed with an equal volume of chloroform: isoamyl alcohol (24:1), vortexed for 1 min, and centrifuged at 3,000 rpm for 5 min at room temperature. To precipitate the DNA the aqueous layer was mixed with an equal volume of isopropyl alcohol, washed with 70% ethanol, air dried, and dissolved in 400-700 µL 1X Tris/EDTA buffer (TE). DNA concentration was measured using Qubit Fluorometric Quantitation (Life Technologies).

Polymerase Chain Reaction (PCR) confirming flanking sequences and characterizing integration site

Primers were designed to confirm the junction regions between the insert and genomic DNA. To characterize the integration site, primers were designed using the Snowden flanking genomic sequences obtained for each side of the insert. A forward primer from the left flank was combined with a reverse primer from the right flank to amplify each integration site (Table 14-1). PCR reaction conditions are described in Table 14-2.. PCR products were visualized following electrophoresis on a 1.0% agarose gel, then extracted using a QIAquick Gel Extraction Kit from Qiagen. PCR products were ligated into pGEM-T Easy Vector (Promega) and transformed into TOP10 *E. coli* cells for plasmid purification and Sanger sequencing.

Table 14-1. PCR primers

Assay	Amplicon size	Primers (5' to 3')
pSIM1278 left flank	1353 bp	CATACATGCTAGTAGCCTAGTA
		CGGGTTATCGGTTCTTAACG
pSIM1278 right flank	1241 bp	ACCGAGTTGGACTACGGTCA
		AAGACCCACAAATGCAGCTT
pSIM1278 insertion site	1443 bp	TCAATGAGAGTCTAAGTCCGTG
		CCCATCTACGGACCACAAAC
pSIM1678 left flank	1469 bp	AGGTAAGATACGTTTACCTCTA
		AGACTCAATTTGTATGTTCCATGA
pSIM1678 right flank	1408 bp	ATCCATAGATGAGAACTTAATGGATAG
		TTCCTGTCAACTATTCTCAATCGATC
pSIM1678 insertion site	1285 bp	AGGTAAGATACGTTTACCTCTA
		ACATAAGTAAATTTACGCAACTTATA

Table 14-2. PCR reaction conditions

Temperature (^{0}C)	Time	Cycle Number
95	2 min	1
95	20 seconds	35
55	20 seconds	
72	3 min	
72	2 min	1

Sanger Sequencing

Plasmids purified using QIAprep Spin Miniprep Kit (Qiagen) were Sanger sequenced at the Sequetech Corporation using the BigDye® Terminator v3.1 Cycle Sequencing Kit and a PRISM 3730xl DNA Analyzer (Applied Biosystems).

Databases

Databases for the potato reference genome (Potato Genome Sequencing Consortium, 2011; Sharma et al., 2013) were utilized for sequence alignments and BLAST searches, obtained from the PGSC Data download site (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml), specifically "PGSC *S. tuberosum* group Phureja DM1-3" Pseudomolecules (v4.03) and Transcripts (v3.4).

Illumina Library Preparation, Capture, and Sequencing

DNA fragment libraries were prepared for Illumina sequencing using the Illumina Nextera Mate Pair Library Preparation Kit (cat. FC-132-1001, version 15035205_d) applying the gel-free option and multiplex adapters. MyBaits capture probes of 80 nucleotide lengths were synthesized every 20 nucleotides across the plasmids pSIM1278 or pSIM1678, including backbone. Mate pair DNA fragments were sequence captured using MyBaits In-Solution Sequence Capture for High-Throughput Targeted Sequencing Kit following the manufacturer's protocol (MYcroarray, v2). Samples were sequenced on a NextSeq 500 (Illumina, performed at the University of Oregon) to obtain paired-end 150 nt reads. Sequences were filtered for quality, demultiplexed, and adapters trimmed using the manufacturer's recommendations (Illumina Inc. (2012); bcl2fastq v2.16).

Illumina Sequence Analysis

Illumina sequencing data was imported into CLC Genomics Workbench (Qiagen, v12.0) in reverse, forward orientation with a pair distance of 2000-9000 bp. After mapping reads to the genome and plasmid sequences, most mapped in the correct mate pair (reverse, forward) orientation with an average pair distance of approximately 4.5 kb across the target sequence, in agreement with the average fragment length determined by Bioanalyzer DNA12000 assay (Agilent) after tagmentation.

To identify insertion loci using NGS data first Burrows-Wheeler Aligner (BWA) alignments were generated (Li and Durbin, 2009), then used with custom junction finding scripts. Junction finding analysis collects sequence read pairs (represented by red lines, with gap between as light grey) associated with inserted T-DNA (Figure 14-1). These programs search alignment files generated by BWA and extract all read pairs in which the reads align >1.5 kb away from the other. The alignment positions of the read pairs are retained and the number of reads in bins across the T-DNA and genome are counted. T-DNA bins were 1 kb in size while genomic bins were 20 kb each. To identify possible insertion sites, minimum reads per junction bin were required to be 20 or greater, with a minimum 3-fold enrichment over other libraries in the analysis (such as the conventional control, and other cultivars with similar insertion cassettes). For visualization of the junction loci, Illumina reads were mapped with CLC Genomics Workbench requiring 85% identity over 50% of the read.

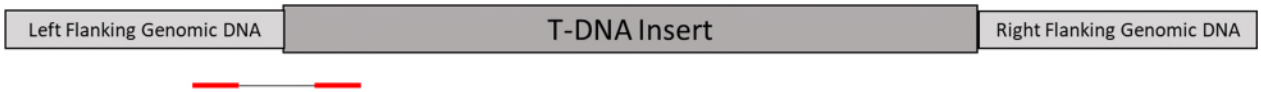

Figure 14-1. Junction Finding Method using Illumina Data
Sequence pairs are collected where one read aligns to the T-DNA and the other read aligns to the reference genome. Loci with >20 reads unique to a given transformation event indicate a T-DNA insertion. Reads mapping to additional loci can arise from potato-derived DNA in the T-DNA cassette, flanking sequence that is repetitive, and insertion sites. Loci identified by junction finding scripts are inspected. Junctions are confirmed with PCR and Sanger sequencing.

Read pairs with one read mapping to the T-DNA cassette of either pSIM1278 or pSIM1678 and the other read mapping to a locus in the genome may not be uniquely associated with insertion sites due to the presence of potato DNA in the T-DNA cassettes. To distinguish between these two possibilities, reads mapped at each potential insertion site were visually inspected. Putative junction loci that were enriched in event Z6 with comparison to Snowden and two other similarly transformed events, Y9 and X17, were inspected. False putative junction loci can arise from capture probes with identity to repetitive sequences or from repetitive insertion sites, as well as from endogenous loci with potato-derived T-DNA elements. For this reason, analysis of enriched junction loci by comparison to wild type controls AND independently transformed lines is essential to reduce noise. Potential junction loci arising from a single amplicon or loci that are shared between Snowden and Z6 were excluded after visual inspection.

After flanking sequences were confirmed by PCR and Sanger sequencing, and the structure confirmed by Southern blotting, the insert and flanking sequences were assembled. Illumina mate pairs were mapped to the assembled sequences with CLC Genomics Workbench, requiring 85% identity over 50% of the read, for confirmation by deep coverage of properly paired reads.

Results showing next generation sequence alignments display reads by orientation, with forward reads (green), reverse reads (red), and pairs (light and dark blue). Blue read pairs indicate pairing has been detected taking into account the reverse/forward orientation associated with mate pair type libraries, and light and dark blue indicate the orientation of the random fragment.

To detect possible vector backbone in the potato genome, reads mapping with low stringency (85% identity over 50% of the read) to the binary vector backbone were extracted. These reads were re-mapped, requiring 99.5% identity over 90% of the read, and the results were inspected. Any reads mapping to the backbone were further analyzed to see whether the mate pair maps to the potato genome or if they might be derived from common laboratory high copy plasmid DNA.

RNA Isolation and Northern Blot

RNA was extracted from tubers using either Plant RNA Reagent (Invitrogen™, ThermoFisher Scientific, Waltham, MA) or TriPure Isolation Reagent (RocheTM) and from roots, leaves, flowers, and stems using Trizol Reagent (Invitrogen™). The concentration of isolated RNA was measured using a Qubit 2.0 fluorometer (Invitrogen™) and RNA quality was evaluated by electrophoresis on 1% agarose gels in 200 mM MOPS buffer containing 50 mM NaOAc and 20 mM EDTA (pH 7.0) for 30-60 min at 90 V. RNA was denatured by heating at 65 °C for 10 min followed by a 5 min incubation on ice. RNA samples were

electrophoresed on 1% agarose gels containing 0.1-0.25 µg/mL ethidium bromide and 2% formaldehyde. Gels were run at 80-85 volts for 2-3 h and imaged using an AlphaImager HP instrument (ProteinSimple, San Jose, CA). The gels were washed twice in 10X Saline Sodium Citrate (SSC) for 15 min to remove formaldehyde. RNA was transferred to a nylon membrane (Roche, Indianapolis, IN) by capillary transfer in 10X SSC buffer for 16-18 h and stabilized by UV cross-linking (UVP, Upland, CA). Transferred membranes were stored at 4 °C until probed.

15.0 Appendix C: pSIM1278 Insert Sequence

pSIM1278 T-DNA Insert in V11 including 1018 nt of left flanking sequence (lowercase), T-DNA insert (uppercase), and 1121 nt right flanking sequence (lowercase). Primer regions are in yellow.

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16.0 Appendix D: pSIM1678 Insert Sequence

pSIM1678 T-DNA Insert in Z6 including 1111 nt of left flanking sequence (lowercase), T-DNA insertion (uppercase), and 1189 nt of right flanking sequence (lowercase). Underlined sequence indicates 957bp genomic DNA duplicated during insertion. Primer regions are in yellow.

aggtaagatacgtttacctctaataagaaaacacattttaatttcaacaacaataatatatctagagataatttcgcaaatgagtttgaaagggtgtgg
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