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STUDY TITLE

Z6 Insert Characterization by Southern Blotting

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[CCI]

Certification Page

I, the undersigned, declare that, to the best of my knowledge, this report provides an accurate evaluation of data in this study

Signed	5/17/2019
[personal information redacted]	Date
Molecular Senior Scientist	

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SUMMARY

Objectives: The objectives of this study were to determine the number of insertion sites and characterize the molecular structures of the pSIM1278 and pSIM1678 inserts in the Z6 genome.

Methods: Southern blot analysis was conducted on genomic DNA extracted from Z6 leaves. Genomic DNA from Snowden and V11 leaves were included as controls. The DNA samples were digested, separated by electrophoresis on agarose gels, transferred to nylon membranes by capillary transfer, and hybridized with multiple DNA probes for insert characterization.

A set of overlapping probes was designed to span the entire T-DNA region of each insert. These probe sets were used to determine the number of inserts associated with each transformation. Additionally, two sets of four probes each were designed to hybridize with elements within the T-DNA (AGP, ASN, GBS, R1 for the pSIM1278 insert and AGP, GBS, INV, VNT for the pSIM1678 insert). These probe sets were used in combination with multiple restriction enzyme digests to determine the structure of the pSIM1278 and pSIM1678 inserts.

The probes were PCR-amplified, DIG-labeled, and separately hybridized to the prepared nylon membranes. V11 and Snowden DNA spiked with a plasmid containing pSIM1278 (1278) or pSIM1678 (1678) T-DNA served as positive controls. Plasmids were spiked into Snowden DNA at a target concentration of one copy per genome equivalent to establish sensitivity of the probes. Snowden served as a negative control.

Droplet digital PCR was used to further confirm the number of insertion sites of the pSIM1278 and pSIM1678 T-DNA in Z6.

Results: Digesting genomic DNA with MfeI restriction enzyme produced a distinct fragment associated with each insert. Hybridization of the digested Z6 genome with specific probes detected one fragment associated with pSIM1278 and one fragment associated with pSIM1678. Droplet digital PCR confirmed the locus number and absolute copy number of pSIM1278 T-DNA and pSIM1678 T-DNA in Z6. These data showed that the event contains a single insert from pSIM1278 and a single insert from pSIM1678.

Southern blots also showed that the pSIM1278 insert consisted of a nearly full-length pSIM1278 T-DNA and the pSIM1678 insert consisted of a nearly full-length pSIM1678 T-DNA.

INTRODUCTION

Event Z6 was developed by transforming Snowden plants with pSIM1278 and then retransforming with pSIM1678. The T-DNA in these plasmids contain cassettes designed to reduce expression of asparagine synthetase, polyphenol oxidase, water dikinase, phosphorylase L, and vacuolar invertase genes in tubers using RNA interference. Reduced expression of these enzymes improves the tuber quality of Z6 for food processing.

The pSIM1678 construct also contains a cassette for expressing the late blight resistance gene, *Rpi-vnt1*, from the wild potato species, *Solanum venturii* (Foster et al., 2009). The *Rpi-vnt1* cassette expresses the full-length coding sequence regulated by its native promoter and terminator.

In this study the numbers and structures of the inserts in Z6 from pSIM1278 and pSIM1678 were analyzed by Southern blot.

Study Objectives

The objectives of this study were to:

1. Determine the number of inserts from pSIM1278 and pSIM1678; and
2. Determine the molecular structure of the pSIM1278 and pSIM1678 inserts.

Study Dates
June 2018 to March 2019

Key Study Personnel
[personal information redacted]

MATERIALS AND 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

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.

Droplet Digital PCR for Insert Detection

Droplet digital PCR (ddPCR) is a quantitative endpoint assay for measuring copy numbers of amplicons in a sample. This was applied to Z6 to assess the inserts from pSIM1278 and pSIM1678. Each droplet is an individual reaction that is cycled to the end of the PCR reaction. The fluorescent signal for each sample is read to determine which droplets are positive for the amplicons. The reliability of ddPCR-based quantification depends upon the number of reactions analyzed and the input concentration of DNA. Some droplets contain no template while others contain one or more template copies. Pinheiro et al. (2012) determined that there is uncertainty in calculating copy number up to about 5%. Expanded

uncertainty increases to 10% when fewer than 1,000 droplets are generated. Therefore, reactions including fewer than 1,000 droplets were excluded from our analysis.

Z6 genomic DNA was analyzed for locus number and absolute copy number using seven sets of primers Table 1. Three primer sets identified junction regions in the pSIM1278 construct (Figure 1, amplicons 1-3). Four primer sets measured junction regions in the pSIM1678 construct (Figure 1, amplicons 4-7). Each primer set was multiplexed with primers that amplify a region of the reference gene, vacuolar invertase, which is present in the potato reference genome (PGSC_DM_v4.03) as a single copy per haploid genome (Potato Genome Sequencing Consortium, 2011).

Table 1. Primer Sets and Probes Used for ddPCR Analysis of Z6 Insert Locus and Copy Number

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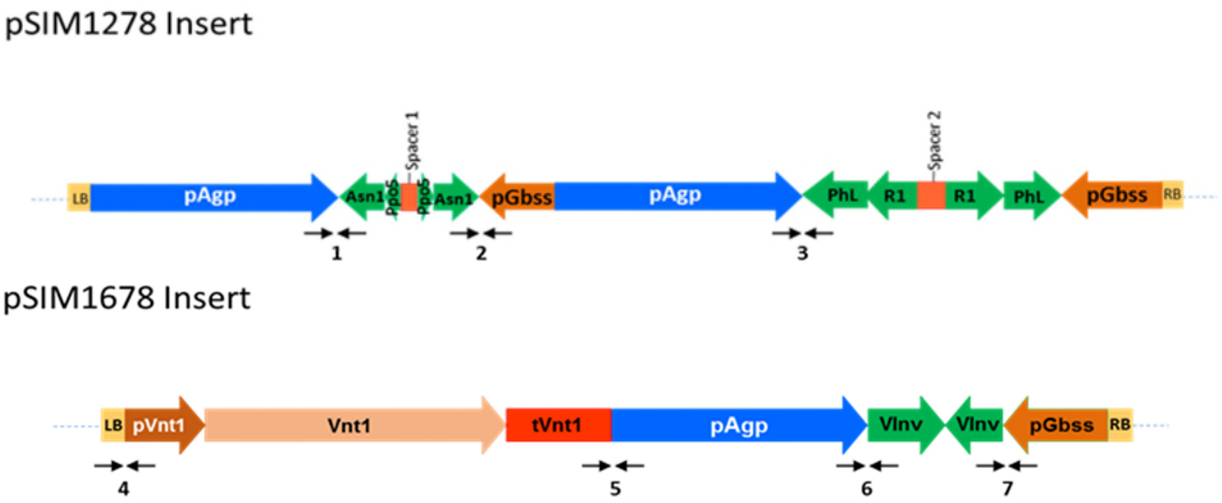

Figure 1. Structure of the Inserts from pSIM1278 and pSIM1678 in Z6 and Regions of the pSIM1278 and pSIM1678 T-DNA Quantitated by ddPCR

The insert from pSIM1278 contains a single copy of the nearly full-length T-DNA consisting of Asn1/Ppo5 and PhL/R1 cassettes flanked by converging Agp and Gbss promoters. The insert from pSIM1678 contains a single copy of the nearly full-length T-DNA consisting of an Rpi-vnt1 expression cassette with the native promoter and terminator sequences, and a VInv down-regulation cassette flanked by converging Agp and Gbss promoters. The left border (LB) and right border (RB) regions contain small deletions compared to the T-DNA, as expected with *Agrobacterium* transformations.

For each reaction, primer pairs (900 nM) were mixed with the ddPCR Supermix for Probes (no dUTP) from BioRad used at 1X for each 20 µL reaction, the corresponding probe (250 nM), and 15 ng of genomic DNA. For absolute copy number determination, restriction enzyme HphI (2-5 units) was added to each 20 µL reaction. Optimized PCR conditions are outlined in Table 2. Copy number (CN) was calculated using the [CCI] software which adjusts for varying template amounts and droplet numbers. The software applies Poisson statistics to calculate 95% confidence intervals, shown here as error bars. Copy number is defined as:

$CN = A/B * N_B$;
A = concentration of target amplicon;
B = concentration of reference gene; and
N_B= number of copies of reference loci in the genome (single per haploid, 4 for tetraploid).

Table 2. PCR Thermal Cycling Conditions

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RESULTS

The data indicate that the inserts from pSIM1278 and pSIM1678 each integrated at a separate, single locus in the Z6 genome. Both consisted of a nearly full-length T-DNA (Figure 1).

Copy Number of Inserts 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 (Figure 2 for 1278 probe set; Figure 4 for 1678 probe set). 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 insertion event. Only a single band is expected for any transformant containing a single insert from pSIM1278. For pSIM1678, MfeI cut once within the T-DNA. This means that one or two distinct bands are expected, 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.

<u>Single Insertion of pSIM1278 T-DNA in the Z6 Genome</u>

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). 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 2). All seven probes identified a 7.6 kb pSIM1678 digestion fragment unique to Z6 (blue arrow, Figure 3). 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). These data were consistent with the characterization of the pSIM1678 insert.

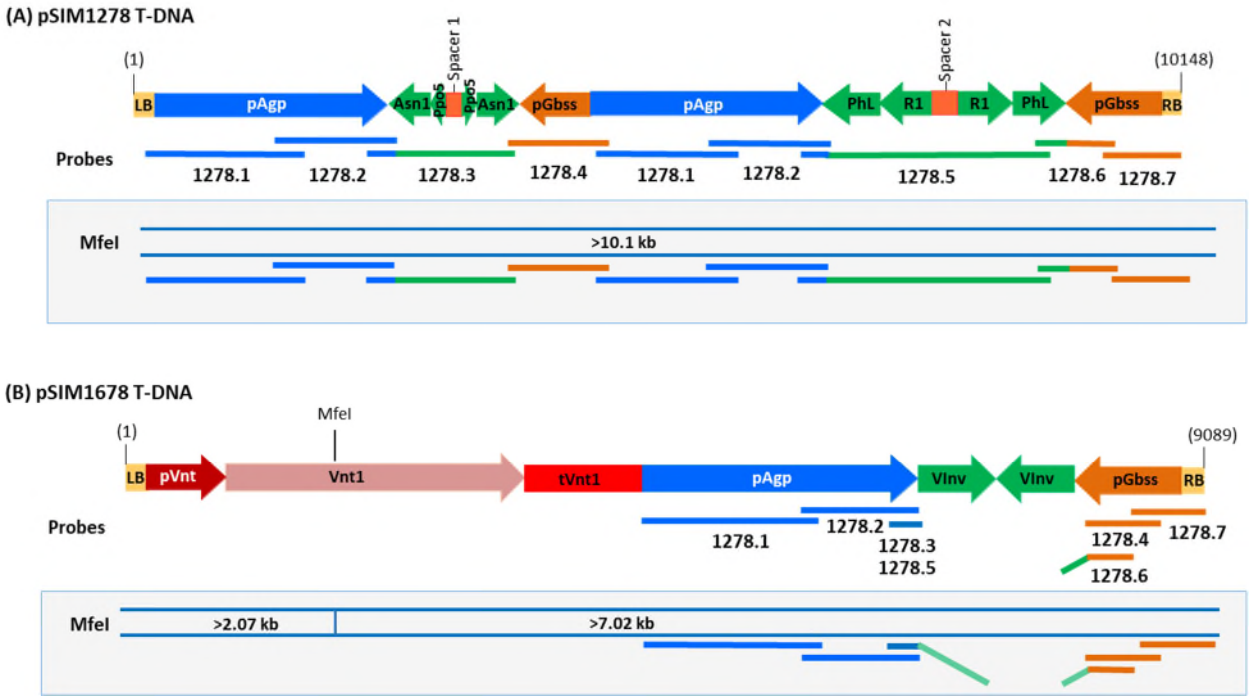


Figure 2. Binding Sites for 1278 Probe Set on pSIM1278 and pSIM1678 T-DNA
(A) Seven probes hybridized across the pSIM1278 T-DNA. Many hybridized to more than one site within the T-DNA as there were two copies of Agp and Gbss promoter elements. The 1278.3, 1278.5 and 1278.6 probes are indicated using 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.

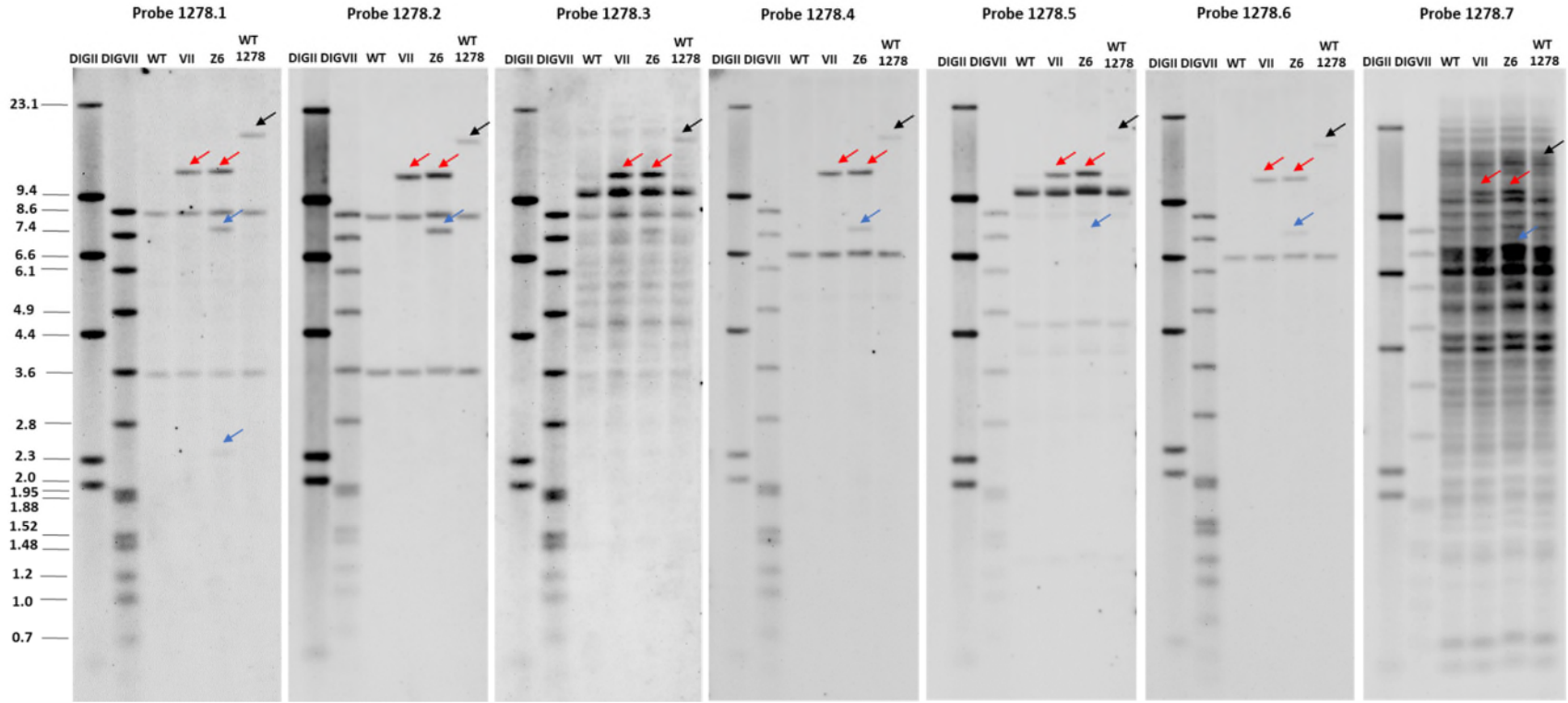


Figure 3. pSIM1278 and pSIM1678 Copy Number in Z6 Using pSIM1278 Probes

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 kilobases (kb) at the left of the first gel.

Single Insertion of pSIM1678 T-DNA in Z6 Genome

Transformation with pSIM1678 resulted in a single insert in the Snowden genome. The MfeI digested fragments from Z6, V11, and WT 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 4). Hybridization with these four probes is 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, is 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 5).

No bands specific to pSIM1278 or pSIM1678 were identified in the WT 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, whereas blue arrows denote the pSIM1678 insert (Figure 5).

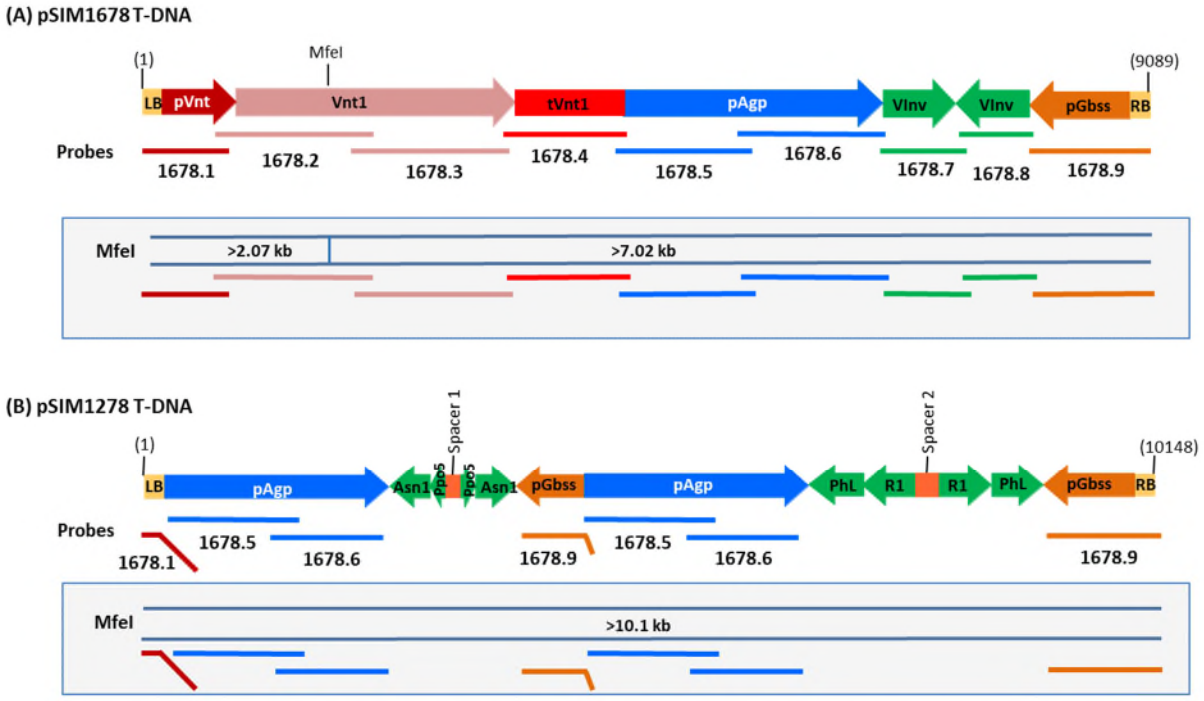


Figure 4. Binding Sites for 1678 Probe Set on pSIM1278 and pSIM1678 T-DNA
A) Nine probes hybridized across the pSIM1278 T-DNA. (B) Hybridization sites for the 1678 probe set on the pSIM1278 T-DNA. Probes 1678.1, 1678.5, 1678.6 and 1678.9 also detected elements in the pSIM1278 insert. Expected digestion products and probe binding sites are shown in boxes below the T-DNA maps.

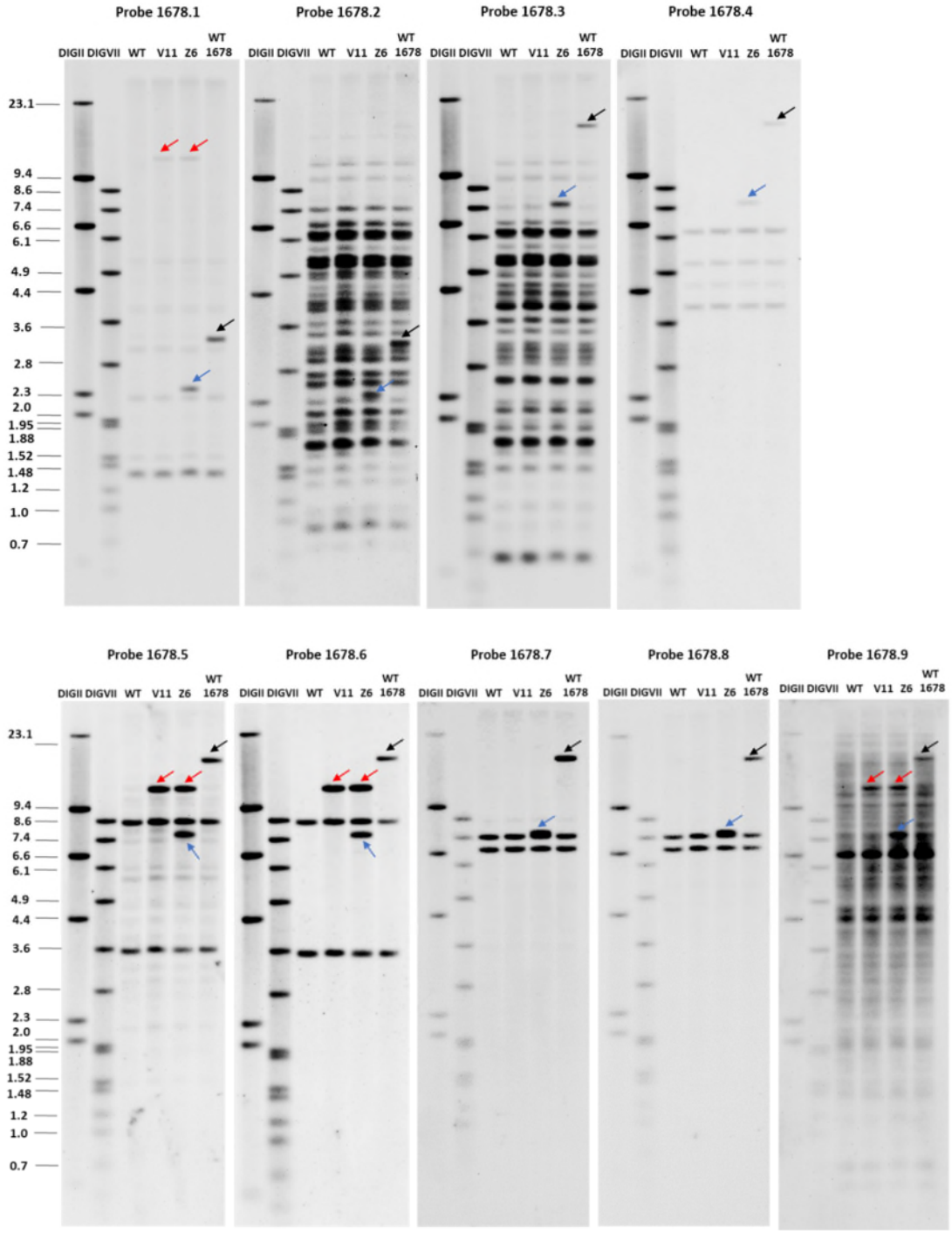


Figure 5. pSIM1278 and pSIM1678 Copy Number in Z6 Using pSIM1678 Probes
Southern blots of MfeI digested genomic DNA isolated from Z6, V11, and Snowden (WT) spiked with pSIM1678 plasmid DNA (WT p1678). Blots were hybridized with the indicated probe (1678.1–1678.9). In addition to endogenous bands common 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 kilobases (kb).

Collectively, all sixteen probes used to hybridize pSIM1278 or pSIM1678 sequences detected only a single insert associated with transformation in Z6.

Droplet Digital PCR Analysis Confirms a Single Copy of the pSIM1278 and pSIM1678 Insert Sites in Z6

The number of the T-DNA insertion sites was confirmed by droplet digital PCR analysis. Primer and probe sets were designed to amplify three unique junction regions (amplicons labeled 1-3) in pSIM1278 and four unique junction regions (amplicons labeled 4-7) in pSIM1678 (Figure 1, Figure 6).

Locus number analysis measured amplicons in undigested Z6 genomic DNA and provided the number of insertion sites containing T-DNA in the genome. Absolute copy number analysis measured the number of each amplicon present in Z6 genomic DNA digested with restriction enzyme ,HphI. This provided the number of copies of each amplicon in Z6.

Locus number and absolute copy number results (Figure 6) indicate that pSIM1278 (amplicons 1-3) and pSIM1678 (amplicon 4-7) inserts are each present at a single locus and as one copy in Z6.

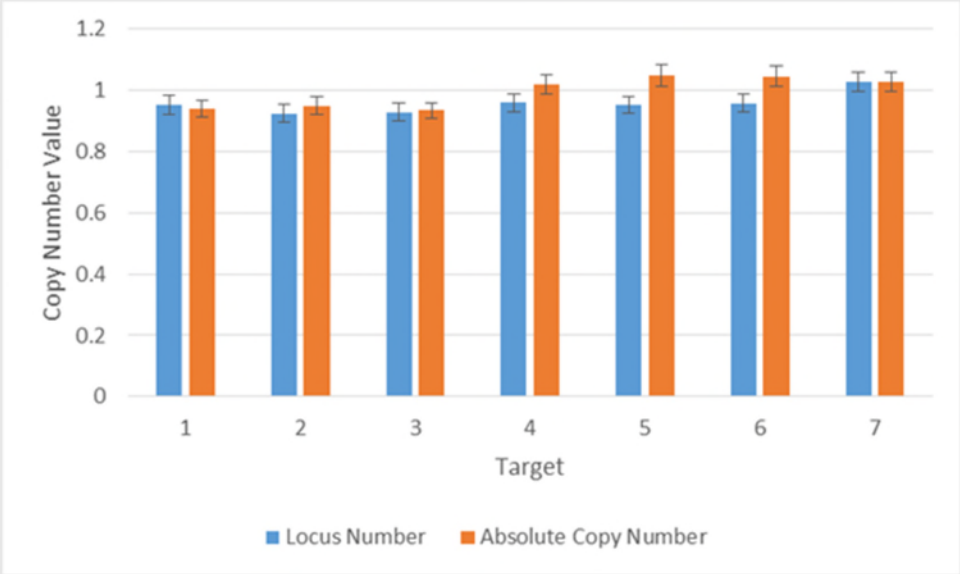


Figure 6. ddPCR Identified a Single Copy of the pSIM1278 and pSIM1678 Insertions
Amplicons 1-7 are present at a single locus (blue bars) and as a single copy (orange bars) in Z6. Error bars indicate 95% confidence intervals.

Structure of the Inserts in Z6

Z6 Insert from pSIM1278

The Southern analysis, shown above in Figure 3, was used to determine that the pSIM1278 insert in Z6 consists of a single, full-length 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, and a double digest with EcoRI and ScaI. The restriction sites, digestion products and sizes, and probe binding sites are shown in Figure 7

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 7). Internal fragments with expected sizes resulted from the restriction digest with EcoRV, HindIII, EcoRI/ScaI, or PacI/XbaI (Figure 7). 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 8, Figure 9). 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 10, Figure 11). 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 12). The 1.3 kb HindIII band and 3.8 kb and 0.8 kb EcoRI/ScaI bands were detected by the R1 probe (Figure 13). Both ASN and R1 probes are specific to pSIM1278 and did not hybridize to pSIM1678 sequences.

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 10). The 7.4 kb MfeI/XbaI junction band was also detected by the GBS probe (Figure 11). The junction bands provide support for the structure of the pSIM1278 insert as they overlap with internal fragments (Figure 7).

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 7. 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 observing how it ran on the gel, and 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 10, Figure 14) and all were consistent with their expected sizes (Figure 7). There were no unexpected bands that would suggest additional inserts of pSIM1278 T-DNA.

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

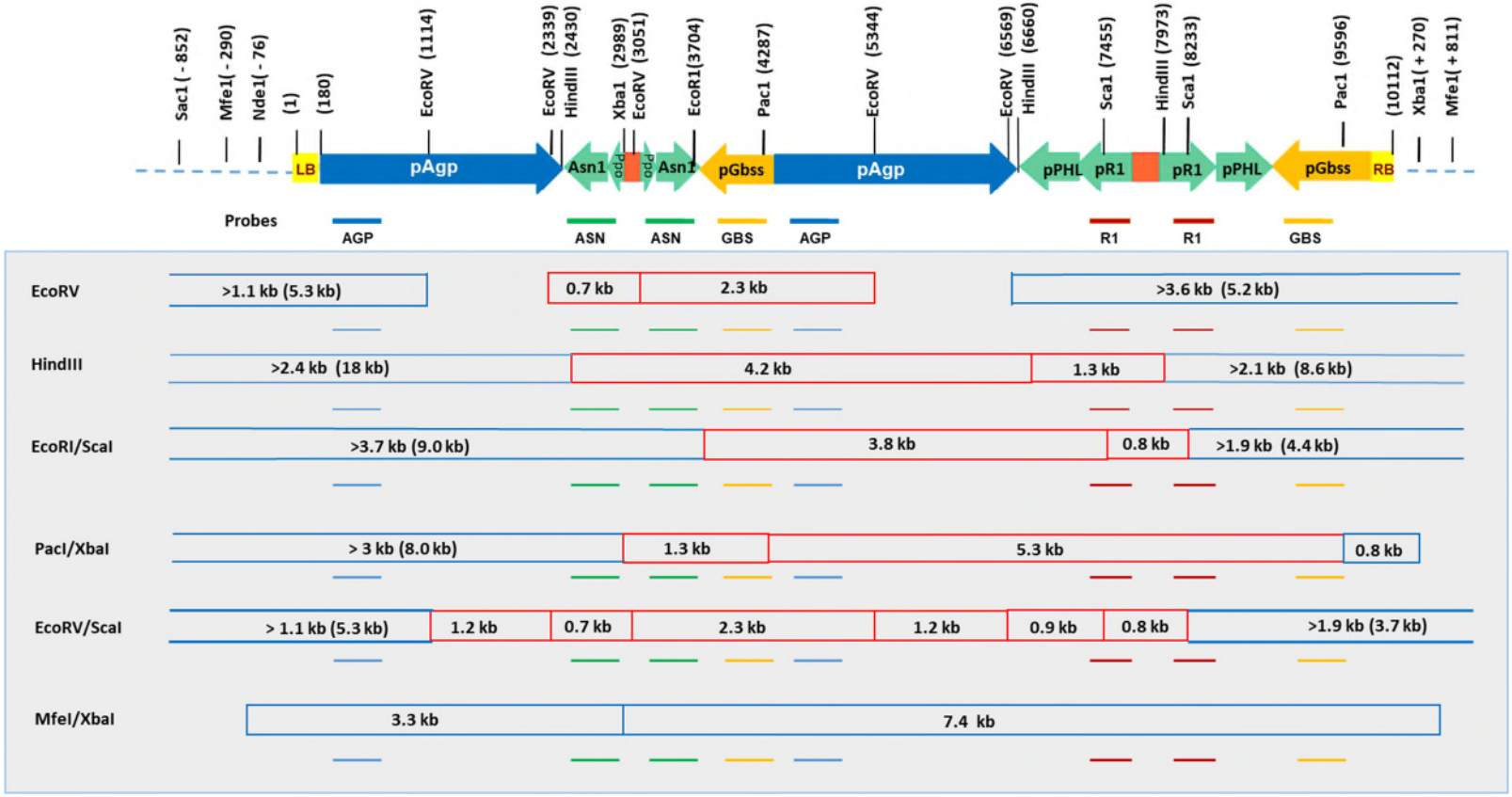


Figure 7. Structure of the pSIM1278 Insert in Z6 with Digestion Patterns and Probe Binding Sites

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).

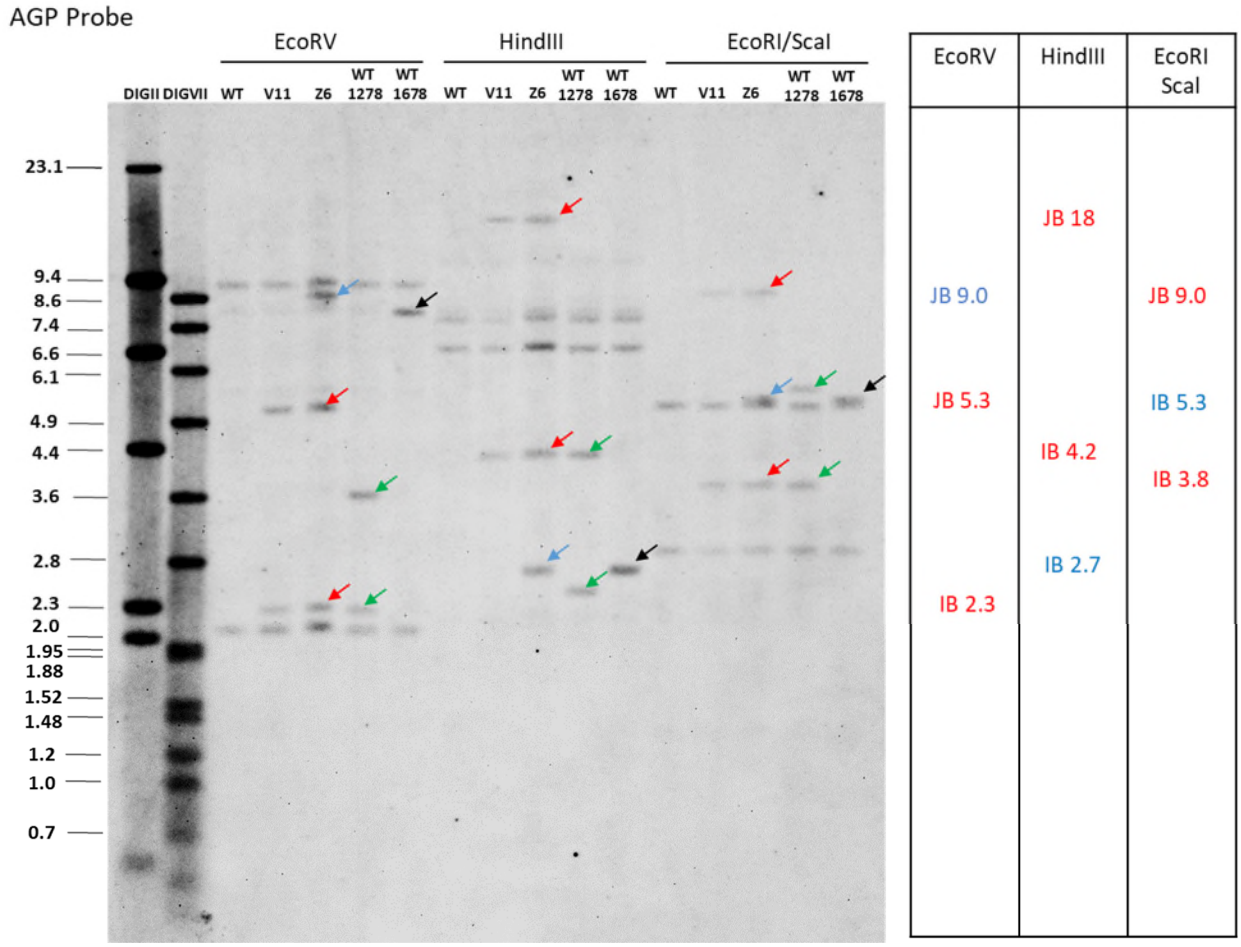
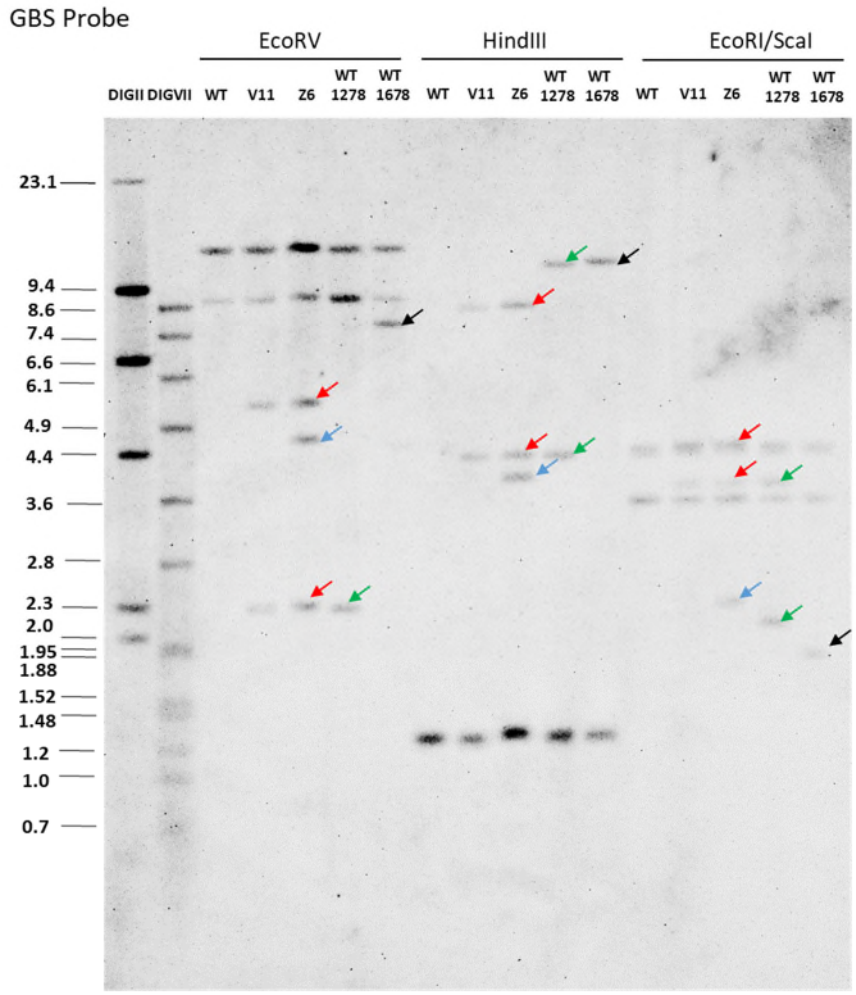


Figure 8. Southern Hybridization of Genomic DNA with AGP Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaII 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 any bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue) and 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).



EcoRV	HindIII	EcoRI ScaI
	JB 8.6	
JB 5.2		
JB 4.6	IB 4.2 JB 4.0	JB 4.4 IB 3.8
IB 2.3		JB 2.3

Figure 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 EcoRV, HindIII, and EcoRI/ScaII 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 any bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue) and 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

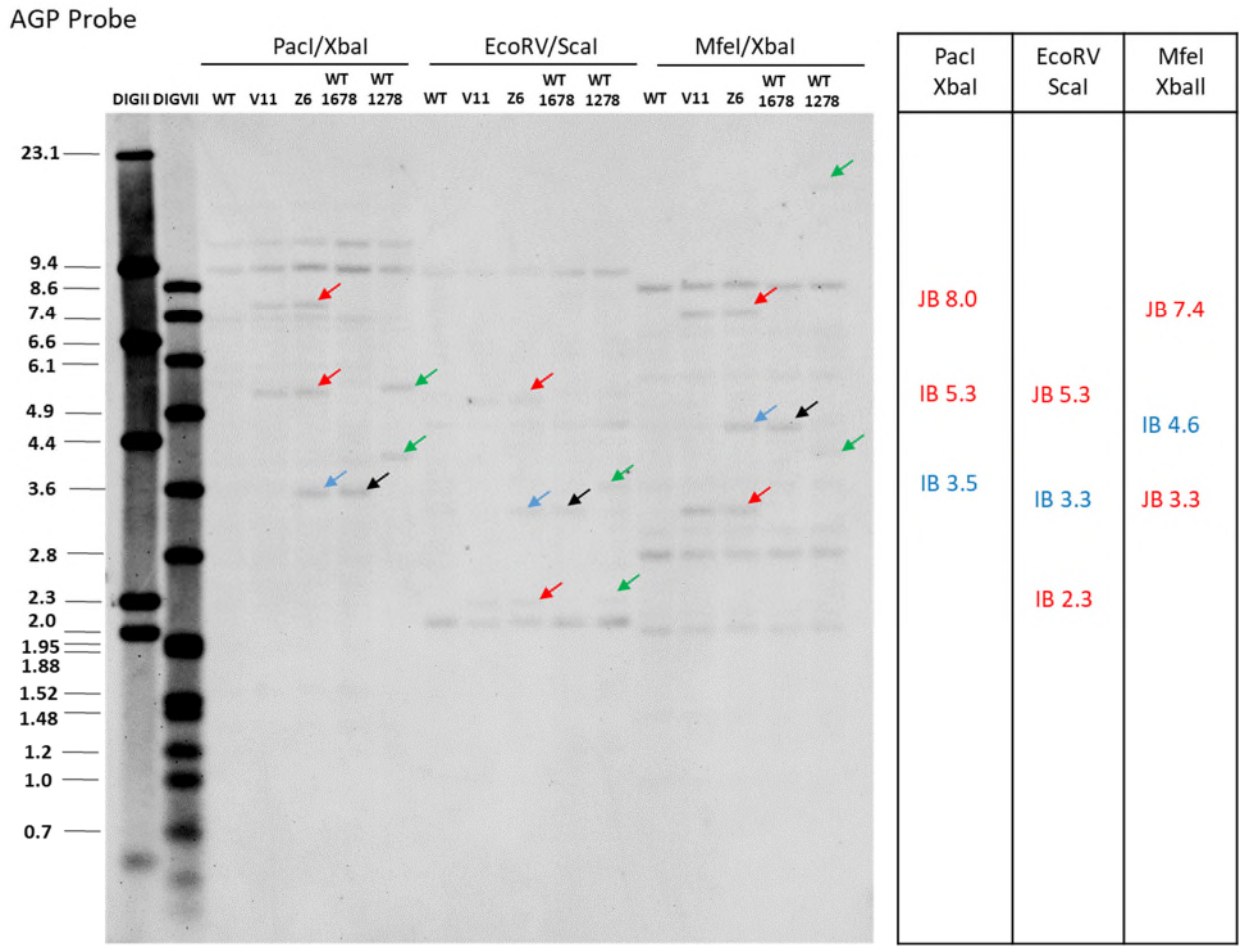


Figure 10. Southern Hybridization of Genomic DNA with AGP Probe
Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with PacI/XbaI, EcoRV/ScaI, and MfeI/XbaI and hybridized with the AGP probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue) and 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

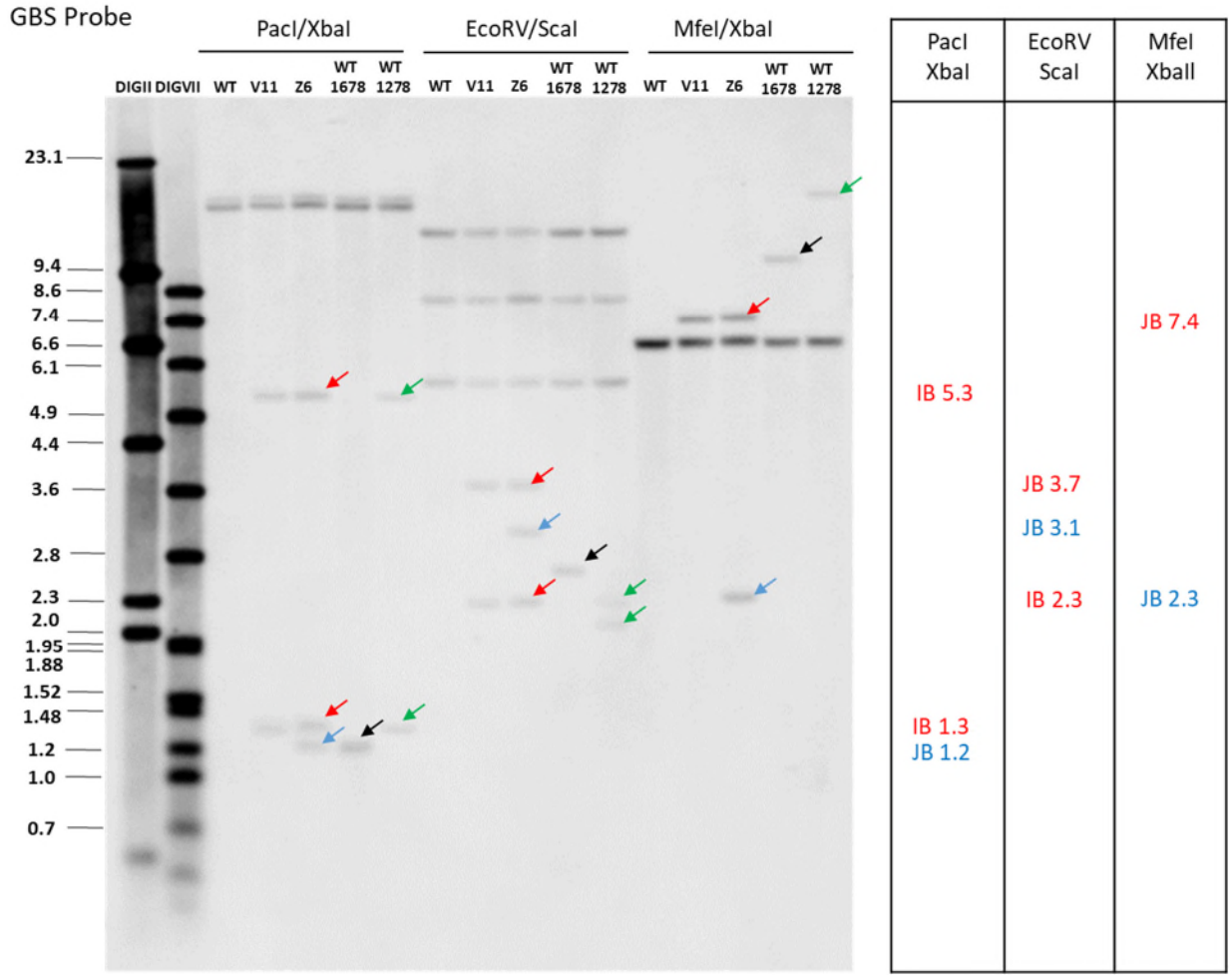
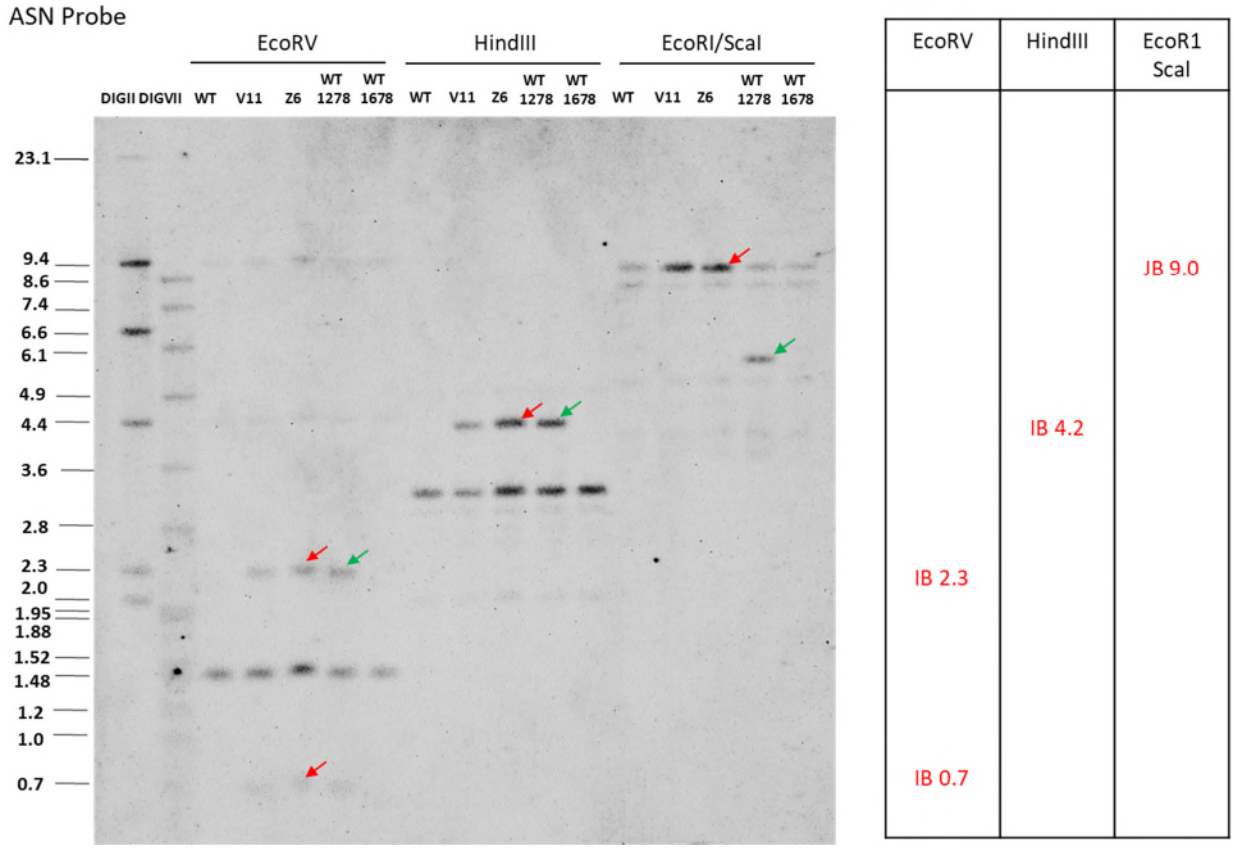
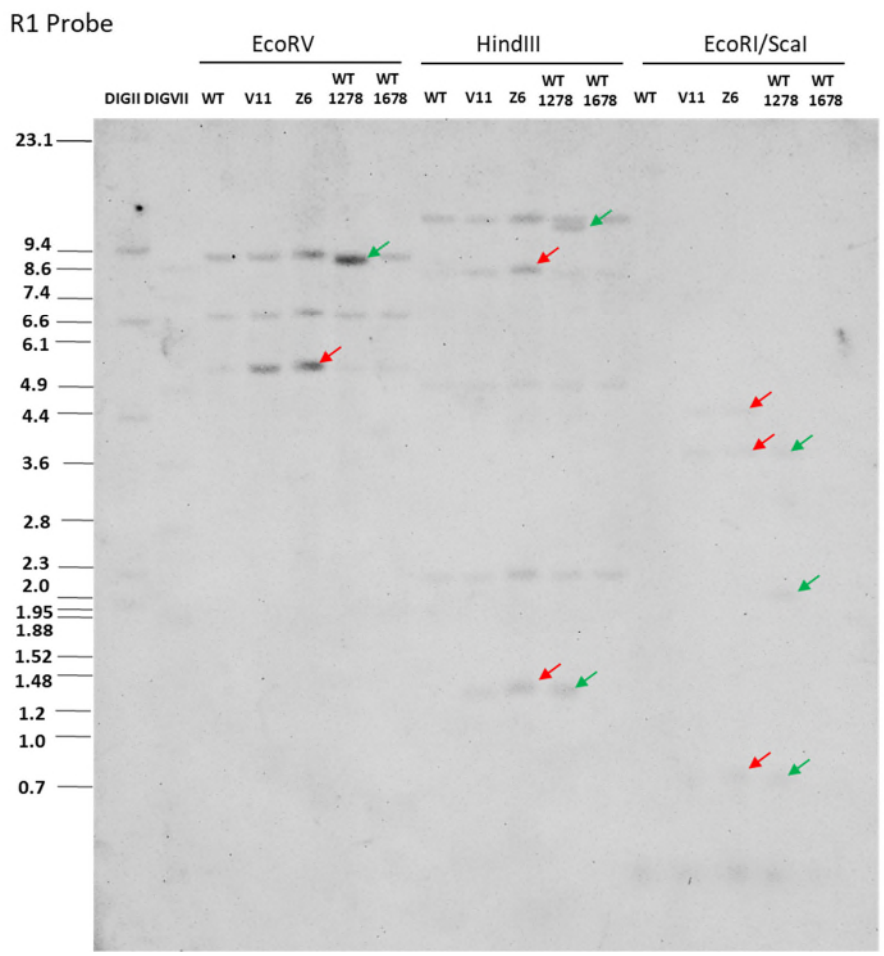


Figure 11. Southern Hybridization of Genomic DNA with GBS Probe
Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1278 or pSIM1678 plasmid DNA was digested with PacI/XbaI, EcoRV/ScaI, and MfeI/XbaI and hybridized with the GBS probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any bands associated with the pSIM1278 insert (red), the pSIM1678 insert (blue) and 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).



EcoRV	HindIII	EcoR1 Scal
		JB 9.0
	IB 4.2	
IB 2.3		
IB 0.7		

Figure 12. 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/ScaII 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 any 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).



EcoRV	HindIII	EcoR1 ScaI
JB 5.2	JB 8.6	JB 4.4
		IB 3.8
	IB 1.3	
		IB 0.8

Figure 13. 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/ScaII 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 any 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

Z6 Insert from pSIM1678

The Southern analysis using MfeI digestion shown above (Figure 5) 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 and digestion products, and probe binding sites are summarized in Figure 14. 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 WT 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 14). The Southern blots probed with AGP and GBS (Figure 8-11) 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 8, 10 and 15). The 2.7 kb HindIII and 3.3 kb EcoRV/ScaI fragments were detected by the AGP probe (Figure 8, Figure 10). The 1.2 kb PacI/XbaI fragment were also detected using the INV probe (Figure 15), which hybridizes to pSIM1678, but not pSIM1278.

Similar to the pSIM1278 insert, the junction regions for the pSIM1678 insert adjoin the plant genome to 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 14).

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 16) connect the left side of the pSIM1678 insert with the flanking region as depicted in Figure 14.

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 WT and V11 samples, by INV probe as expected (Figure 15, Figure 17). 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 15). 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 15, Figure 17) 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 Z6 consists of a single, nearly full-length T-DNA from pSIM1678.

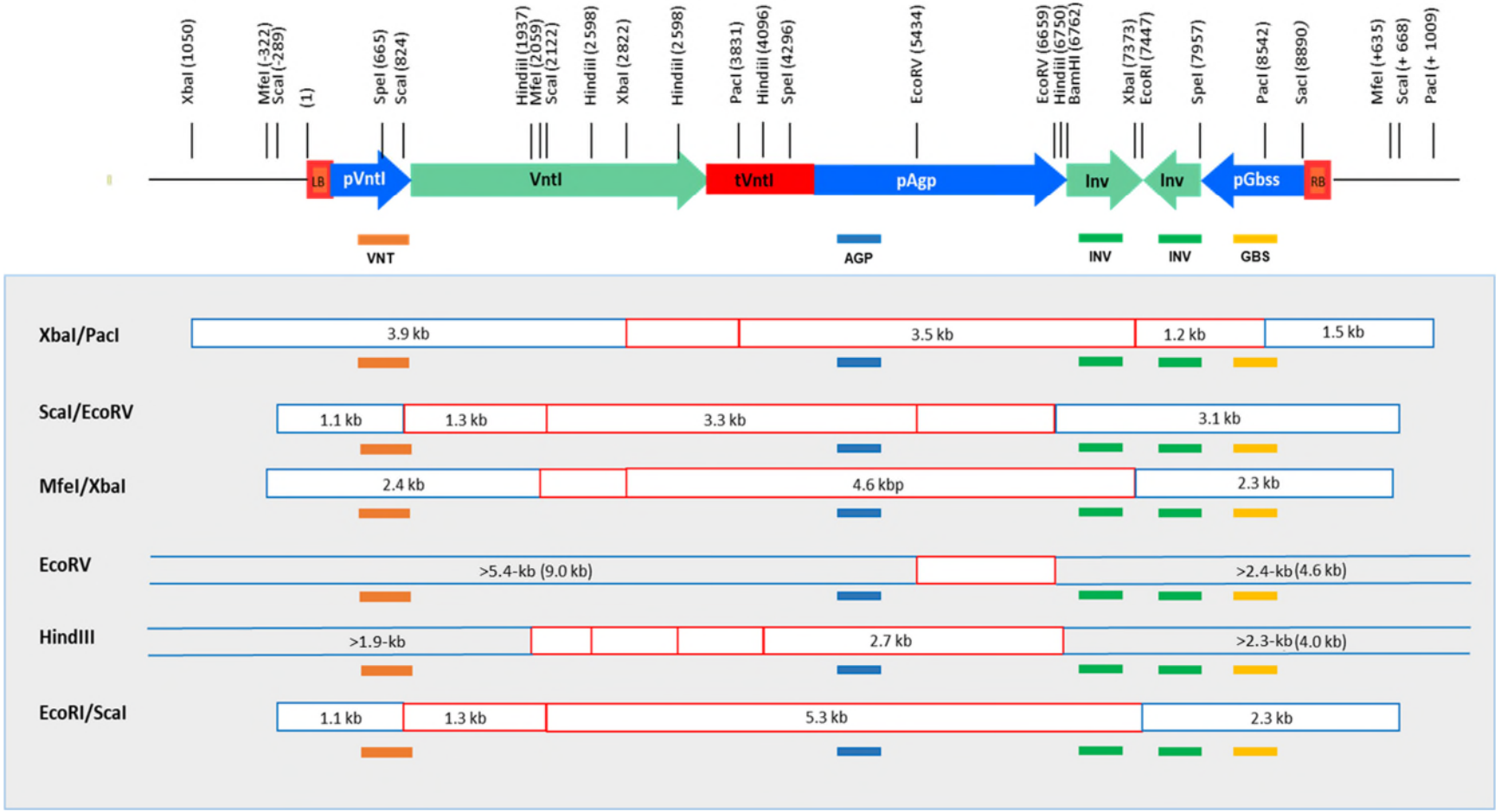


Figure 14. Structure of the pSIM1678 Insert in Z6 with Digestion Patterns and Probe Binding Sites

The figure represents the structure of the insert associated with the pSIM1678 construct, including designated restriction sites. The digestion pattern for selected enzymes is shown as colored boxes with the digest and fragment size indicated. The probes that are expected to detect each digestion product are indicated below the fragment with 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 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).

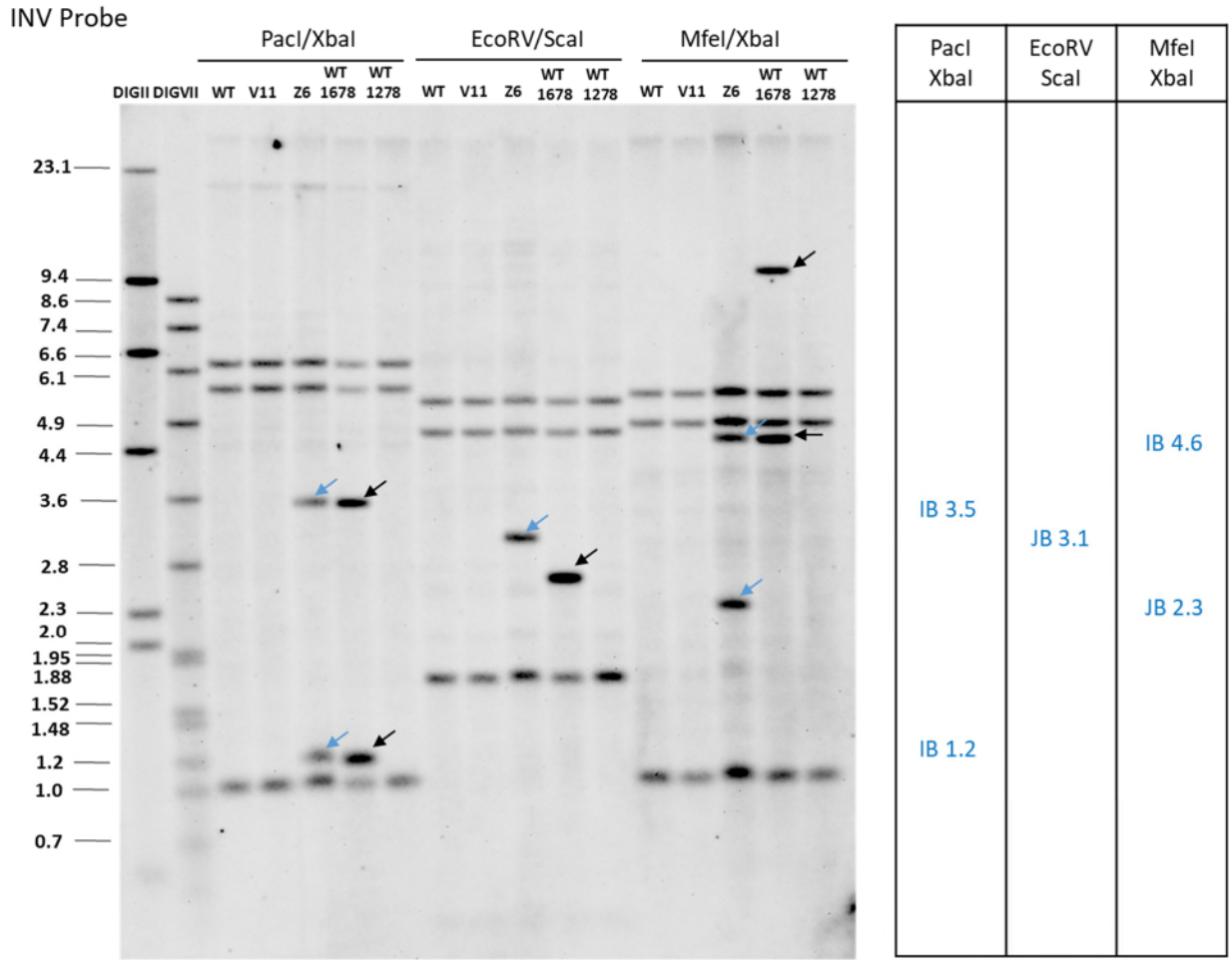


Figure 15. Southern Hybridization of Genomic DNA with INV Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278plasmid DNA was digested with PacI/XbaI, ScaI/EcoRV, and XbaI/MfeI 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 any 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

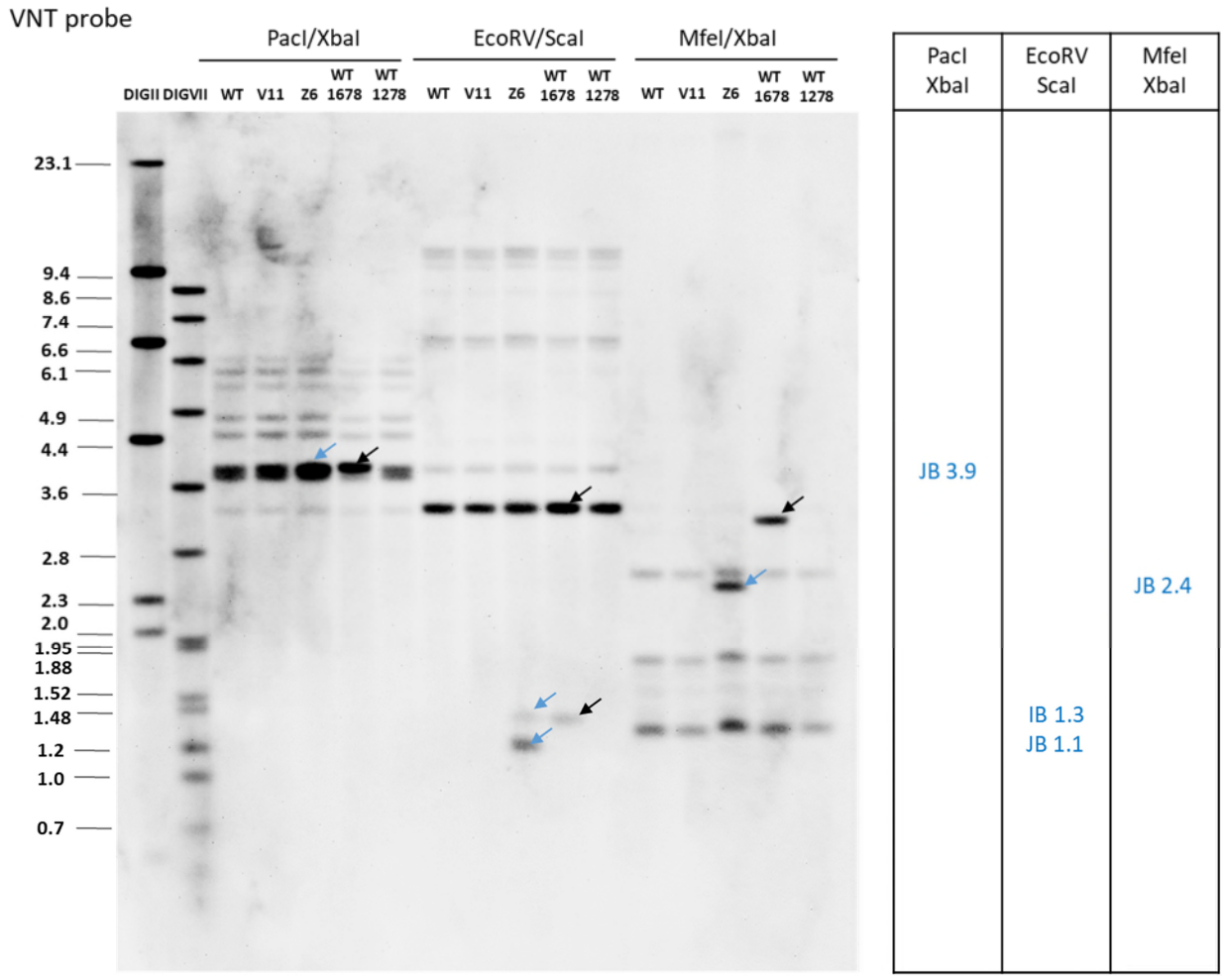


Figure 16. Southern Hybridization of Genomic DNA with VNT Probe

Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278plasmid DNA was digested with PacI/XbaI, ScaI/EcoRV, and XbaI/MfeI 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 any 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

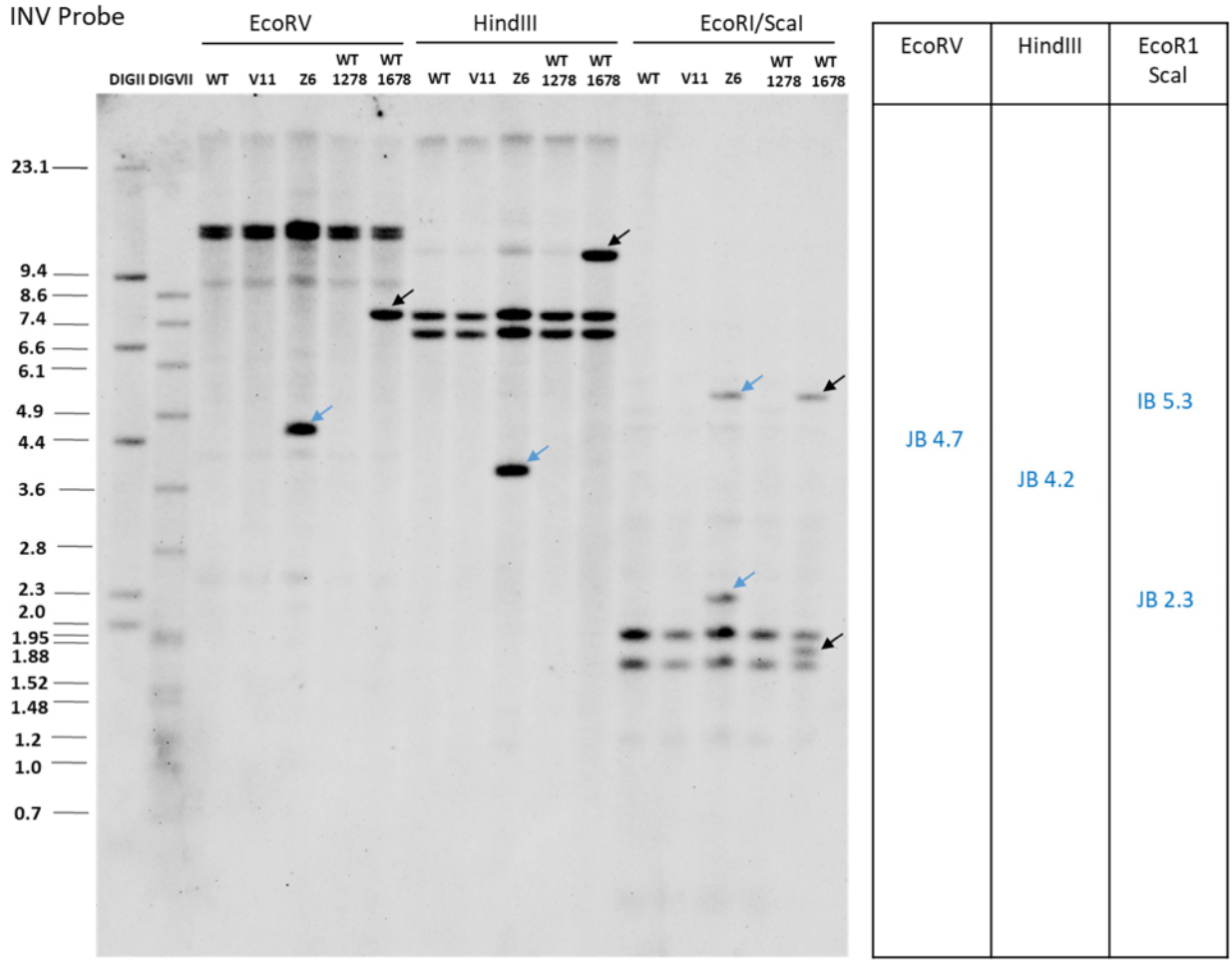


Figure 17. Southern Hybridization of Genomic DNA with INV Probe
Genomic DNA of Snowden (WT), V11, Z6, and WT spiked with pSIM1678 or pSIM1278plasmid DNA was digested with EcoRV, HindIII, and EcoRI/ScaI and hybridized with the INV probe. Size of the DigII and DigVII molecular weight markers are indicated adjacent to the blot image. Colored arrows were used to distinguish between any 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 coloring convention. (JB) junction bands, (IB) internal bands. All molecular weights are presented in kilobases (kb).

CONCLUSION

A combination of Southern blot and DNA sequencing analysis was used to show that Z6 contains single, nearly full-length inserts from both pSIM1278 and pSIM1678. The Southern blot data generated for the pSIM1278 insert in Z6 matched the previous Southern blot data generated for its V11 parent. The transformation of V11 with pSIM1678 resulted in a single T-DNA integration in Z6, which consists of a single *Rpi-vnt1* expression cassette and a single vacuolar invertase down-regulation cassette.

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