Personal information and commercial confidential information [CCI] have been redacted

STUDY TITLE

Stability of Inserts in Vegetatively Propagated Z6

AUTHORS

[personal information redacted]

REPORT DATE

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PERFORMING LABORATORIES

Simplot Plant Sciences, Boise, ID

STUDY NUMBER

[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	2/19/2019
[personal information redacted] Senior Scientist	Date

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SUMMARY

Objective: To evaluate the stability of the inserts in Z6 after multiple rounds of vegetative propagation.

Methods: Leaves from vegetative propagations of Z6 and Snowden were harvested for DNA isolation and Southern blot analysis. Snowden and Z6 G0 plants were grown in the greenhouse. G1, G2, and G3 plants were also grown in the greenhouse from previously propagated G0, G1, and G2 seed. The structure of the inserts was assessed by Southern blot following digestion of genomic DNA (4 µg) using different restriction enzymes, EcoRV and MfeI/XbaI, and hybridization with six probes. Two probes (ASN and R1) were unique to pSIM1278, two were unique to pSIM1678 (INV and VNT), and two probes (AGP and GBS) hybridized to both inserts due to shared sequence between the pSIM1278 and pSIM1678 T-DNA.

Results: Digestion of Z6 genomic DNA by EcoRV and MfeI/XbaI lead to an expected digestion pattern that was assessed by Southern blot using six probes that detected fragments associated with each insert. All four expected pSIM1278 fragments and both pSIM1678 fragments were detected by the appropriate probes following digestion with EcoRV. Similarly, the two expected pSIM1278 fragments and three pSIM1678 fragments were detected by the appropriate probes following digestion with MfeI/XbaI. Since T-DNA inserts contain potato DNA detectable by the probes, Snowden was included as a negative control.

The banding pattern was consistent between vegetatively propagated Z6 plants following digestion with multiple enzymes and assessment by six probes.

Conclusion: The same banding pattern was observed in all Southern blots for each of the vegetatively propagated Z6 plants. All plants exhibited the expected digestion pattern. The data in this study demonstrated the inserts from pSIM1278 and pSIM1678 were stable in vegetatively propagated Z6.

INTRODUCTION

The Snowden potato variety was transformed with pSIM1278 and pSIM1678 to produce Z6, which contains a single insert from each plasmid. Commercial potatoes are vegetatively propagated. Vegetative progeny do not undergo meiotic recombination and are genetically and phenotypically stable. Consequently, it was not applicable to evaluate insert stability by examining inheritance using Mendelian segregation analysis.

In this study, the stability of the inserts was assessed by Southern blot analysis of plants that had undergone multiple rounds of vegetative propagation. Restriction enzyme digests were used for the analysis. The results confirmed that the structure of the Z6 inserts was the same and the inserts were maintained stably in vegetatively propagated Z6 plants.

STUDY OBJECTIVES

The objective of this study was to confirm that the pSIM1278 and pSIM1678 inserts in Z6 remain stable during vegetative propagation.

STUDY DATES

11/2018 – 01/2019

KEY PERSONNEL

[personal information redacted]

MATERIALS AND METHODS

Plant Material

Snowden and Z6 G0 plants were grown in the greenhouse. G1, G2, and G3 plants were also grown in the greenhouse from previously propagated G0, G1, and G2 seed (Figure 1).

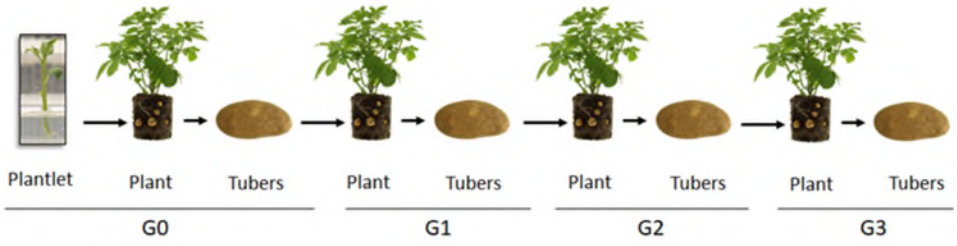

Figure 1. Vegetative Propagation of Potato Plants
Tissue culture plantlets were planted in soil to produce tubers, designated G0. G0 tubers were planted to produce G1 plants and tubers. G1 tubers were planted to produce G2 plants and tubers, and so on.

DNA Isolation

DNA was isolated from leaves of plants grown from plantlets (G0) and from G1-G3 plants. A 1.0 g sample 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. 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 and 20 mg/mL CTAB hexadecyl trimethyl ammonium bromide) and 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. 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). DNA quality was confirmed by running the DNA on a 0.8% agarose gel in 1X Tris/Acetate/EDTA (TAE) for 30-40 min at 80 volts.

DNA Restriction Digestion

A 4 µg sample of plant DNA was digested overnight in 400 µL final volume with at least 5 µL (10 units/µL) EcoRV or a combination of MfeI and XbaI restriction enzymes (Invitrogen) at 37 °C. Digested DNA was concentrated by ethanol precipitation (40 µL of 3M NaOAc, pH 5.3, and 1 mL ethanol) at -80 °C for 10 min and washed with 70% ethanol. Samples were normalized based upon the lowest concentration sample after precipitation to ensure equal loading.

Membrane Preparation and Transfer

Digested plant DNA was loaded on a 0.7% agarose gel containing 1X Tris/Acetate/EDTA (TAE) buffer with 3-5 µL ethidium bromide (10 mg/mL) and run at 35 volts for 18 h. The gel was photographed using a gel documentation system from Alpha Innotech (Santa Clara, California), then depurinated by submerging in 0.25 N HCl for 2 x 10 min. The gel was placed in denaturation solution (0.5 M NaOH / 1.5 M NaCl) for 2 x 15 min then neutralization solution (1.5 M NaCl and 0.5 M Tris-HCl pH 7.5) for 2 x 15 min on a shaker at room temperature. The gel was equilibrated with 10X SSC for 10 min. The transfer of DNA to the nylon membrane was carried out using 10X SSC according to a standard capillary transfer method.

DIG-Labeled Probe Preparation

The labeling of the PCR-derived probe used Hotmaster *Taq* enzyme and buffer (Fisher BioReagents) according to Roche's DIG labeling instructions. A standard 50 µL reaction consisted of 1x Hotmaster *Taq* Buffer, 10 µM each of forward and reverse primers (Table 1), 5 µL DIG-labeled dNTP (Roche), 10 ng plasmid template, and 0.75 µL Hotmaster *Taq* polymerase. The PCR amplification conditions were dependent on each DIG-labeled probe. PCR with regular dNTP instead of DIG labeled dNTP was used as control. Quality of the DIG-labeled probe was assessed by running a small amount of the probe on a 1% agarose DNA gel. The probe was denatured before use by incubating at 95 °C for 5 min followed by placing on ice for 2 min.

Table 1. Primer Sequences used for Probe Preparation

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Probe Hybridization

The cross-linked nylon membrane was prehybridized in 40 mL pre-warmed DIG Easy Hybridization solution (Roche) at 42 °C for 1-4 h in a bottle using a standard hybridization oven (Amerex Instruments Inc.) at 20-25 rpm. Hybridization was carried out by replacing the prehybridization buffer with fresh preheated prehybridization solution, containing 25-50 µL denatured DIG labeled probe. The membrane was incubated at 42 °C, 20-25 rpm for 16 h. The hybridization solution was stored at -20 °C and reused up to three times. The reused hybridization solution was heated at 68 °C for 10 min before use.

Detection

The hybridization solution was removed and replaced with 100 mL washing solution I (2X SSC/0.1% SDS). The membrane was washed twice in washing solution I for 10 min at room temperature. This low stringency buffer was poured off and preheated high stringency washing solution II (0.5X SSC/0.1% SDS, 60 °C) was added immediately. The membrane was washed twice in washing solution II at 68 °C for 20 min each at 25-30 rpm, followed by a brief rinse with 2X SSC to remove SDS. The membrane was rinsed with 150 mL 1X DIG Washing Solution (Roche) in a tray for 2 min and incubated in 1X Blocking solution (Roche) for 0.5-3 h on a low-speed shaker. The blocked membrane was incubated with DIG antibody solution (1:10,000 dilution of Anti-DIG-alkaline phosphate conjugate with 1X Blocking solution) for 30 min on a shaker at room temperature. The membrane was washed twice (15 min each) with 1X DIG Washing Solution (Roche) and equilibrated with 1X detection buffer. The detection reaction was carried out with 2 mL CDP-Star solution (1:100 diluted stock of CDP-Star with 1X detection buffer) for

5 min. The membrane was wrapped in a plastic film and developed on an Amersham Imager 600 (GE Healthcare Life Sciences).

RESULTS

The stability of the pSIM1278 and pSIM1678 inserts in Z6 was demonstrated by Southern blot analysis of plants from successive rounds of vegetatively propagated Z6 plants (G0 to G3). Stability was assessed by verifying that the structure of the inserts was consistent with the expected digestion patterns of both inserts and that the banding patterns were consistent between each of the vegetatively propagated plants. Probes from each of the inserts were used to assess the structure following digestion with different restriction enzymes (EcoRV and MfeI/XbaI combination). A schematic diagram of the expected restriction patterns, including fragment sizes and probe specificity, for each digest is provided (Figure 2).

The pSIM1278 insert was assessed using four probes AGP, GBS, ASN, and R1 that hybridized to genetic elements of the insert. Similarly, the pSIM1678 insert was assessed using four probes, AGP, GBS, VNT, and INV, that hybridized to elements of the insert. Each probe recognized one or more fragments following digestion with either EcoRV or MfeI/XbaI. The AGP and GBS probes hybridized to fragments associated with both inserts as each contains pAgp and pGbss elements. For clarity, bands associated with each insert are labeled in distinct colors in the accompanying Southern blots (blue, pSIM1278; green, pSIM1678). Bands shared between Snowden and Z6 samples were not labeled as they represent endogenous DNA fragments and are not related to the pSIM1278 or pSIM1678 inserts.

Digestion of the Z6 pSIM1278 insert was expected to produce four fragments (4.9 kb, 0.7 kb, 2.3 kb, and 5.2 kb) after digestion by EcoRV, and two fragments (3.3 kb and 7.4 kb) after digestion by MfeI/XbaI that were detected by the probes. Digestion of the Z6 pSIM1678 insert was expected to produce two fragments (8.6 kb and 4.5 kb) following digestion with EcoRV, and three fragments (2.4 kb, 4.6 kb, and 2.3 kb) when digested by MfeI/XbaI. These fragments were detected by the probes.

Six Southern blots were performed following digestion of Z6 DNA with EcoRV (Figure 3). The banding pattern on each Southern blot was identical for each of the vegetatively propagated Z6 plants (from G0-G3) when hybridized separately with the six probes. The two expected pSIM1278 fragments (4.9 kb and 2.3 kb) and one pSIM1678 fragment (8.6 kb) were observed for each sample when hybridized with AGP. The GBS probe detected two pSIM1278 fragments (2.3 kb and 5.2 kb) and one pSIM1678 fragment (4.5 kb) in each Z6 sample. The ASN and R1 probes are unique to the pSIM1278 insert. The expected fragments (0.7 kb and 2.3 kb) were observed with ASN, while R1 detected the 5.2 kb fragment, which has higher intensity compared to the Snowden band. The pSIM1678-specific probes, INV and VNT, identified the expected 4.5 kb and 8.6 kb fragments, respectively.

A second set of Southern blots was performed following digestion with restriction enzymes, MfeI/XbaI, to increase the rigor of the structural comparison between samples (Figure 4). Again, the same banding pattern was observed for each of the vegetatively propagated plants (from G0-G3) when hybridized with the six probes. The two expected pSIM1278 fragments (3.3 kb and 7.4 kb) and one pSIM1678 fragment (4.6 kb) were observed for each sample when hybridized with AGP. The one expected pSIM1278 fragment (7.4 kb) and the one pSIM1678 fragment (2.3 kb) were detected by the GBS probe. The pSIM1278-specific probes, ASN and R1, detected the expected fragments (3.3 kb and 7.4 kb, ASN; 7.4 kb, R1) in each Z6 sample. The expected 2.4 kb fragment was observed with pSIM1678-specific VNT probe.

Two expected pSIM1678 fragments (4.6 kb and 2.3 kb) were also detected by the pSIM1678-specific INV probe.

The same banding pattern was observed in all Southern blots for each of the vegetatively propagated Z6 plants (Figure 3, Figure 4), which corresponded to the expected digestion pattern in all cases (Figure 2)**.**

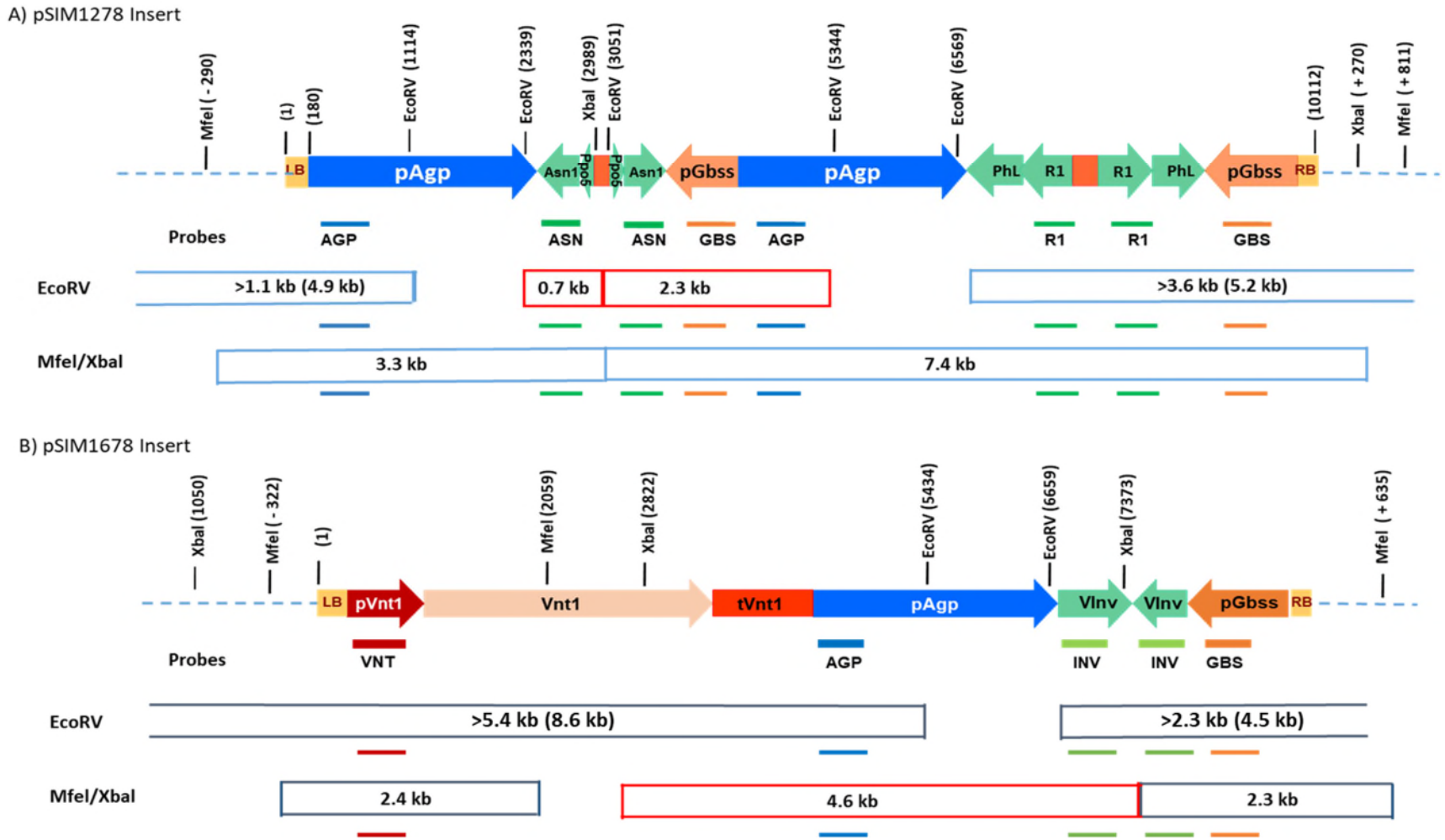

Figure 2. Structure and Expected Digestion Pattern for Z6 Inserts from pSIM1278 and pSIM1678
Structure of (A) pSIM1278 and (B) pSIM1678 inserts in Z6 shown with EcoRV and MfeI/XbaI restriction sites. Digestion products detected by the six probes are indicated below the insert maps. Closed boxes denote fragments fully contained within the insert, whereas open-ended boxes denote fragments extending into the genomic flanking region. A colored bar notes the binding site for the AGP, ASN, GBS, R1, INV, and VNT probes to each digestion product.

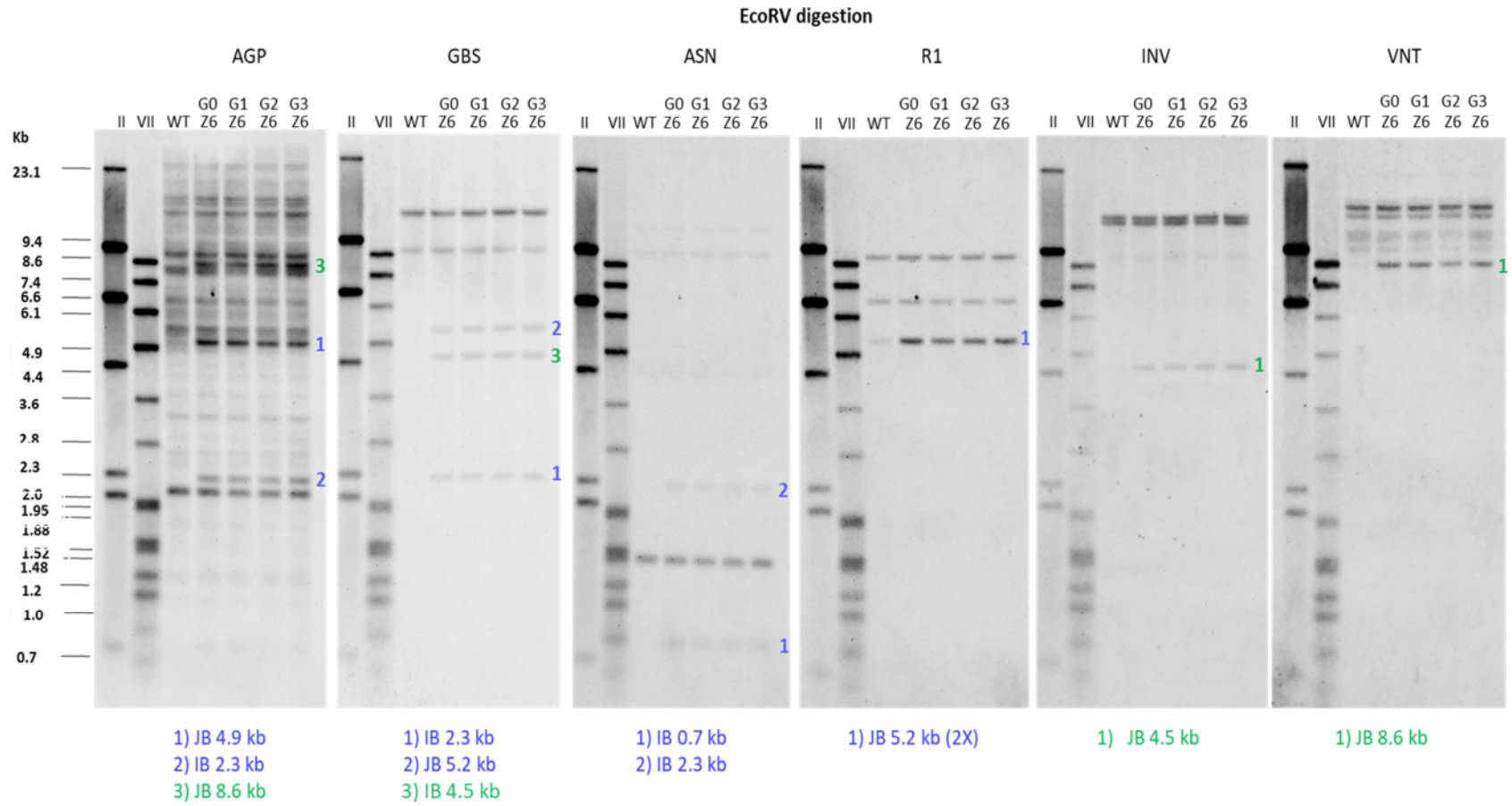

Figure 3. EcoRV Digests: pSIM1278 and pSIM1678 Z6 Inserts are Stable during Vegetative Propagation

Southern blots of Snowden (WT) and Z6 (G0 to G3) genomic DNA following digestion with EcoRV. Southern blots hybridized with AGP, GBS, ASN, R1, INV, or VNT probe. Sizes of insert bands from pSIM1278 (blue) and pSIM1678 (green) are given below the blots. Band sizes correspond to expected products (Figure 2). Molecular weight markers, Dig II (II) and Dig VII (VII), were included in each gel and sizes are labeled to the left of the first gel.

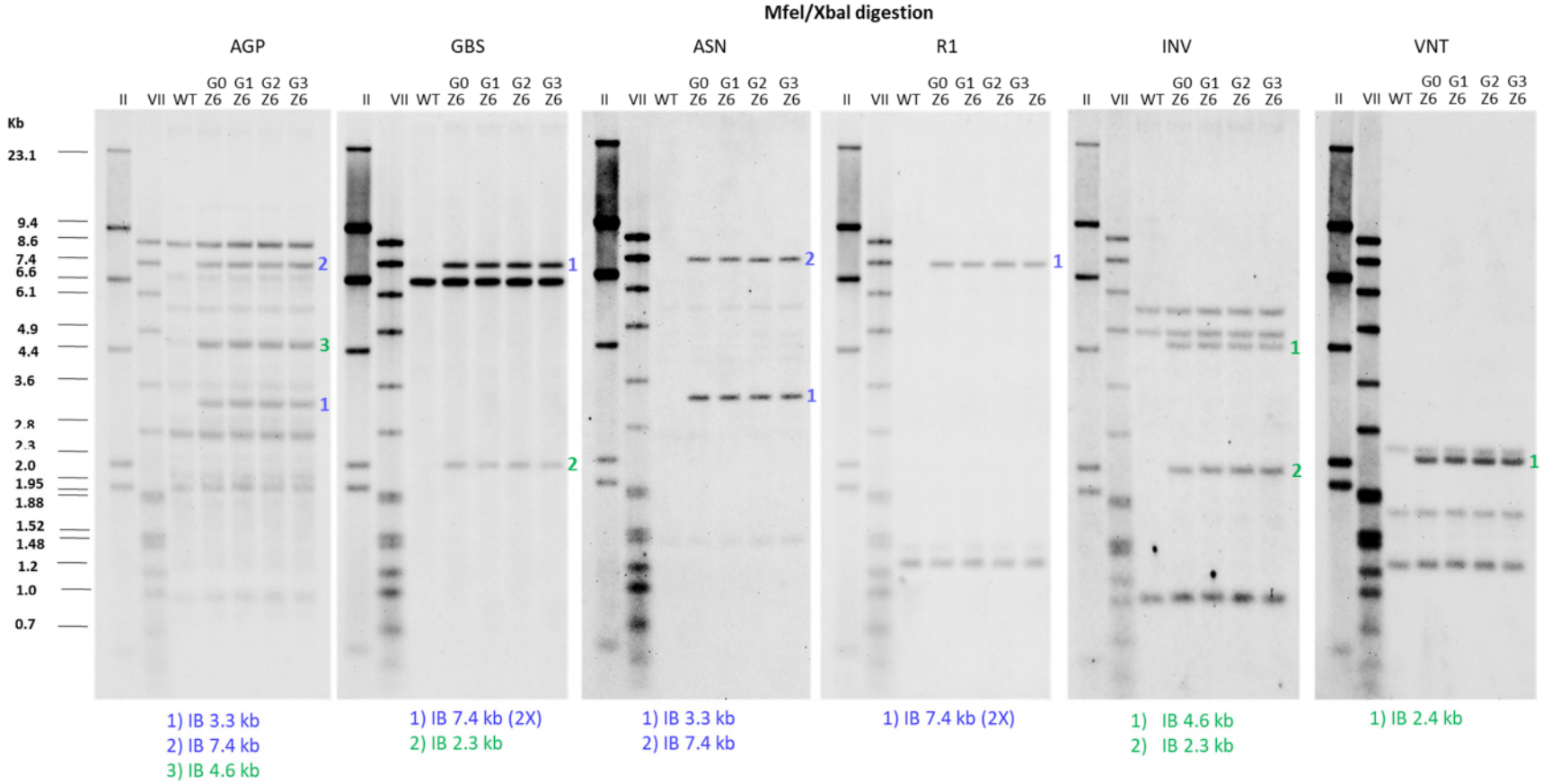

Figure 4. MfeI/XbaI Digests: pSIM1278 and pSIM1678 Z6 Inserts are Stable during Vegetative Propagation

Southern blots of Snowden (WT) and Z6 (G0 to G3) genomic DNA following digestion with MfeI/XbaI. Southern blots hybridized with AGP, GBS, ASN, R1, INV, or VNT probe. Sizes of insert bands from pSIM1278 (blue) and pSIM1678 (green) are given below the blots. Band sizes correspond to expected products (Figure 2). Molecular weight markers, Dig II (II) and Dig VII (VII), were included in each gel and sizes are labeled to the left of the first gel.

CONCLUSION

Insert stability was assessed using Southern blots following digestion with different restriction enzymes and hybridization with six probes that detected one or more fragments from each insert. The same banding pattern was observed in all Southern blots for each of the vegetatively propagated Z6 plants. The data in this study demonstrated that the inserts from pSIM1278 and pSIM1678 were stable in vegetatively propagated Z6.