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STUDY TITLE

Evidence for the Absence of Plasmid Backbone DNA in Event Z6

AUTHORS

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PERFORMING LABORATORIES

SPS Regulatory Lab

STUDY NUMBER

[CCI]

Quality Control Statement

This report was reviewed to assure that it accurately reflects the raw data of this study. The raw data were audited for compliance with the protocol, study notebook, and Standard Operating Procedures were applicable.

Signed	9/19/18
[Personal information redacted] Associate Molecular Scientist	Date

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	9/19/18
[Personal information redacted] Molecular Senior Scientist	Date

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Summary

Objective: The objective of this study was to verify that backbone sequences from pSIM1278 and pSIM1678 were not integrated in the genome of the Z6 event.

Methods: Southern blot analysis was used to screen for the presence of backbone sequences in Z6. The pSIM1278 and pSIM1678 transformation vectors 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.

Results: There were no hybridization signals observed with Z6 genomic DNA that corresponded to backbone sequences from pSIM1278 and pSIM1678.

Conclusion: Southern blot hybridization confirmed the absence of backbone DNA in the Z6 genome.

Introduction

Snowden Z6 event was developed by two sequential transformations of the Snowden potato variety using *Agrobacterium*-mediated transformation. Snowden was first transformed with pSIM1278 and an event from this transformation, V11, was transformed with pSIM1678.

Agrobacterium-mediated transformation can lead to the integration of plasmid backbone sequences into the host genome along with the desired T-DNA. The plasmid backbone is not intended to transfer into the genome during transformation. The two transformation plasmids, pSIM1278 and pSIM1678, share the same backbone sequence. The absence of backbone in Z6 was assessed by Southern blots using a series of eight probes that span the shared backbone of the two plasmids. There was no evidence of backbone DNA in the Z6 genome.

Study Objective

The objective of this study was to verify that backbone sequences from pSIM1278 and pSIM1678 were not integrated in the genome of the Z6 event.

Study Dates
8/2018-9/2018

Key Study Personnel
[Personal information redacted]

Materials and Methods

To detect backbone DNA, which may be present in Z6, genomic DNA isolated from Snowden (WT) and Z6 plants was digested with EcoRI or EcoRI/ScaI and analyzed by Southern blotting. A set of eight probes was used that spanned the backbone sequence, which was identical for pSIM1278 and pSIM1678. The pSIM1278 plasmid (1278) was spiked (prior to digestion) into Snowden genomic DNA as a positive control at a targeted concentration of one copy/genome equivalent (23.4 pg). This concentration ensured the probes were sensitive enough to detect a single copy of backbone DNA in the genome.

Plant material

Snowden and Z6 (G0) plants were grown in two-gallon pots in Sunshine Mix #1 (www.sungro.com) in a temperature- (18 °C minimum/27 °C maximum) and light-controlled (16 h photoperiod; intensity approximately 1,500 µmol/m^2/s) greenhouse. After one to two months of growth, genomic DNA was isolated from leaf tissue samples.

DNA Isolation

A 1 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. The powdered tissue was mixed with 10 mL extraction buffer (0.35 M sorbitol, 0.1 M Tris-HCl (pH 8.0), and 0.05 M EDTA) and the sample centrifuged at 3,000 rpm for 15 min at room temperature (RT). The pellet was suspended in 2 mL extraction buffer containing 200 µg RNase A. After incubation at 65 °C for 20 min in 2 mL nuclear lysis buffer (0.2 M Tris-HCl (pH 7.5), 0.005 M EDTA, 20 mg/mL CTAB, and 800 µL 5% sarcosyl), an equal volume of chloroform: isoamyl alcohol (24:1) was added. The sample was mixed by vortex 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). The DNA concentration was measured using a Qubit Fluorometric Quantitation (Life Technologies) system, and quality was confirmed by electrophoresis using a 0.8% agarose gel in 1X Tris/Acetate/EDTA (TAE) run for 30-40 min at 80 volts.

DNA Restriction Digestion

The pSIM1278 plasmid, which contains the full backbone sequence, served as a positive control. A 4 µg sample of plant DNA was digested using 50 units of restriction enzyme incubated overnight at 37 °C in a final volume of 400 µL. 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 and washed with 70% ethanol. The DNA pellet was dissolved in 20 µL 1X TE followed by addition of 2 µL DNA gel loading buffer (40% sucrose, 0.35% Orange G (Sigma)) in water.

Gel Preparation

Digested DNA was electrophoresed on a large 0.7% agarose gel containing Tris-acetate-EDTA (TAE) buffer for 24 h using 30 volts. The gel was stained with ethidium bromide (10 mg/mL), photographed using the Alpha Innotech (Santa Clara, California) gel documentation system, and depurinated by submersion in 0.25 N HCl for 20 min. After subsequent denaturation in 0.5 M NaOH/1.5 M NaCl for 2x 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 a nylon membrane was carried out with 10X SSC using capillary transfer.

DIG-Labeled Probe Preparation

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

Table 1. The primers used for backbone probes

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Hybridization

After transfer, the nylon membrane 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.) rotating at 20-25 rpm. The nylon membranes cross-linked with EcoRI-digested DNA were hybridized independently with each of the BB1 to BB6 probes, and membranes with EcoRI/ScaI-digested DNA were hybridized independently with the BB7 and BB8 probes. Hybridization was carried out by replacing the prehybridization buffer with a fresh amount of the same preheated solution containing 25-50 µL denatured DIG labeled probe and continuing the incubation with rotation (20-25 rpm) at 42 °C for about 16 h. The probe-containing hybridization solution was stored (-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 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. This low stringency buffer was removed 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 60-63 °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 on a low-speed shaker. The blocked membrane was incubated with DIG antibody solution (1:10,000 dilution of Anti-DIG-alkaline phosphate conjugate with 1X Blocking solution) for 30 min on a shaker. The membrane was washed twice (15 min each) with 1X DIG Washing Solution (Roche) and equilibrated with 1X detection buffer. The detection reaction was carried out with 2 mL CDP-Star solution (1:100 diluted stock of CDP-Star with 1X detection buffer) for 5 min. The membrane was wrapped in plastic film. Depending on the experiment 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 (0.2M 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. Each membrane was stripped once and probed twice.

Results

Digestion of pSIM1278 with EcoRI produced three bands (12.5 kb, 1.57 kb, and 5.6 kb) detectable by the probe set (Figure 1A). A linear representation of the plasmid backbone shows the expected fragment sizes following digestion and probe binding sites (Figure 1A). 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 1B).

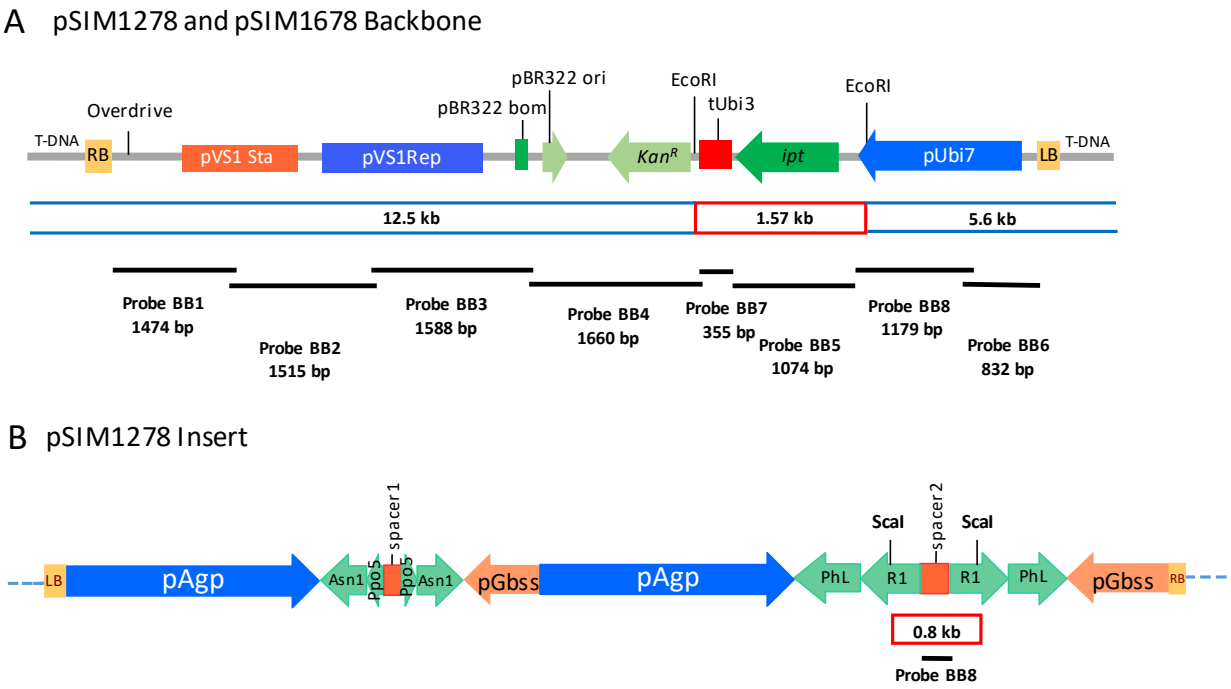

Figure 1. Probe Alignment for the pSIM1278/pSIM1678 Backbone and pSIM1278 T-DNA
(A) Linear map of backbone for pSIM1278 and pSIM1678. Eight probes (BB1-8) to detect backbone are indicated with the size of probes in base pairs (bp). EcoRI sites and expected band sizes for the pSIM1278 control are indicated 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 2). 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 2).

There were no backbone fragments detected in the genome of Z6 when hybridized with probes BB5-BB8 (Figure 3). The 1.57 kb EcoRI plasmid fragment described in Figure 1A 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 1A) was detected in positive control samples by probes BB6 and BB8 (Figure 3). The 0.8 kb band (Figure 1B) was detected in the Z6 and pSIM1278 control sample in the blot probed with BB8 (Figure 3). This band corresponded to the spacer 2 element of the pSIM1278 insert (Figure 1B). Endogenous bands not related to the transformations were observed in all samples in the blots hybridized with probes BB6–BB8 (Figure 3).

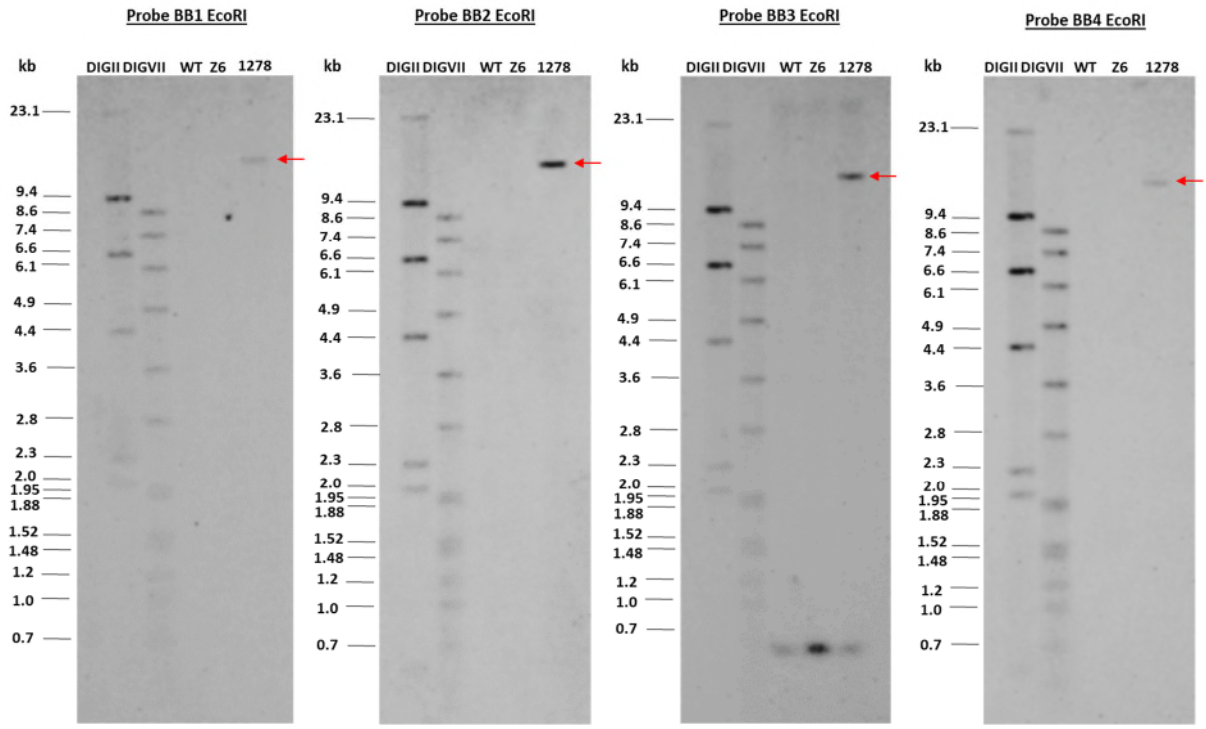

Figure 2. Southern Blots Probed with BB1-BB4 Show No Evidence of Backbone DNA in Z6
Southern blots of genomic DNA isolated from Snowden (WT), Z6, and WT spiked with pSIM1278 (1278). Molecular weight mark markers, DIGII and DIGVII, are labeled in kb. Red arrows indicate expected 12.5 kb bands unique to the plasmid control (1278).

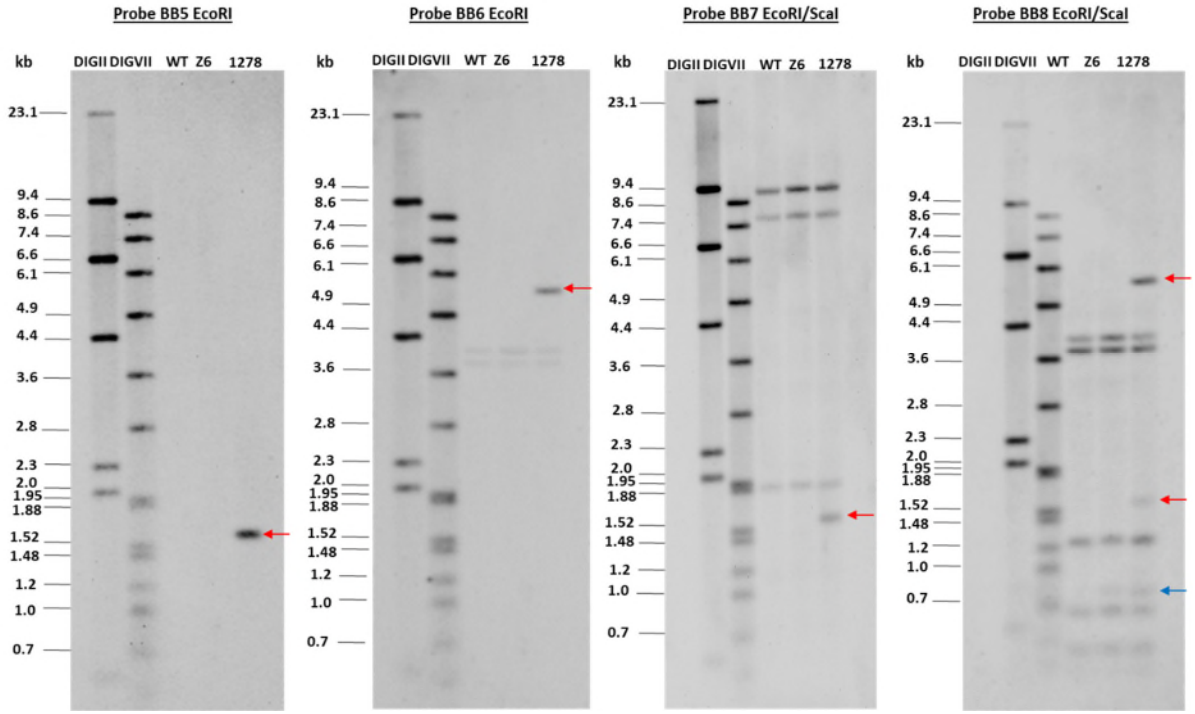

Figure 3. No Evidence in Z6 of Backbone DNA Corresponding to Probes BB5-BB8
Southern blots of genomic DNA isolated from Snowden (WT), Z6, and WT spiked with pSIM1278 (1278). Molecular weight markers, DIGII and DIGVII, are labeled in kb. Red arrows indicate expected bands (1.57 kb and 5.6 kb) unique to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

Conclusion

Southern blots were used to detect the presence of backbone DNA in the Z6 genome. Using probes that spanned the length of the backbone, no hybridization signal was detected corresponding to backbone DNA in the Z6 genome. Fragments from the pSIM1278 positive control samples were correctly identified using the designed probes. The results of the study confirmed the absence of pSIM1278 and pSIM1678 plasmid backbone DNA in Z6.