Personal Information and Commercial Confidential Information [CCI] redacted

STUDY TITLE

Expression of RNAi Targeted Transcripts in Snowden V11

AUTHORS

[personal information]

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PERFORMING LABORATORY

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CERTIFICATION PAGE

This report is an accurate and complete representation of the study activities.

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ABSTRACT

V11 was developed by transforming Snowden with pSIM1278. The inserted cassettes were designed to reduce expression of asparagine synthetase, polyphenol oxidase, phosphorylase L, and water dikinase genes in tubers without introducing unwanted developmental or agronomic characteristics. The pSIM1278 construct consists of two inverted repeats containing sequences isolated from members of these gene families expressed in tubers: *Asn1*, *Ppo5*, *R1*, and *PhL*. The cassettes were designed to lower expression of the target transcripts primarily in tubers through the use of the *Gbss* and *Agp* promoters. The efficacy of each cassette was evaluated by assessing relative expression of each set of transcripts in tubers, leaves, roots, stems, and flowers by northern blot. The results show that transcripts associated with each target were expressed at lower levels in V11 tubers.

INTRODUCTION

Event V11 was developed by transforming Snowden with pSIM1278. The inserted cassettes were designed to use RNA interference (RNAi) to reduce expression of asparagine synthetase, polyphenol oxidase, phosphorylase L, and water dikinase genes in tubers without introducing unwanted developmental or agronomic characteristics. The pSIM1278 construct consists of two inverted repeats containing sequences isolated from members of these gene families expressed in tubers: *Asn1*, *Ppo5*, *R1*, and *PhL*. The cassettes were designed to lower expression of the target transcripts primarily in tubers through the use of the *Gbss* and *Agp* gene promoters, which are primarily active in tubers and stolons (Nakata et al., 1994; Visser et al., 1991). The effectiveness of the cassettes to reduce expression of target transcripts was evaluated by northern blot analysis.

STUDY OBJECTIVES

Objectives of this study are to:

1. Determine whether cassettes reduced expression of target transcripts in V11 tubers
2. Evaluate expression of target transcripts in samples isolated from leaves, roots, flowers, and stems from V11 and WT plants.

STUDY DATES

01/2008-02/2016

KEY STUDY PERSONNEL

[personal information]

MATERIALS AND METHODS

Plant Material

Tubers, leaves, stems, roots, and flowers of field grown V11 and Snowden plants were collected from Parma, ID. Tubers were collected at harvest, all other samples were collected at the flowering stage. All samples were flash frozen in liquid nitrogen and stored at -80 °C. Three biological replicates were analyzed, with each northern blot lane containing RNA from an individual plant.

RNA Isolation

RNA was extracted from tubers and roots using [CCI].

These methods are briefly described below.

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RNA was precipitated with an equal volume of isopropyl alcohol and incubated for 10 min at RT. RNA was pelleted by centrifugation at 6,000 x g for 10 min at 4 °C, rinsed with 70% ethanol, suspended in RNase-free H_2O, and stored at -80 °C.

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 Transfer

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.

Probe Labeling

Probes were made for the target genes and for the 18S rRNA control. Probes were designed to hybridize to the target transcripts but could hybridize to other members of the same gene family. Probes were DIG labeled using PCR. Primers used for PCR amplification and probe lengths are shown in Table 1. A typical reaction (50 µL) contained 5 µL HotMaster™ Taq Buffer (ThermoFisher), 0.4-1 µM forward primer, 0.4-1 µM reverse primer, 5 µL DIG-labeled dNTP (Roche), 5-30 ng plasmid template, and 0.5-0.75 µL HotMaster™ Taq polymerase. PCR conditions were specific to each probe. The size and purity of the DIG-labeled probe was confirmed by agarose gel electrophoresis. As expected, the labeled probe migrated slower than the unlabeled control. The probe was denatured before use (5 min at 95 °C) and quenched on ice.

Table 1. Primers and Probes Used for Northern Analysis of Potato Transcripts

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* Probes were designed by PCR amplification of cDNA clones of each indicated gene

Hybridization

Hybridization solution was heated to 68 °C for 10 min prior to use. Membranes were incubated in 40 mL DIG Easy Hybridization solution (Roche) at 42 °C for 1-4 h with rotation at 20-25 rpm. The hybridization solution was replaced with 40 mL, 42 °C hybridization solution containing 25-50 µL of the DIG-labeled probe and incubated for 3-16 h at 42 °C.

Detection

Blots were washed twice with Solution I (2X SSC/0.1% SDS) for 10 min at RT with rotation at 25-30 rpm. Blots were washed twice with Solution II (0.5X SSC/0.1% SDS) for 20 min at 60 °C with rotation at 25-30 rpm. A final wash step was carried out in solution III (0.1X SSC/0.1% SDS) for 20 min at 65 °C. Blots were rinsed with 2X SSC to remove SDS. The membrane was rinsed with 100 mL 1X DIG Washing Solution (Roche) for 2 min at RT and then blocked with 1X Blocking solution (Roche) for 30 min to 3 h at RT with constant shaking. A 1:10,000 dilution of anti-DIG-alkaline phosphate conjugate was added and blots incubated for 30 min at RT with constant shaking. Membranes were washed twice (15 min each) with 1X DIG Washing Solution and equilibrated in 1X detection buffer. A 1:100 dilution of CDP-Star was added. After 5 min the membrane was wrapped in plastic and developed at exposure times ranging from 1 to 25 min using an Amersham™ Imager 600 instrument (GE Healthcare Life Sciences, Pittsburgh, PA).

RESULTS

Northern blotting was used to compare the relative level of targeted transcripts of V11 and Snowden (WT) using samples isolated from tuber, leaf, stem, root, and flower. Northern probes were designed using the sequence of the particular genes (e.g. *Asn1*) that were used to design the pSIM1278 inverted repeats (Table 1); however the probes may detect related genes (e.g. *Asn1* and *Asn2*) due to sequence conservation. This approach allowed us to assess differences in transcripts associated with a particular enzymatic activity (e.g. asparagine synthetase) across the sample types.

Probes were hybridized to RNA blots from each of the sample types containing three biological replicates from both V11 and WT. An internal control was provided by the 18S probe, which hybridizes to 18S ribosomal RNA (rRNA), and shows similar loading between samples.

The results showed reduced expression of all targeted transcripts in V11 tubers (Figure 1). The 18S rRNA levels provided a loading control and allowed for direct comparisons of transcripts between samples of the same type. A summary of the transcript expression results are provided in Table 2, where an arrow denotes an observable decrease in the V11 sample relative to Snowden controls of the same type.

Table 2. Summary of Samples with Reduced Transcript Expression in V11

Transcripts	Sample types				
	Tuber	Leaf	Stem	Root	Flower
Asparagine synthetase	↓	-	-	-	-
Polyphenol oxidase	↓	-	-	-	-
Phosphorylase L	↓	-	-	-	-
Water dikinase	↓	-	-	-	-

(↓) reduced expression, (-) no change.

Tuber

As shown by the ASN probe (Figure 1), asparagine synthetase transcripts were expressed at lower levels in V11 than in the Snowden controls (WT). Similarly, polyphenol oxidase, phosphorylase L, and water dikinase transcripts were reduced in V11 compared to WT (PPO, PHL, and R1 probes, respectively).

The cassettes containing inverted repeats in pSIM1278 were designed to use RNA interference to reduce levels of transcripts necessary for asparagine synthetase, polyphenol oxidase, water dikinase, and phosphorylase L enzymatic activity in tubers. This was observed for all four targets in V11 (Figure 1).

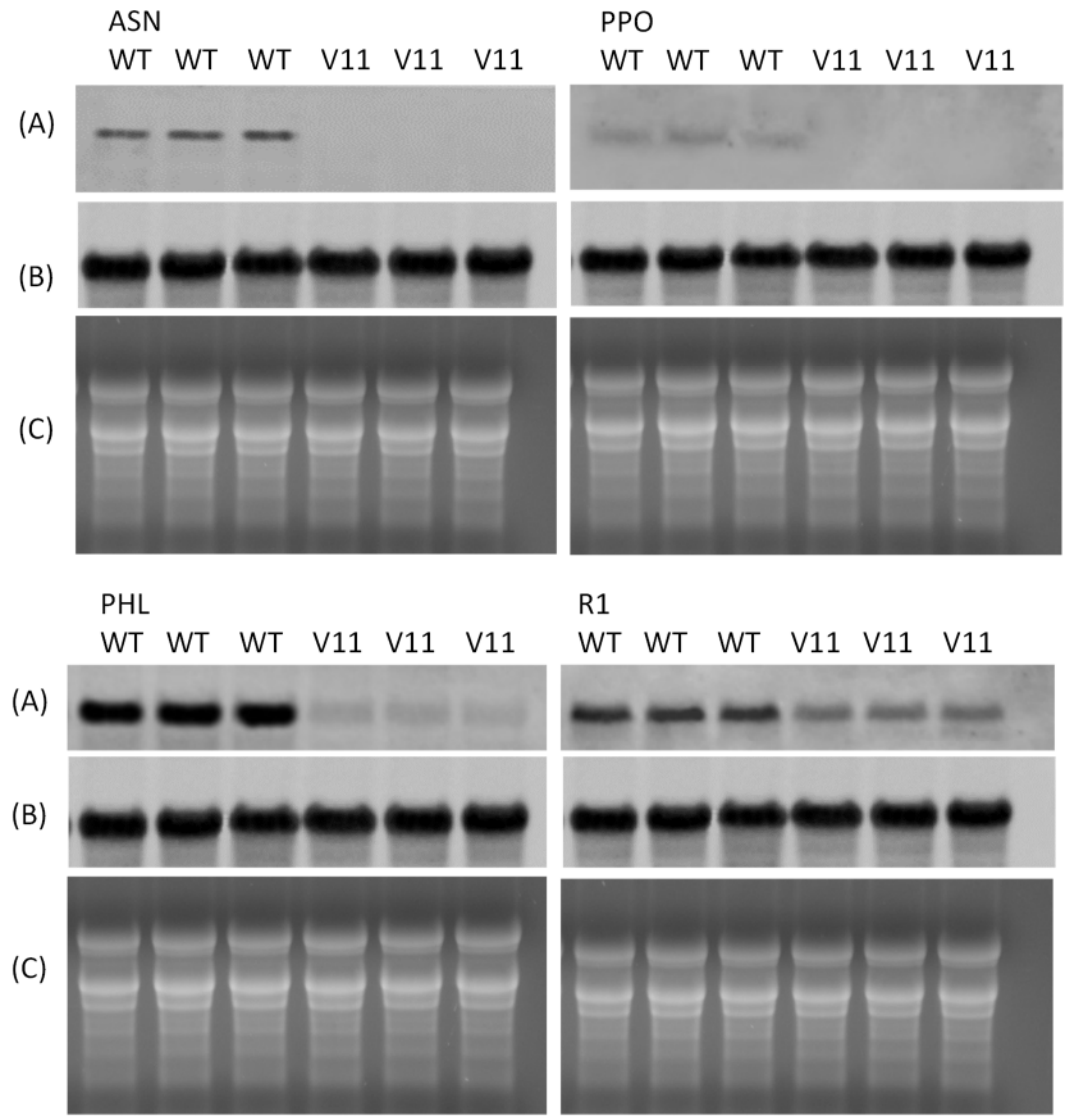

Figure 1. Reduced Expression of Target Transcripts in V11 Tubers
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

Leaf

The northern blots did not identify a reduction in transcript levels between WT and V11 leaf samples using any of the probes: ASN, PPO, PHL, and R1 (Figure 2).

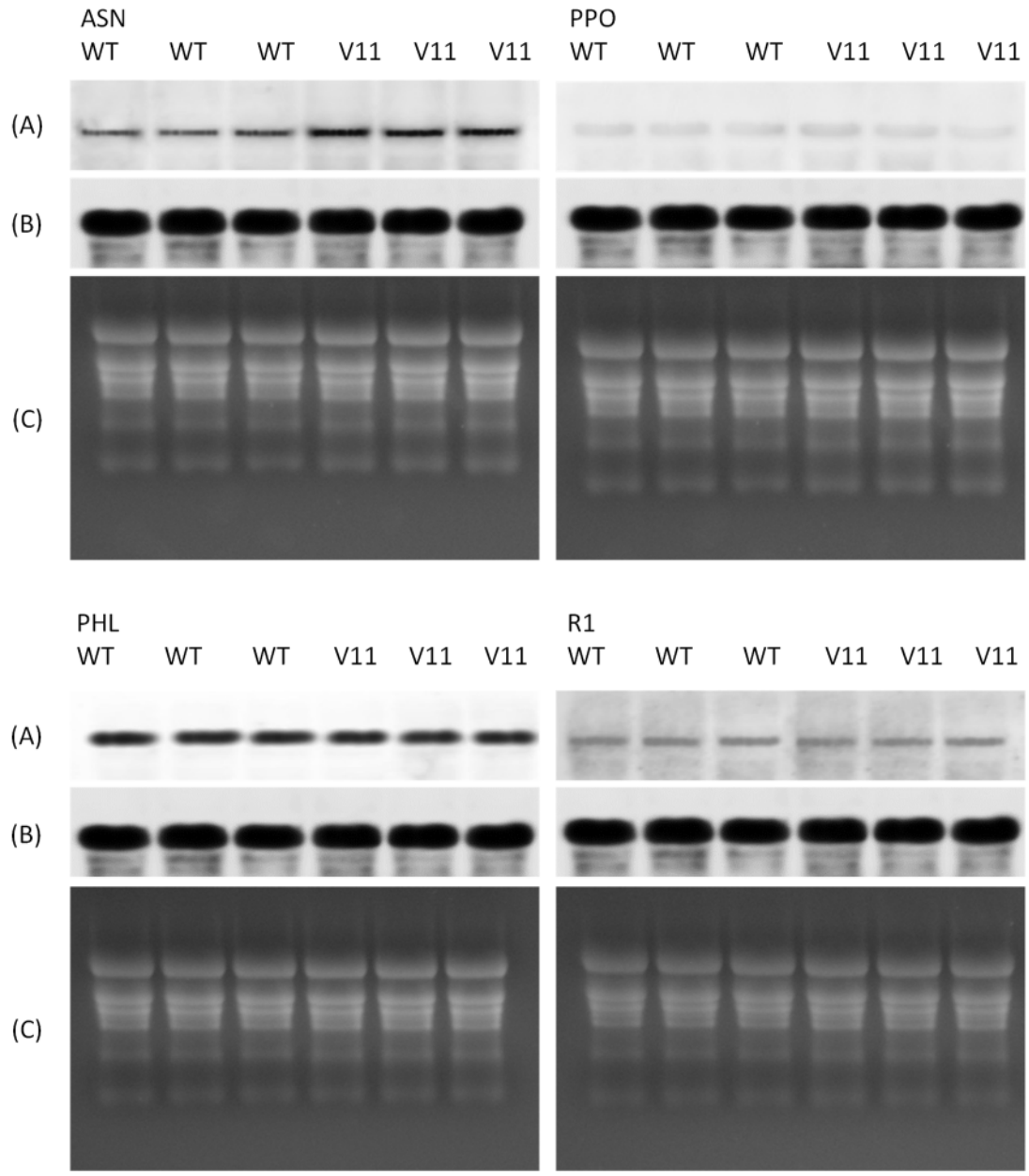

Figure 2. No Reduction in Target Transcript Expression in V11 Leaves
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

Stem

The northern blots did not identify a reduction in transcript levels between WT and V11 stem samples using any of the probes: ASN, PPO, PHL, and R1 (Figure 3).

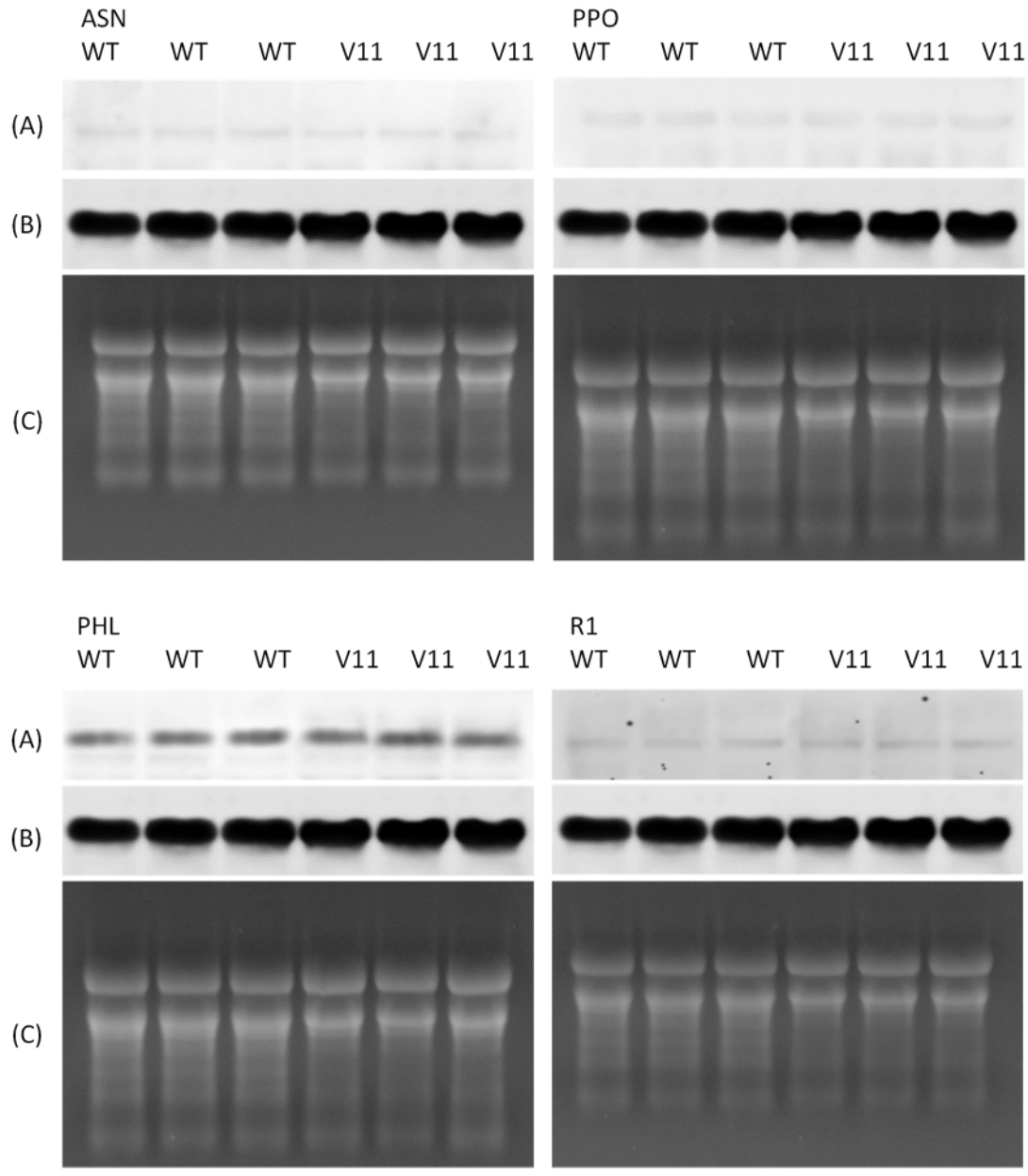

Figure 3. No Reduction in Target Transcript Expression in V11 Stems
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

Root

The northern blots did not identify a reduction in transcript levels between WT and V11 root samples using any of the probes: ASN, PPO, PHL, and R1 (Figure 4).

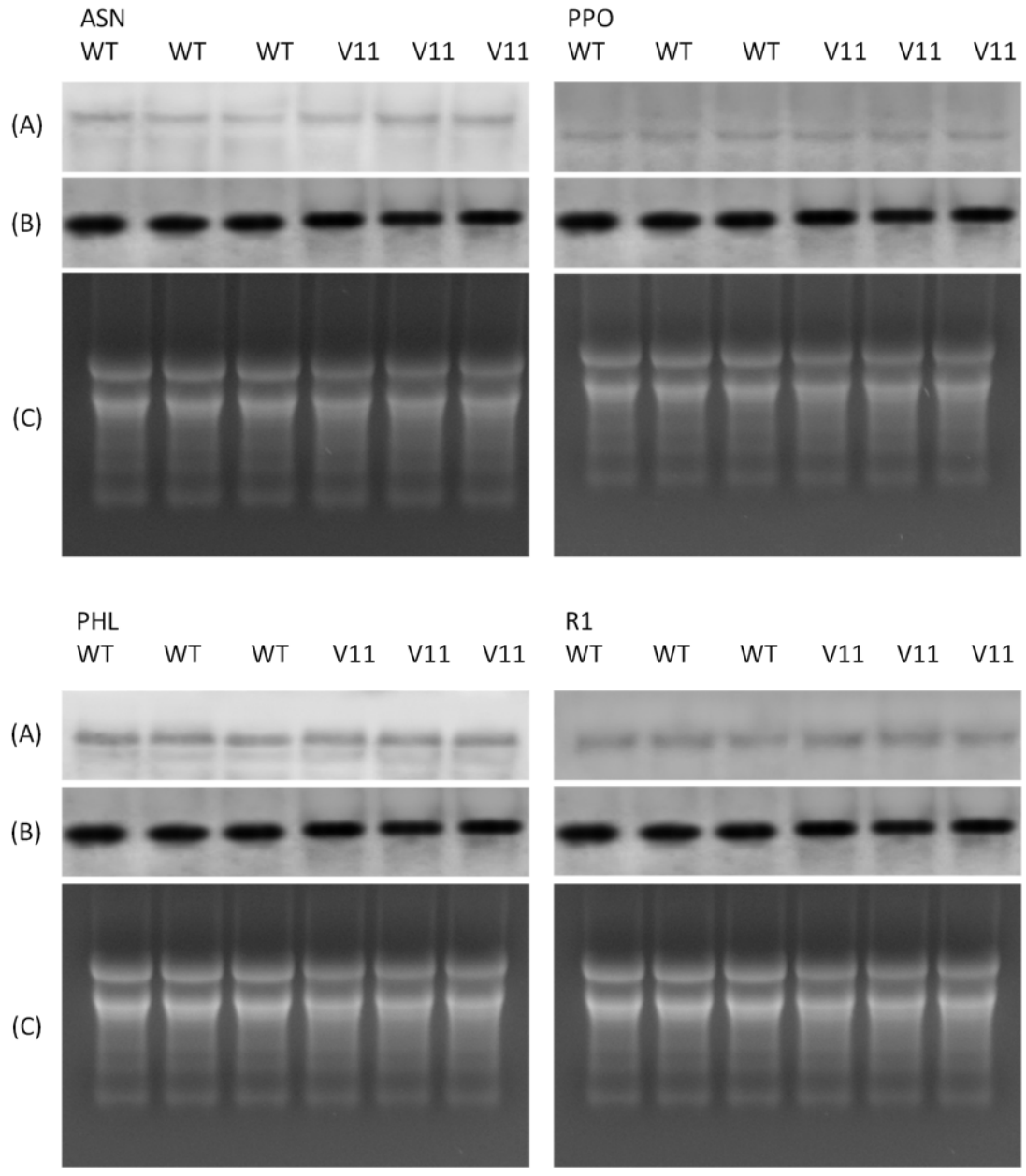

Figure 4. No Reduction in Target Transcript Expression in V11 Roots
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

Flower

The northern blots did not identify a reduction in transcript levels between WT and V11 flower samples using any of the probes: ASN, PPO, PHL, and R1 (Figure 5).

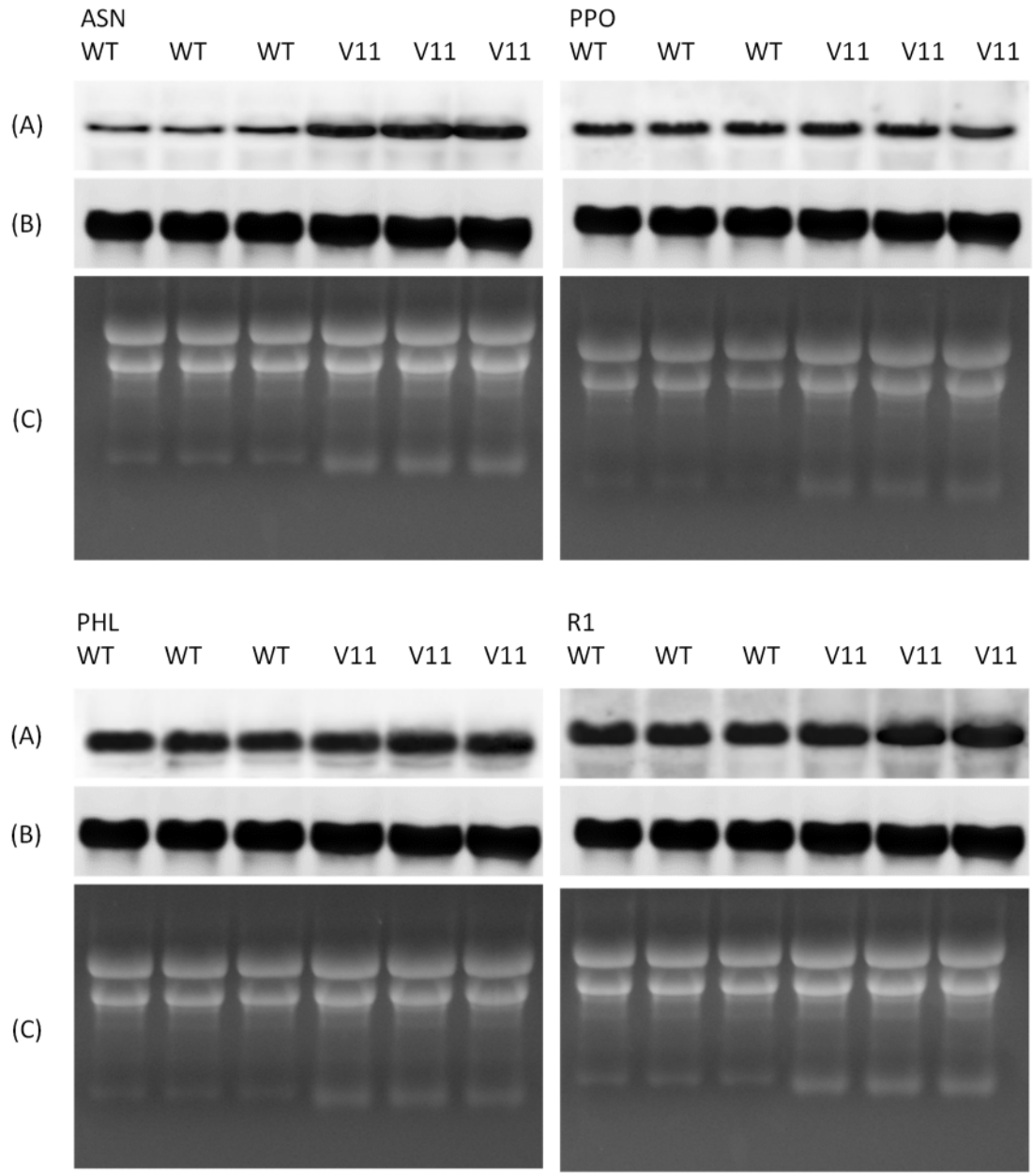

Figure 5. No Reduction in Target Transcript Expression in V11 Flowers
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide.

CONCLUSION

This study showed the inserted cassettes were successful in reducing expression of asparagine synthetase, polyphenol oxidase, phosphorylase L, and water dikinase transcripts in V11 tubers.

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