Personal information and commercial confidential information have been redacted

**STUDY TITLE**

Expression of RNAi-targeted Transcripts in Z6

**AUTHORS**

[personal information redacted]

**REPORT DATE**

5/27/2020

**PERFORMING LABORATORY**

SPS Molecular Lab

**STUDY NUMBER**

[CCI]

**CERTIFICATION PAGE**

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

| Signed | 4/9/2019 |
| --- | --- |
| [personal information redacted]<br>Senior Molecular Scientist | Date |

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**SUMMARY**

**Objective:** The pSIM1278 insert consists of two inverted repeats, designed to reduce expression of asparagine synthetase (ASN), polyphenol oxidase (PPO), glucan water dikinase R1 (R1), and phosphorylase L (PHL) transcripts. The pSIM1678 insert contains an additional inverted repeat to reduce expression of vacuolar invertase (INV) transcripts. The inverted repeats were all designed to lower expression of the target transcripts in tubers using RNA interference (RNAi). The objective of this study was to evaluate reduction of target transcript levels in Z6 samples compared to Snowden.

**Methods:** Tubers, leaves, roots, flowers, and stems of field grown Z6 and Snowden plants were collected for total RNA extraction. The concentration of isolated RNA was measured using a fluorometer and RNA quality was evaluated by electrophoresis. Equal amounts of total RNA samples from three Z6 and Snowden plants were loaded on formaldehyde gels, separated by electrophoresis, and transferred to nylon membranes for northern blot analysis.

Probes ASN, PPO, R1, PHL, and INV were designed to detect the five target transcripts. Probe 18S was designed to detect endogenous potato rRNA and served as an RNA sample loading control. These probes were PCR-amplified, DIG-labeled, and separately hybridized to the prepared nylon membranes.

**Results:** Total RNA staining with ethidium bromide and 18S blot control showed similar loading for all samples.

Through the use of the Gbss and Agp promoters, the inverted repeats were designed to lower expression of the target transcripts primarily in tubers. The northern blot results showed that asparagine synthetase, polyphenol oxidase, phosphorylase L, glucan water dikinase R1, and invertase transcripts were expressed at lower levels in Z6 tubers when compared to Snowden samples.

**Conclusion:** Northern blot analysis confirmed the reduction of asparagine synthetase, polyphenol oxidase, phosphorylase L, glucan water dikinase R1, and vacuolar invertase transcripts in Z6 tubers compared to Snowden.

**INTRODUCTION**

Z6 was developed by transforming Snowden with pSIM1278 and pSIM1678. The inserted cassettes were designed to use RNA interference (RNAi) to reduce expression of asparagine synthetase, polyphenol oxidase, glucan water dikinase R1, phosphorylase L, and vacuolar invertase transcripts 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 pSIM1678 construct contains an additional inverted repeat containing sequence from the acid vacuolar invertase gene, *VInv*. 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 Z6 tubers; and
2. Evaluate expression of target transcripts in samples isolated from leaves, stem, root, and flower from Z6 and Snowden plants.

**STUDY DATES**

12/2018 to 3/2019

**KEY STUDY PERSONNEL**

[personal information redacted]

**MATERIALS AND METHODS**

Plant Material

Tubers, leaves, stems, roots, and flowers of field grown Z6 and Snowden G2 plants were collected from Jerome, 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 either [CCI] or [CCI] and from leaves, flowers, and stems using TriPure Isolation Reagent (Roche™). These methods are briefly described below.

[CCI]

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 five 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. [CCI]

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**

| [CCI] | | | |
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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 Z6 and Snowden using samples isolated from tuber, leaf, stem, root, and flower. Northern probes were designed using

the sequence of the target transcripts (Table 1); however, the probes may detect transcripts from related genes due to sequence conservation.

Probes were hybridized to RNA blots from each of the sample types containing three biological replicates from both Z6 and Snowden. 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 asparagine synthetase, polyphenol oxidase, phosphorylase L, glucan water dikinase R1, and invertase transcripts in Z6 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 Z6 sample relative to Snowden control of the same type.

**Table 2. Summary of RNA Expression Changes in Z6**

| Transcripts | Sample types | | | | |
|---|---|---|---|---|---|
| | Tuber | Leaf | Stem | Root | Flower |
| Asparagine synthetase | ↓ | - | - | - | - |
| Polyphenol oxidase | ↓ | - | - | - | - |
| Phosphorylase L | ↓ | - | - | - | - |
| Glucan water dikinase R1 | ↓ | - | - | - | - |
| Vacuolar invertase | ↓ | - | - | - | - |

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

**Tuber**

The probes showed asparagine synthetase, polyphenol oxidase, phosphorylase L, glucan water dikinase R1, and vacuolar invertase transcripts were expressed at lower levels in Z6 tubers than in Snowden (Figure 1).

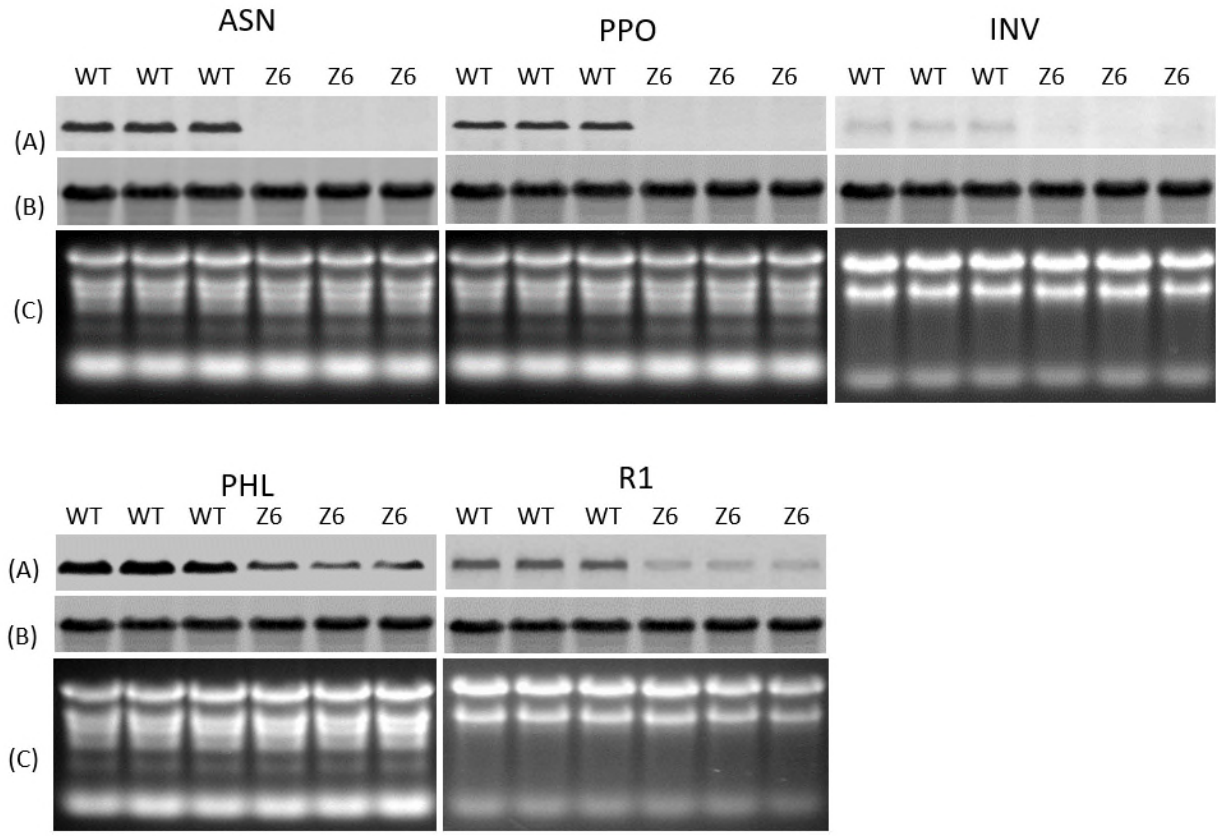


**Figure 1. Reduced Expression of Target Transcripts in Z6 Tubers**
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, INV, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium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

**Leaf**

The northern blots did not identify any differences in transcript levels between Snowden and Z6 leaf samples using any of the five probes: ASN, PPO, PHL, INV, and R1 (Figure 2).

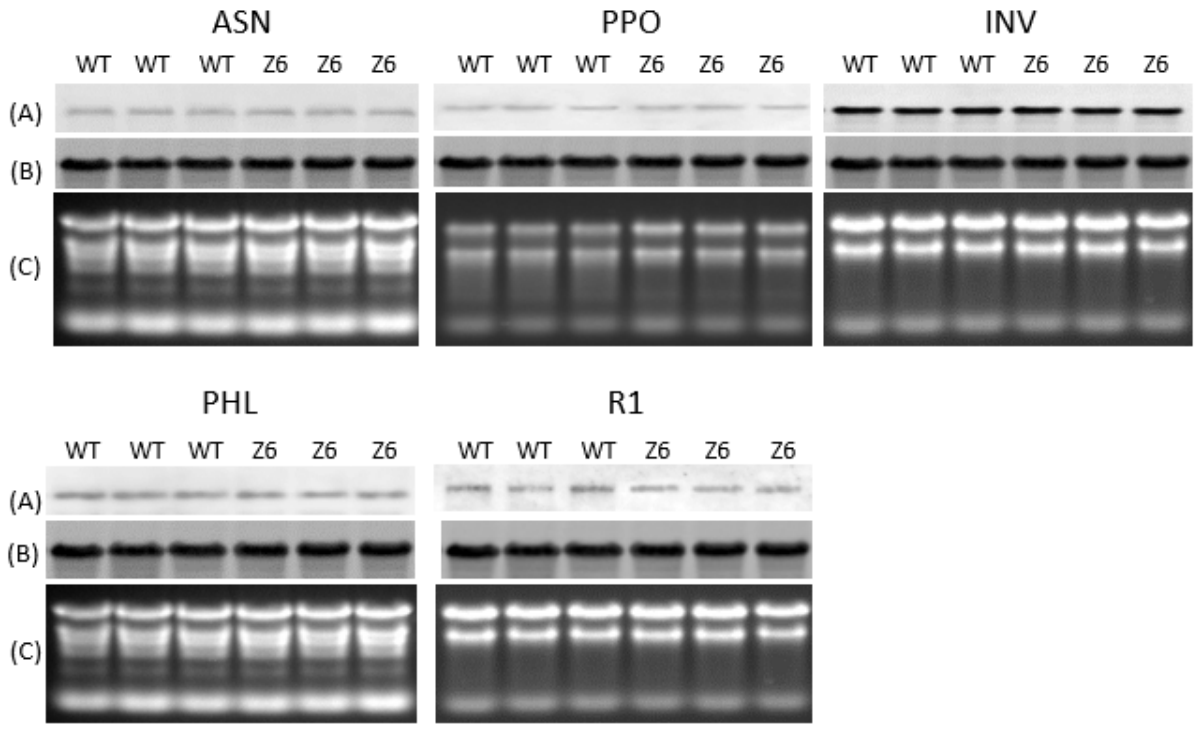


**Figure 2. No Changes in Target Transcript Expression in Z6 Leaves**
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, INV, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium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

**Stem**

The northern blots did not identify any differences in transcript levels between Snowden and Z6 stem samples using any of the five probes: ASN, PPO, INV, PHL, and R1 (Figure 3).

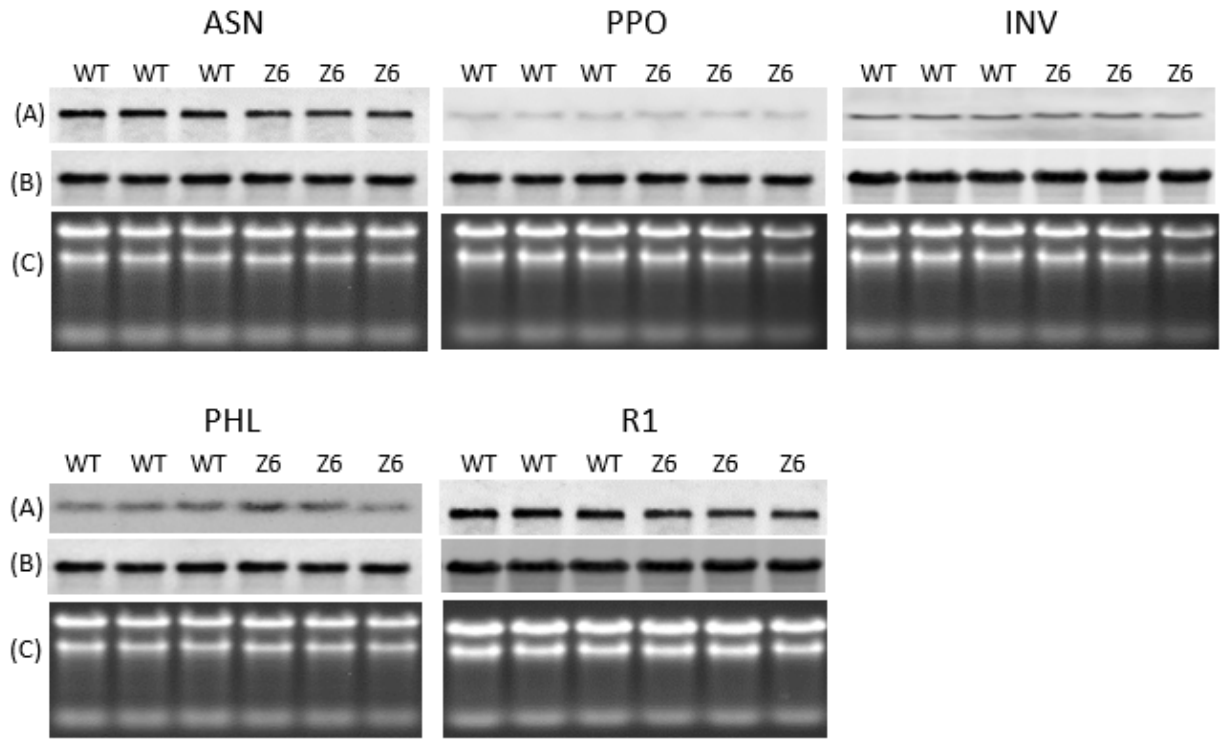


**Figure 3. No Changes in Target Transcript Expression in Z6 Stems**
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, INV, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide. WT = Snowden.

**Root**

The northern blots did not identify any differences in transcript levels between Snowden and Z6 root samples using any of the five probes: ASN, PPO, INV, PHL, and R1 (Figure 4).

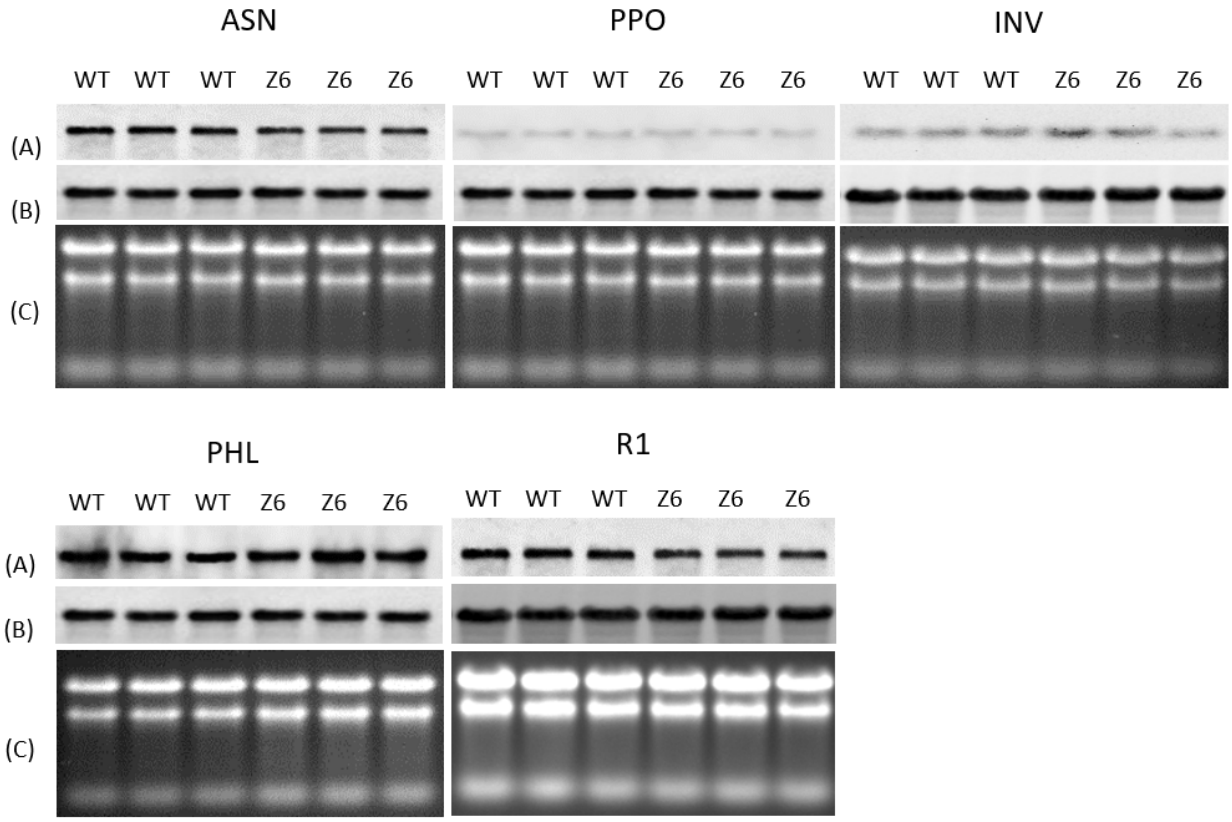


**Figure 4. No Changes in Target Transcript Expression in Z6 Roots**
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, INV, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium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

**Flower**

The northern blots did not identify any differences in transcript levels between Snowden and Z6 flower samples using any of the five probes: ASN, PPO, PHL, INV, and R1 (Figure 5).

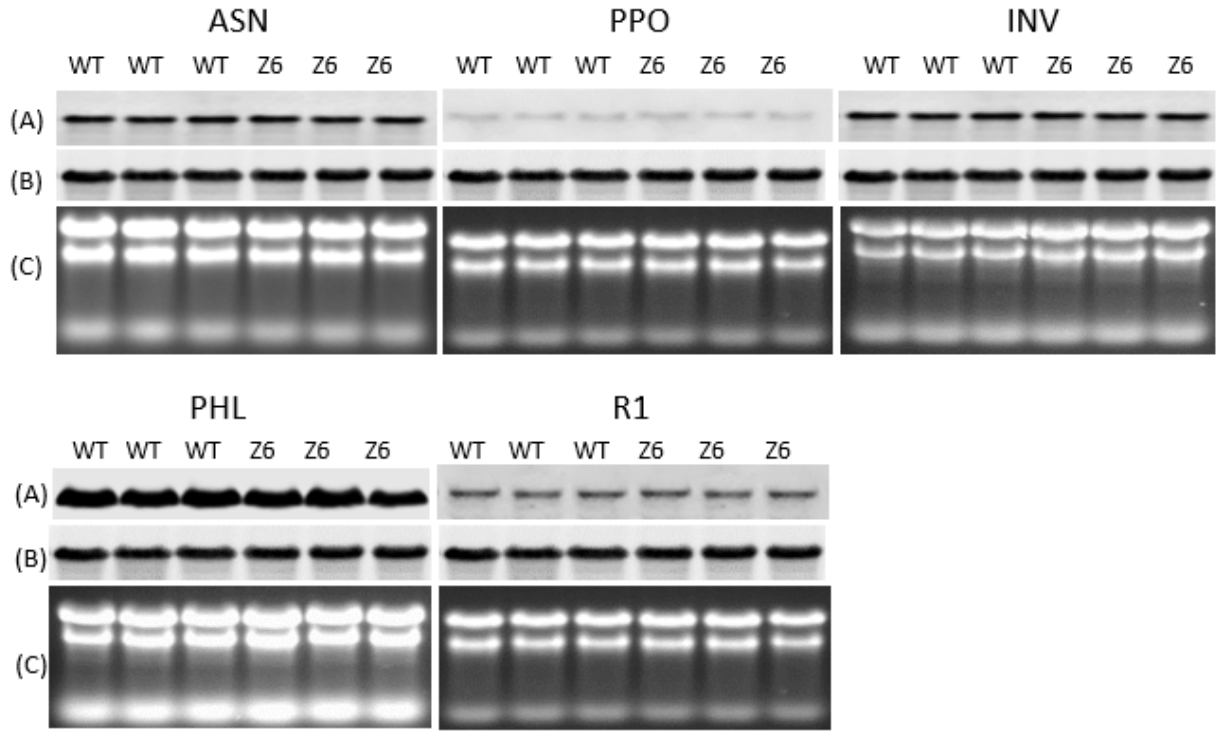


**Figure 5. No Changes in Target Transcript Expression in Z6 Flowers**
(A) Northern blots were hybridized to detect targeted transcripts with the indicated probes: ASN, PPO, INV, PHL, or R1. (B) Same blots probed for 18S rRNA provide a gel loading control. (C) Total RNA is shown after staining with ethidium bromide. WT = Snowden.

**CONCLUSION**

This study showed the inserted cassettes were successful in reducing expression of asparagine synthetase, polyphenol oxidase, phosphorylase L, glucan water dikinase R1, and vacuolar invertase transcripts in Z6 tubers. Down regulation of the targeted transcripts was not observed in Z6 leaves, stems, roots, or flowers.

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