Personal information and commercial confidential information [CCI] redacted

STUDY TITLE

VNT1 Protein and *Rpi-vnt1* Gene Expression in Z6

AUTHORS

[personal information redacted]

REPORT DATE

29 January 2019

PERFORMING LABORATORY

SPS Regulatory Lab

STUDY NUMBER

[CCI]

CERTIFICATION PAGE

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

Signed	19 Feb 2019
[personal information redacted] Regulatory Affairs Manager	Date

TABLE OF CONTENTS

SUMMARY

Objectives: To determine VNT1 protein and *Rpi-vnt1* transcript levels in Z6 plants.

Methods: Western blots using a VNT1-specific antibody were used to detect VNT1 levels in Z6 tuber and leaf samples. Extracted proteins from three biological replicates of field grown Snowden and Z6 tuber and leaf tissues were analyzed for VNT1 expression. The VNT1 protein standard was included as a positive control.

RT-qPCR was used to measure *Rpi-vnt1* transcript levels in Z6 leaf, stem, root, flower, and tuber samples from field-grown Z6 and Snowden plants. Greenhouse grown *Solanum venturii* leaf samples were used as positive controls. Total RNA was isolated from these samples and cDNA were synthesized using reverse transcriptase. A Taqman-based PCR assay was designed to measure *Rpi-vnt1* transcript levels using primers and a probe specific to the LRR region of *Rpi-vnt1.* Endogenous reference genes, *Elongation Factor 1α* and *adenine phosphoribosyltransferase* (*APRT*), were multiplexed with the *Rpi-vnt1* assay and used to normalize the qPCR results.

Results: Using western blots and a VNT1-specific antibody, endogenous VNT1 was not detected in Z6 tuber and leaf tissues. This indicated that VNT1 levels in Snowden and Z6 plants were below the estimated LOQ of 220 ppb in tuber tissues and 450 ppb in leaf tissues.

Due to the undetectable levels of VNT1 in Z6 leaf and tuber samples, *Rpi-vnt1* transcript expression levels were measured by RT-qPCR in Z6 plants. *Rpi-vnt1* transcript expression was detected at low levels in *S. venturii* leaf samples and in Z6 leaf, stem, root, flower, and tuber tissues. No *Rpi-vnt1* transcript expression was detected in any Snowden tissues.

Conclusions: The *Rpi-vnt1* transcript expression results together with the western blot analysis show that *Rpi-vnt1* is expressed in Z6 tissues and suggests that expression levels are low.

INTRODUCTION

Z6 was developed by transforming Snowden potato variety with pSIM1278 followed by transformation with pSIM1678. The pSIM1278 T-DNA consists of two inverted repeats designed to reduce the expression of asparagine synthetase, polyphenol oxidase, water dikinase (R1), and phosphorylase L in tubers. The pSIM1678 T-DNA consists of the late blight resistance gene, *Rpi-vnt1* from *Solanum venturii,* and an inverted repeat to reduce expression of vacuolar invertase in tubers. The gene product, VNT1, is predicted to be an 891-amino acid R-protein of the coiled-coil (CC), nucleotide-binding site (NBS), and leucine-rich repeat (LRR) class involved in the plant hypersensitive response. It functions in the protection of potato plants against late blight infection from *Phytophthora infestans* (Foster et al., 2009). Numerous R-protein homologs are present in potato and tomato varieties and wild *Solanum* species (Jupe et al., 2012). To reduce metabolic costs of aberrant R-protein activation that results in plant cell death associated with the hypersensitive response, R-proteins are tightly regulated and expressed at low levels in plants (Marone et al., 2013).

Immunoblot methods able to detect low picogram (pg) quantities of VNT1 protein were developed with limits of quantitation (LOQ) conservatively established at 500 parts per billion (ppb) in potato tubers and leaves. To avoid underestimating the levels of VNT1 in Z6 plants, the LOQ was set at 500 ppb even though lower limits were possible in leaf (450 ppb) and tuber (220 ppb) tissues. These methods were applied for endogenous detection of VNT1 in Z6 plants. VNT1 was not detected in Z6 leaf or tuber tissues, determining the VNT1 protein levels to be below the established LOQ.

Because the VNT1 protein levels were undetectable in the immunoblots, a reverse transcription quantitative PCR (RT-qPCR) method was developed to determine *Rpi-vnt1* transcript levels in Z6 plants and *S. venturii* leaf material. *S. venturii* is a wild potato species containing the *Rpi-vnt1* gene. Leaf material from *S. venturii* was used as a positive control, as these plants typically do not produce tubers large enough for this type of measurement. Analyses of field grown potato leaf, stem, flower, root, and tuber samples using this RT-qPCR method demonstrated the low expression levels of the *Rpi-vnt1* gene in Z6 plants.

STUDY OBJECTIVES

Objectives of this study:

1. Determine VNT1 protein levels in Z6 potato leaves and tubers.
2. Assess *Rpi-vnt1* transcript expression in Z6 plants.

STUDY DATES

12/2018-01/2019

KEY STUDY PERSONNEL

[personal information redacted]

MATERIALS AND METHODS

Western Blot to Determine VNT1 Protein Expression Levels

Plant Material for Western Blot Analyses

Leaf and tuber samples were collected from field grown Snowden and Z6 G2 plants (three biological replicates labeled -1, -2, and -3, each a separate plant) from a field trial in August 2018 (Table 1). Prior to analysis, all samples were flash frozen in liquid nitrogen, ground to powder, and stored at -80 °C. The Electronic Notebook (ELN) unique identifier provided in Table 1 is for material tracking purposes.

Table 1. Leaf and Tuber Samples used for Western Blot Analysis

Variety	Tissue	Sample I.D.	ELN Unique I.D.
Snowden	Leaf	SN-1	B1
		SN-2	B2
		SN-3	B3
	Tuber	SN-1	B12 7
		SN-2	B12 9
		SN-3	B12 11
Z6	Leaf	Z6-1	B13
		Z6-2	B14
		Z6-3	B15
	Tuber	Z6-1	B24 5
		Z6-2	B24 7
		Z6-3	B24 9

Protein Extraction for Western Blot Analyses

Tuber and leaf proteins were extracted in [CCI].

The supernatant was collected and centrifuged again. The total protein concentration was measured by Bradford assay.

Western Blot (Immunoblot) Assay

Proteins were denatured in 4x Laemmli Buffer (Bio-Rad) for 3 min at 85 °C, separated on 4-20% TGX Mini-PROTEAN® Stain-Free Gels (Bio-Rad), and transferred to PVDF membranes using Bio-Rad Trans-Blot® Turbo™ Blotting System. The membrane was [CCI].

VNT1 antibody, α-VNT1-602, was used at a 1:500 (v/v) dilution in 5% Bio-Rad blotting grade milk and TBST for leaf samples. The membranes were incubated with primary antibody for 1 h at RT, and then

washed three times for 10 min, each with TBST. The secondary antibody (anti-rabbit-IgG-peroxidase; Sigma-Aldrich) was added [CCI].

SuperSignal® West Femto Maximum Substrate (Thermo Scientific) was added, and after 5 min blots were imaged with a Bio-Rad Chemi-Doc™ MP Imaging system.

RT-qPCR to Determine *Rpi-vnt1* Expression Levels

Plant Material for RT-qPCR Gene Expression Analysis

Flower, leaf, stem, root, and tuber samples were collected from field grown Snowden and Z6 plants (three biological replicates) from a field trial in August 2018 (Table 2). Leaf, root, stem, and flower samples were flash-frozen in liquid nitrogen and immediately placed on dry ice before shipment. Tubers were bagged separately and shipped to SPS immediately following harvest. Tubers were chopped and flash-frozen in liquid nitrogen. Leaf samples from greenhouse grown *S. venturii* (three biological replicates) were used as positive controls. Prior to analysis, all samples were flash-frozen in liquid nitrogen, ground to powder, and stored at -80 °C. The ELN unique identifier provided in Table 2 is for material tracking purposes.

Table 2. Tissue Samples used for RT-qPCR Gene Expression Analysis

Variety	Tissue	Sample	ELN Unique I.D.
Snowden	Leaf	SN-1 – SN-3	B1, B2, B3
	Stem	SN-1 – SN-3	B4, B5, B6
	Root	SN-1 – SN-3	B7, B8, B9
	Flower	SN-1 – SN-3	B10, B11, B12 1
	Tuber	SN-1 – SN-3	B12 6, B12 9, B12 11
Z6	Leaf	Z6-1 – Z6-3	B13 1, B14, B15
	Stem	Z6-1 – Z6-3	B16, B17, B18
	Root	Z6-1 – Z6-3	B19, B20, B21
	Flower	Z6-1 – Z6-3	B22, B23, B24
	Tuber	Z6-1 – Z6-3	B24 4, B24 6, B24 8
S. venturii	Leaf	1 – 3	458367-2 (1), 458367-2 (2), 458367-3 (1)

RNA Isolation for RT-qPCR Analysis

RNA was extracted from leaf, stem, roots, flowers, and tuber material using [CCI]

Samples were mixed and incubated with TriPure Isolation Reagent for 5 min at RT. Insoluble plant material was removed by centrifugation at 4 °C for 10 min at 6,000 x g and the supernatant transferred to a new tube. [CCI],

RNA was precipitated by adding an equal volume of isopropyl alcohol and incubating for 10 min at RT. RNA was precipitated by centrifugation at 4 °C for 15 min at 6,000 x g. The RNA pellet was rinsed with 70% ethanol, suspended in RNase-free H_2O, and stored at -80 °C.

RNA Quality Analysis

The concentration of isolated RNA was measured by absorbance (A_{260}/A_{280}) using the BioTek Eon™ Microplate Spectrophotometer. RNA quality was evaluated by gel 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. Samples were treated using the TURBO DNA-free DNase (Ambion) to remove DNA.

Taqman-based RT-qPCR Analysis of *Rpi-vnt1* Transcript Levels

cDNA was synthesized from total RNA using an iScript Reverse Transcription kit (Bio Rad). Reactions were incubated for 5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C.

Endogenous reference genes (*Elongation Factor 1α* and *adenine phosphoribosyltransferase [APRT]*) were multiplexed with the *Rpi-vnt1* assay and used to normalize the qPCR results (Table 3). qPCR reactions contained [CCI]). Cycling parameters are shown in Table 4. Negative control samples (without reverse transcriptase or template) were included to ensure absence of DNA contamination in the reaction. The level of *Rpi-vnt1* mRNA was calculated from raw quantitation cycle (Cq) values after PCR amplification of *Rpi-vnt1* cDNA using gene-specific primers. *S. venturii* leaf material from greenhouse plants was used as a positive control in the *Rpi-vnt1* analysis. Experiments were performed on three biological replicates of Snowden and Z6 material with three technical replicates each. Replicate values were averaged, and standard deviations were determined. Standard curves were used to measure the PCR efficiency for each gene (Table 5).

Table 3. Primers used for qPCR

[CCI]		

Table 4. Cycling Parameters

qPCR Cycling Parameters		
[CCI]		

Table 5. PCR Efficiency and Linearity in Snowden and Z6 Tissues

Tissue	Target Gene	Efficiency	Linearity
Leaf, Stem, Root, Flower, and Tuber	*Rpi-vnt1*	109.2%	0.928
	APRT	92.7%	0.961
	EF1α	92.8%	0.957

Expression of the target gene (*Rpi-vnt1*) was normalized to endogenous reference genes (*Elongation Factor 1α* and *APRT*) by calculating the ΔCq value using the geometric mean of the reference genes ($[EF1\alpha * APRT]^{1/2}$). The Cq value corresponding to the geometric mean of the endogenous reference genes was subtracted from the *Rpi-vnt1* Cq value ($Cq_{Rpi\text{-}vnt1} - Cq_{\text{endogenous gene geometric mean}}$), resulting in the ΔCq. The ΔCq for each replicate was exponentially transformed to the ΔCq Expression by $2^{-\Delta Cq}$, which is described as *Rpi-vnt1* relative expression.

RESULTS

VNT1 Protein Expression Analysis by Western Blot

VNT1 levels were determined by western blot using VNT1 antibodies. A full-length VNT1 protein standard was expressed in *E. coli* and used for VNT1 quantitation and detection purposes. Expression, purification, and quantification of the full-length VNT1 protein standard was described in report [CCI]. The limit of quantitation (LOQ) of the western blot method was established using samples of Russet Burbank initially transformed with pSIM1678. The VNT1 western blot methods were then applied to other pSIM1678 transformed plants, including Z6.

<u>Determining Endogenous VNT1 Concentrations using Western Blot</u>

The VNT1 protein standard was used as a positive control to compare endogenous levels of VNT1 in Z6 plants. The VNT1 protein standard was analyzed alone or spiked into Z6 and Snowden lysates at two different concentrations (2.5 ng and 1.25 ng). Proteins extracted from Z6 and Snowden tuber and leaf tissues (10 µg) were mostly consistent in yield and banding patterns, as shown in the stain-free gels (left panels, Figure 1). There were some slight differences observed between the lanes in the leaf samples, which do not appear to impact VNT1 detection. Other bands that are not VNT1 are observed on the blots. These bands are in both the Z6 and SN samples and have different sizes compared to the VNT1 standard (VNT1 STD) (right panels, Figure 1). These data indicate that VNT1 concentrations in Z6 are below the LOQ of 500 ppb in tuber and leaf.

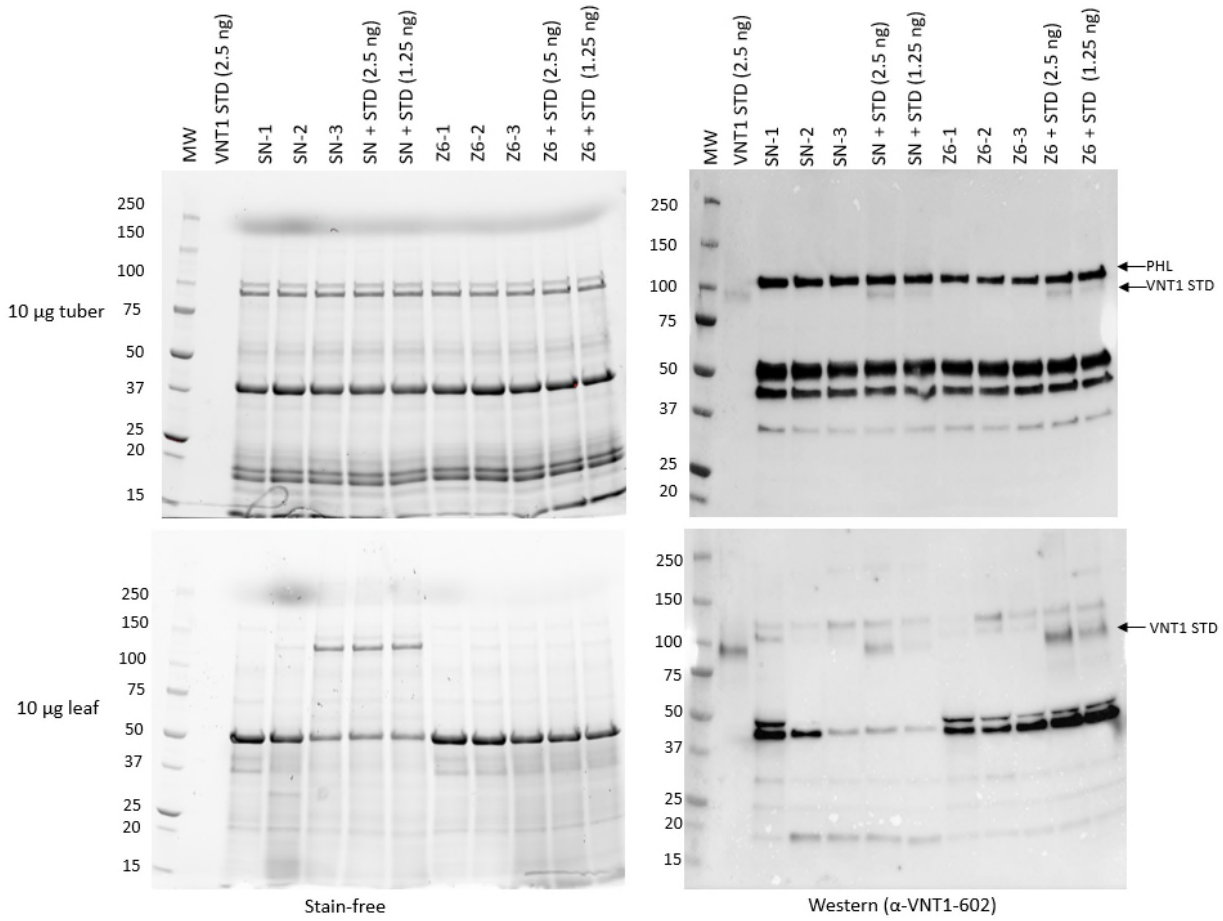

Figure 1. VNT1 was Undetected in Z6 by Western Blot
The VNT1 protein was undetected in three biological replicates of Z6 leaf and tuber tissues following western blot analysis using the α-VNT1-602 antibody. Z6 and Snowden (SN) samples were spiked with 2.5 ng and 1.25 ng of the VNT1 protein standard. Stain free SDS-PAGE gels show the amount of protein loaded in each lane (left). VNT1 was not detected in Z6 or SN tissue samples (right). Tuber is shown in the top panels and leaf in the bottom panels.

Analysis of *Rpi-vnt1* Transcript Expression by RT-qPCR

RT-qPCR was used to measure *Rpi-vnt1* transcript levels in Z6 and Snowden leaf, stem, root, flower, and tuber tissues. Primers and probes were specific to the LRR region of *Rpi-vnt1*. Achieving specificity in the assay was a challenge due to *Rpi-vnt1* homologs present in Snowden, however, amplification was largely limited to Z6 and *S. venturii*, which served as a positive control (Figure 2).

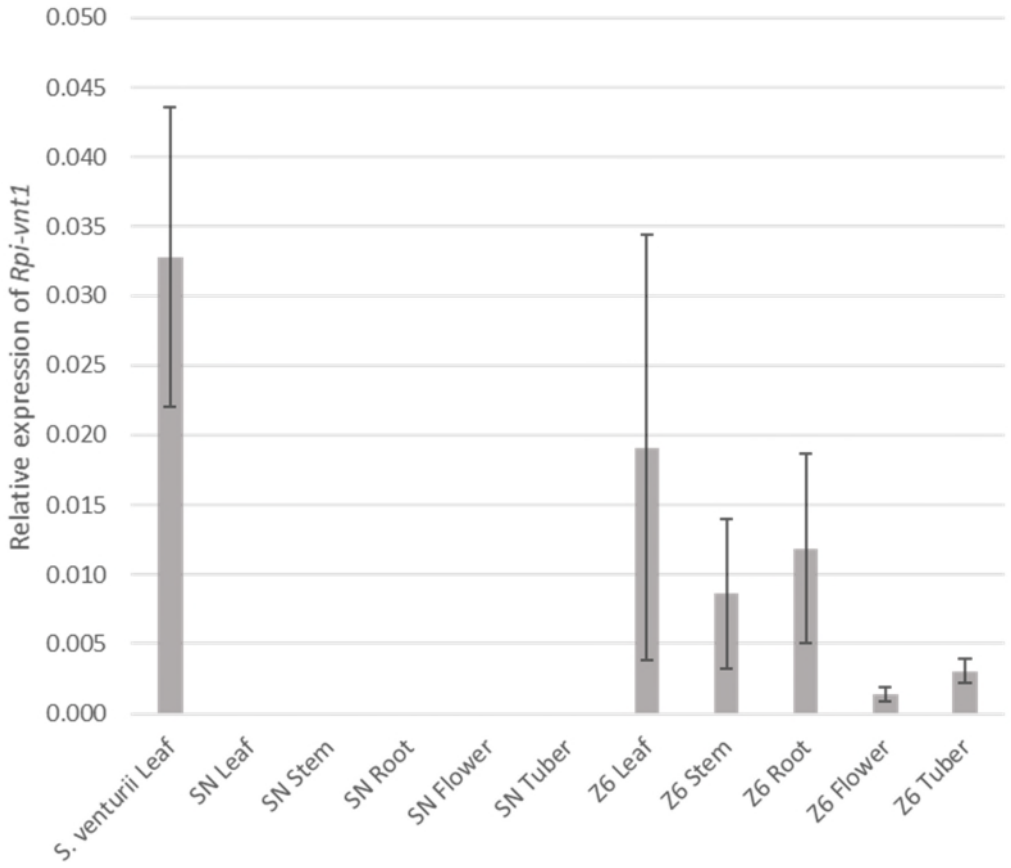

Figure 2. *Rpi*-vnt1 Transcript Levels in Z6 Measured by RT-qPCR
The *Rpi-vnt1* RT-qPCR assay was performed on three biological replicates of Z6 and Snowden (SN) leaf, stem, root, flower, and tuber tissues, analyzed in triplicate. Expression of *Rpi-vnt1* was normalized to endogenous reference genes (*Elongation Factor 1α* and *APRT)* by calculating a ΔCq value. The ΔCq for each replicate was exponentially transformed to ΔCq-Expression by $2^{-\Delta Cq}$, showing relative expression of *Rpi-vnt1* for all samples analyzed. *S. venturii* leaf tissue was used as a positive control.

CONCLUSION

The *Rpi-vnt1* gene, which encodes the VNT1 protein and confers resistance to late blight, was introduced into Snowden to generate Z6. A western blot assay, with a VNT1-specific antibody, was used to detect VNT1 in Z6 plants. VNT1 was not detected in Z6 leaf and tuber field samples, and the amount of VNT1 is estimated to be below the LOQ, which was conservatively established to be less than 500 ppb. To show that *Rpi-vnt1* is expressed in Z6, even though VNT1 was not detected in Z6 leaf and tuber tissues, an *Rpi-vnt1* transcript expression analysis was performed using RT-qPCR. The transcript expression results show that *Rpi-vnt1* is expressed in Z6 tissues and suggests that expression levels are low.

REFERENCES

Foster, S.J., Park, T.-H., Pel, M., Brigneti, G., Śliwka, J., Jagger, L., van der Vossen, E., and Jones, J.D.G. (2009). *Rpi-vnt1.1*, a *Tm-2*^{2} Homolog from *Solanum venturii*, Confers Resistance to Potato Late Blight. Molecular Plant-Microbe Interactions *22*, 589–600.

Jupe, F., Pritchard, L., Etherington, G.J., MacKenzie, K., Cock, P.J.A., Wright, F., Sharma, S.K., Bolser, D., Bryan, G.J., Jones, J.D.G., et al. (2012). Identification and Localisation of the NB-LRR Gene Family within the Potato Genome. BMC Genomics *13*, 75.

Marone, D., Russo, M.A., Laidò, G., Leonardis, A.M. De, and Mastrangelo, A.M. (2013). Plant Nucleotide Binding Site–Leucine-Rich Repeat (NBS-LRR) Genes: Active Guardians in Host Defense Responses. 7302–7326.