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STUDY TITLE

Characterization of VNT1 Protein Expression in Y9

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STUDY NUMBER

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CERTIFICATION PAGE

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

signed	8/29/2017
[personal information] Regulatory Science Manager	Date

signed	8/29/17
[personal information] Senior Regulatory Science Manager	Date

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SUMMARY

Objectives: To establish the limit of quantitation (LOQ) for VNT1 protein and determine VNT1 protein levels in Y9 potato leaves and tubers.

Methods: Full-length VNT1 protein was expressed in *E. coli* to use as a standard protein for VNT1 quantitation and detection purposes. Western blots were used to detect VNT1 levels in Y9 tuber and leaf samples. A serial dilution of VNT1 protein standard spiked into potato protein extract was used to establish an LOQ for VNT1 in leaf and tuber tissues. Extracted proteins from three biological replicates of field grown Atlantic and Y9 tuber and leaf tissues were analyzed by western blot. The VNT1 protein standard was included as a positive control.

Liquid chromatography-mass spectrometry (LC-MS) was used to determine VNT1 levels in potato leaf samples. Three peptides (IIITSR, SFELFTK, and ILSALSPVPR) were identified as VNT1 proteotypic peptides. They were isotopically labeled at the C-terminus and used as signature peptides for VNT1 detection. These isotope-labeled peptides were spiked into protein extracts from Russet Burbank leaf samples to establish an LOQ of VNT1 by liquid chromatography-multiple reaction monitoring-mass spectrometry (LC-MRM-MS). Total protein was extracted from leaf tissues of field grown plants transformed with the same plasmids as Y9 and greenhouse grown *S. venturii* in three biological replicates. These leaf tissue lysates were analyzed for detection of endogenous VNT1 peptides (IIITSR, SFELFTK, and ILSALSPVPR) and also spiked with labeled versions of these same peptides as controls.

Results: Using western blots with antibodies generated from VNT1 peptides, an LOQ for VNT1 was estimated at 220 ppb in tuber samples and 450 ppb in leaf (ng of VNT1 per g of tissue). Extracted proteins from Atlantic and Y9 tuber and leaf tissues (10 µg and 40 µg) were analyzed by western blot, but endogenous VNT1 was undetected. This indicated that VNT1 levels were below the estimated LOQ in Atlantic and Y9.

Each peptide in potato leaf protein extracts was quantitated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, establishing the VNT1 LOQ as 500 ppb (ng of VNT1 per g of leaf tissue) using this method. The VNT1 signature peptides IIITSR, SFELFTK, and ILSALSPVPR were not detected in leaf protein extracts from pSIM1678 transformed plants and donor organism, *S. venturii*, indicating that VNT1 was present at amounts below the established LOQ (500 ppb).

Conclusions: A western blot assay using a VNT1-specific antibody was developed that detected and quantified VNT1 protein with an estimated LOQ of 220 ppb in tuber tissues and 450 ppb in leaf tissues. Using the western blot method, VNT1 was not detected in Y9 leaf and tuber field samples indicating the level of VNT1 is below the LOQ. An LC-MS method was developed to detect and quantify VNT1 protein with an LOQ of 500 ppb in leaf tissue. A conservative estimate for VNT1 in Y9 tissues was established to be less than 500 ppb.

INTRODUCTION

Y9 was developed by transforming Atlantic potato variety with pSIM1278 followed by retransformation 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, and protects 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). Because the hypersensitive response results in plant cell death and to reduce metabolic cost, R-proteins are tightly regulated and expressed at low levels in plants (Marone et al., 2013).

Immunoblot and liquid chromatography-mass spectrometry (LC-MS) 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.

STUDY OBJECTIVES

Objectives of this study:

1. Establish the LOQ for detection of VNT1 protein in potato leaves and tubers; and
2. Determine VNT1 protein levels in Y9 potato leaves and tubers.

STUDY DATES

03/2017-07/2017

KEY STUDY PERSONNEL

[personal information]

MATERIALS AND METHODS

Western Blot to Determine VNT1 Protein Expression Levels

Expression and Purification of VNT1 Standard from *E. coli*

Plasmid harboring full-length *Rpi-vnt1* sequence [CCI].

For induction of recombinant protein expression, [CCI].

E. coli cells were harvested by centrifugation (3,300 x g) and cell pellets were stored at -80 °C.

The VNT1 protein was primarily identified [CCI]

analyzed on an SDS-PAGE gel for molecular weight assessment.

[CCI].

The gels were Coomassie stained using 0.05% Coomassie solution in water. The band corresponding to approximately 102 kDa, which would be the correct molecular weight based on amino acid sequence, was cut from the gel, washed with water, and cut into 1 mm^3 pieces. These small pieces were transferred to [CCI].

The collected protein samples were quantified by both Bradford and BCA assays, and aliquots were stored at -80 °C until use.

Plant Material for Western Blot Analyses

Leaf samples were collected from field grown Russet Burbank, W8, Atlantic, and Y9 plants [CCI].

Tuber samples were collected from the same trial at harvest in August 2016 (Table 1). Prior to analysis, all samples were flash frozen in liquid nitrogen, ground to powder, and stored at -80 °C.

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

Variety	Tissue	Sample I.D.
Russet Burbank	Leaf	RB-1
		RB-2
		RB-3
	Tuber	RB-1
		RB-2
		RB-3
W8	Leaf	W8-1
		W8-2
		W8-3
	Tuber	W8-1
		W8-2
		W8-3
Atlantic	Leaf	Atl-1
		Atl-2
		Atl-3
	Tuber	Atl-1
		Atl-2
		Atl-3
Y9	Leaf	Y9-1
		Y9-2
		Y9-3
	Tuber	Y9-1
		Y9-2
		Y9-3

Protein Extraction for Western Blot Analyses

Tuber and leaf proteins were extracted in [CCI].

The total protein concentration was measured by Bradford assay. These concentrations were used to determine that 9.5 µL (tuber) and 4.8 µL (leaf) of Russet Burbank protein extracts were spiked with VNT1 protein standard to bring the amount of total protein to 10 µg loaded onto SDS-PAGE gels for LOQ determinations.

Western Blot (Immunoblot) Assay

Proteins were denatured in [CCI].

The gels were soaked in transfer buffer for 15 min. Membranes were soaked in methanol for 10 min and then in transfer buffer for 10 min. The transfer apparatus was assembled according to manufacturer's

instructions and run at 150 V for 1 h. The membrane was placed in 1X TBS with 0.1% Tween-20 (TBST) and 5% non-fat milk added (blocking buffer) for 1 h at room temperature. VNT1 antibody, [CCI].

VNT1 protein standard characterization was conducted using a slightly different western blotting procedure than that stated above and is described as follows. [CCI].

Determining LOQ of VNT1 using Western Blot Assay

The LOQ, in ppb, for VNT1 was based on the amount of the *E. coli*-expressed VNT1 protein standard spiked into a Russet Burbank protein extract. The LOQ is presented as the amount of VNT1 protein standard in fresh tissue (ng/g fresh weight) containing 10 µg of potato protein.

Calculation of VNT1 LOQ in tuber samples:

- Protein was extracted from 150 mg of fresh weight tuber in 500 µL of extraction buffer (150 mg/500 µL = 0.30 mg fw tuber / µL). VNT1 standard was spiked into 9.5 µL (i.e. 10 µg of total protein) of extracts (0.30 mg fw tuber / µL * 9.5 µL * 0.001 g/mg = 0.0029 g).
- Lowest concentration of spiked VNT1 standard detected in extracts was 0.625 ng.
- 220 ppb = 0.625 ng spiked VNT1 / 0.0029 g fw tuber.

Calculation of VNT1 LOQ in leaf samples:

- Protein was extracted from 150 mg of fresh weight leaf in 500 µL of extraction buffer (150 mg/500 µL = 0.30 mg fw leaf / µL). VNT1 standard was spiked into 4.8 µL (i.e. 10 µg of total protein) of extracts (0.30 mg fw leaf / µL * 4.8 µL * 0.001 g/mg = 0.0014 g).
- Lowest concentration of spiked VNT1 standard detected in extracts was 0.625 ng.
- 450 ppb = 0.625 ng spiked VNT1 / 0.0014 g fw leaf.

LC-MS to Determine VNT1 Protein Expression Levels

The LC-MS method was developed using the Russet Burbank potato variety and two events, W3 and W8, derived from transformation with the same plasmids, pSIM1278 and pSIM1678, used to develop Y9.

Plant Material used for LC-MS Protein Expression Analysis

Leaf samples were collected from Russet Burbank, W3, and W8 field grown plants (three biological replicates), and greenhouse grown *S. venturii* plants (Table 2). Prior to analysis, all samples were flash frozen in liquid nitrogen, ground to powder, and stored at -80 °C.

Table 2. Leaf Samples used for LC-MS Analysis

Variety	Tissue	Sample I.D.
Russet Burbank	Leaf	RB-A
		RB-B
		RB-C
W3	Leaf	W3-A
		W3-B
		W3-C
W8	Leaf	W8-A
		W8-B
		W8-C
S. venturii	Leaf	*S. venturii*-1
		S. venturii-2
		S. venturii-3

Protein Extraction for LC-MS Analysis

Proteins from 150 mg frozen leaf tissue of Russet Burbank, W3, W8, and *S. venturii* were extracted in [CCI].

The remaining 1 mL of supernatant was precipitated with 4 mL of cold 100% acetone and incubated at -20 °C overnight. Precipitated protein was centrifuged at 21,600 x g for 15 min and all acetone was removed. The pellet was air dried and sent to UC Davis for LC-MS/MS analysis.

In-solution Tryptic Digests and LC-MRM-MS Analysis to Determine Limit of Quantitation (LOQ)

Samples were digested by trypsin in solution and analyzed by LC-MRM-MS (liquid chromatography-multiple reaction monitoring-mass spectrometry) at UC Davis Proteomics Core facility. Specifically, samples were reconstituted in approximately [CCI]

[CCI]

Both endogenous peptides and isotopically labeled peptides were selected sequentially for analysis.

RESULTS

VNT1 Protein Expression Analysis by Western Blot

VNT1 levels were determined using western blotting with VNT1 antibodies. A full-length VNT1 protein standard expressed in *E. coli* was used for VNT1 quantitation and detection purposes in these assays. Initial pSIM1678 transformations were done in Russet Burbank. Russet Burbank and W8 samples were assessed for VNT1 limit of quantitation (LOQ) and detection. The VNT1 western blot identification methods were then applied to other pSIM1678 transformed plants, including Y9.

VNT1 Standard Production and Characterization

A VNT1 protein standard was expressed and purified from *E. coli* cells for use as a positive control in western blot assays. The expression plasmid contained full-length VNT1 protein with no additional tags, so the molecular weight was expected to be approximately 102 kDa. Since the purified protein appeared to migrate slightly faster than the predicted size of 102 kDa relative to the protein molecular weight standards, it was characterized using western blotting and Edman degradation (Figure 1). Antibodies produced against the SQKGYQHVTFPKK peptide (α-VNT1-602), FHSSSKLPFGVWESKIL peptide (α-VNT1-5363), and KKLFLVQGPNISPISLR peptide (α-VNT1-5365) from the LRR domain of VNT1 were used to assess the VNT1 protein standard. VNT1 at the same molecular weight was detected by each antibody (Figure 1) confirming the presence of the C-terminus in the purified standard. Edman degradation was used to assess the N-terminus of the protein standard, which identified a single N-terminus in the purified VNT1 protein standard that corresponded to the predicted sequence of the 891 amino acid protein. These data suggest that the VNT1 purified protein was full-length, but migrated slightly faster than predicted when compared to the molecular weight protein markers (Figure 1). The concentration of the VNT1 protein standard was 10 ng/µL based on quantitation from standard curves using bovine serum albumin (BSA) and green fluorescent protein (GFP) (Appendix A). This concentration was used for LOQ estimations of VNT1 in Y9.

The VNT1 protein standard expressed from *E. coli* cells was an insoluble, unfolded version of the protein. This purified VNT1 served as a marker in western blot analysis and for use in quantitation experiments. Due to the unfolded nature of the protein, it is an unsuitable candidate for use in digestion, toxicity, and equivalency studies. Long-chain unfolded polypeptides, like the VNT1 protein standard, are targeted differently by digestive enzymes than properly folded polypeptides and proteins.

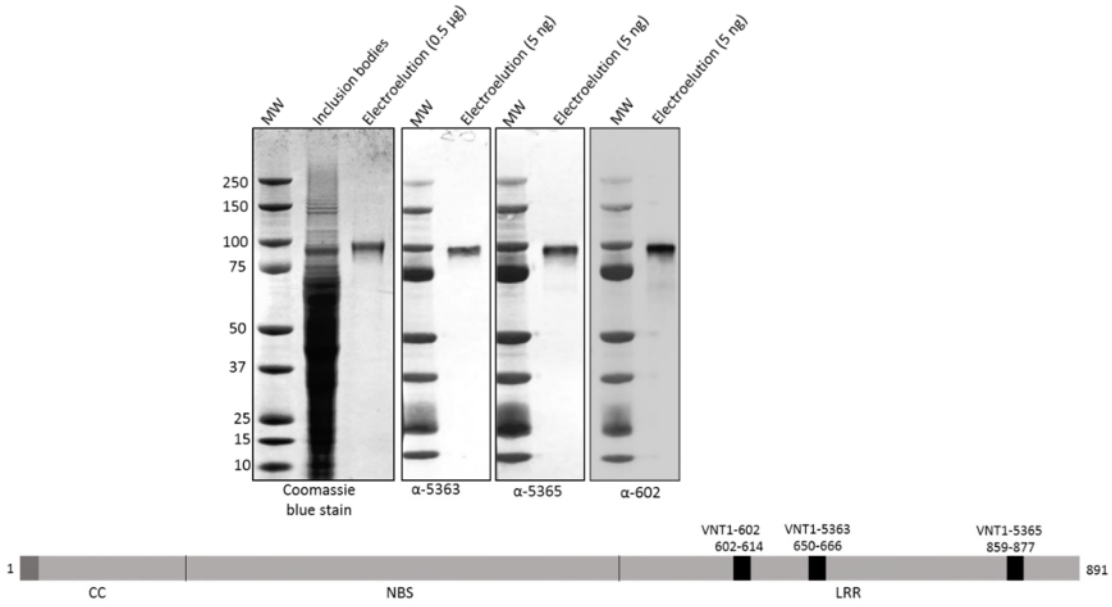


Figure 1. Expression, Purification, and Characterization of Recombinant VNT1 from *E. coli*
VNT1 protein standard was expressed and purified by electroelution from inclusion bodies in *E. coli* cells. It was analyzed using SDS-PAGE analysis and Coomassie blue staining. This confirmed the molecular weight of VNT1 to be close to 102 kDa in size. Three anti-VNT1 antibodies (α-VNT1-5363, α-VNT1-5365, and α-VNT1-602) with epitopes in the LRR domain of VNT1 were used to indicate the electroeluted VNT1 protein standard was the expected protein. N-terminal sequencing was conducted to ensure the N-terminal portion of the VNT1 standard was intact.

Determine LOQ for VNT1 using Western Blot

The VNT1 protein standard expressed and purified from *E. coli* was used to establish the LOQ for the α-VNT1-602 antibody. A serial dilution of the VNT1 protein standard spiked into Russet Burbank background was used to establish an LOQ of approximately 0.625 ng (Figure 2). Westerns using the α-VNT1-5363 antibody were included for comparison, as this antibody was used in previous studies.

Western blots using the α-VNT1-5363 antibody established an LOQ for the VNT1 standard of roughly 1.88 ng when spiked in 10 µg of leaf or tuber protein extract. The α-VNT1-602 improved specificity and sensitivity compared to the α-VNT1-5363 antibody. The LOQ for VNT1 using α-VNT1-602 was estimated at 0.625 ng (i.e. 220 ppb) in tuber and 0.625 ng (i.e. 450 ppb) in leaf, using VNT1-spiked protein extracts for analysis. The LOQ estimations for leaf and tuber were based on establishing a protein extraction method that yielded the greatest number of proteins extracted from both tissue types (Appendix B). Although the VNT1 LOQ was estimated as 0.625 ng in these experiments for both leaf and tuber, the lowest concentration of VNT1 standard used in future experiments was 1.25 ng for optimal resolution using western blot detection. Based on these data, α-VNT1-602 antibody was used for all future VNT1 western blot analyses.

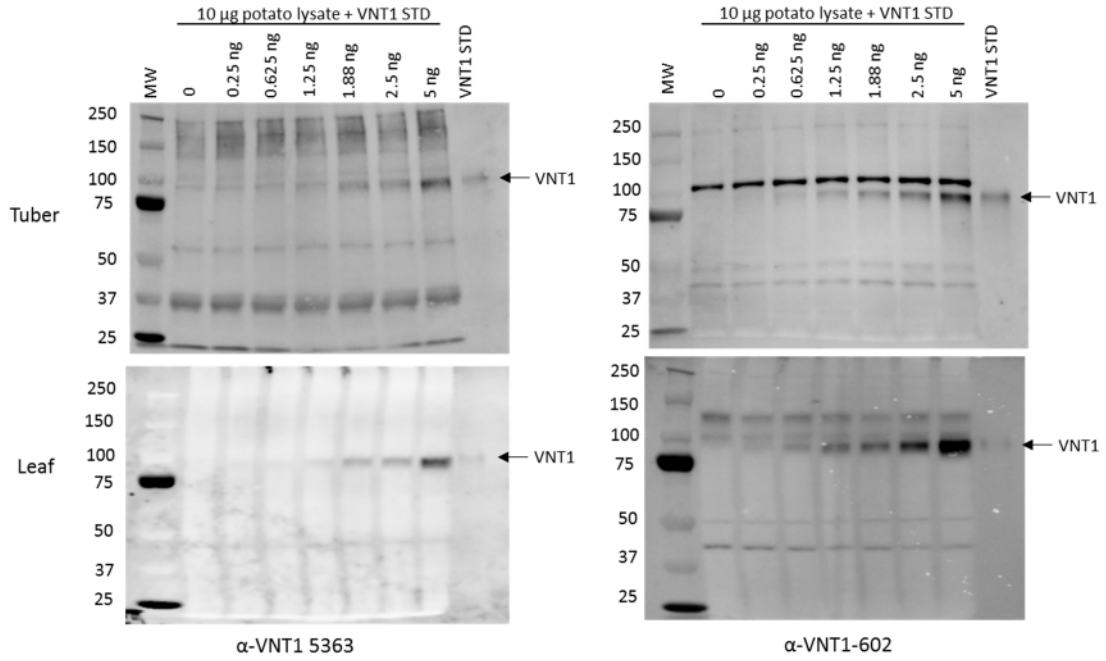


Figure 2. Limit of Quantitation for VNT1 in Tuber and Leaf using Western Blotting
A serial dilution of VNT1 protein standard spiked into Russet Burbank background was used to establish an LOQ. Western blots using α-VNT1-602 antibody (right) showed increased VNT1 specificity compared to α-VNT1-5363 antibody (left). The VNT1 protein standard (VNT1 STD) was loaded on its own in each western blot at an amount of 2.5 ng in tuber blots and 0.25 ng in leaf blots. The lowest amount of VNT1 detected in leaf and tuber samples containing 10 µg of protein was 0.625 ng. This equated to an estimated LOQ of 220 ppb for tuber and 450 ppb for leaf (see Methods for calculations).

Determining Endogenous VNT1 Concentrations using Western Blot

The VNT1 protein standard was used to determine endogenous levels of VNT1 present in W8 and Y9 plants by western blot. Protein extracts were analyzed by western blot using the α-VNT1-602 antibody. The VNT1 protein standard purified and quantified from *E. coli* provided a positive control when ran alone or when spiked into both Russet Burbank and W8 lysates at two different concentrations (2.5 ng and 1.25 ng).

Determining VNT1 Levels in W8 Samples

Extracted proteins from the Russet Burbank and W8 tuber and leaf tissues (10 µg) were analyzed by western blot using the α-VNT1-602 antibody. The protein yield and banding patterns were consistent between samples of the same tissue type as shown in the stain-free gels (left panels, Figure 3). Western blots on Russet Burbank and W8 tuber lysates showed little background, but identified a non-specific band recognized by the α-VNT1-602 antibody around 100 kDa, specifically in tuber lysates (upper right panel, Figure 3). This band was present in Russet Burbank, but not in the W8 tuber lysates. As described in Appendix C, the band was confirmed to be phosphorylase L protein (PHL), which is expressed at lower levels in W8 due to the pSIM1278 insert. The finding that the PHL band was observed in stain-free gels indicates it is an abundant protein in the lysate.

The predicted size of VNT1 is smaller than the 109 kDa PHL protein (Accession Number P04045), consistent with the migration of the VNT1 protein standard (Figure 3). Although the pSIM1278 insert reduces expression of phosphorylase L in W8 and Y9, the reduction is more pronounced in W8. The reduction of PHL in W8 tubers led to a nearly complete loss of the cross-reactive PHL protein in western blots (Figure 3), confirming that endogenous VNT1 is not co-migrating with PHL. These data showed the concentration of VNT1 in tuber and leaf samples of W8 was less than the LOQ (Figure 3).

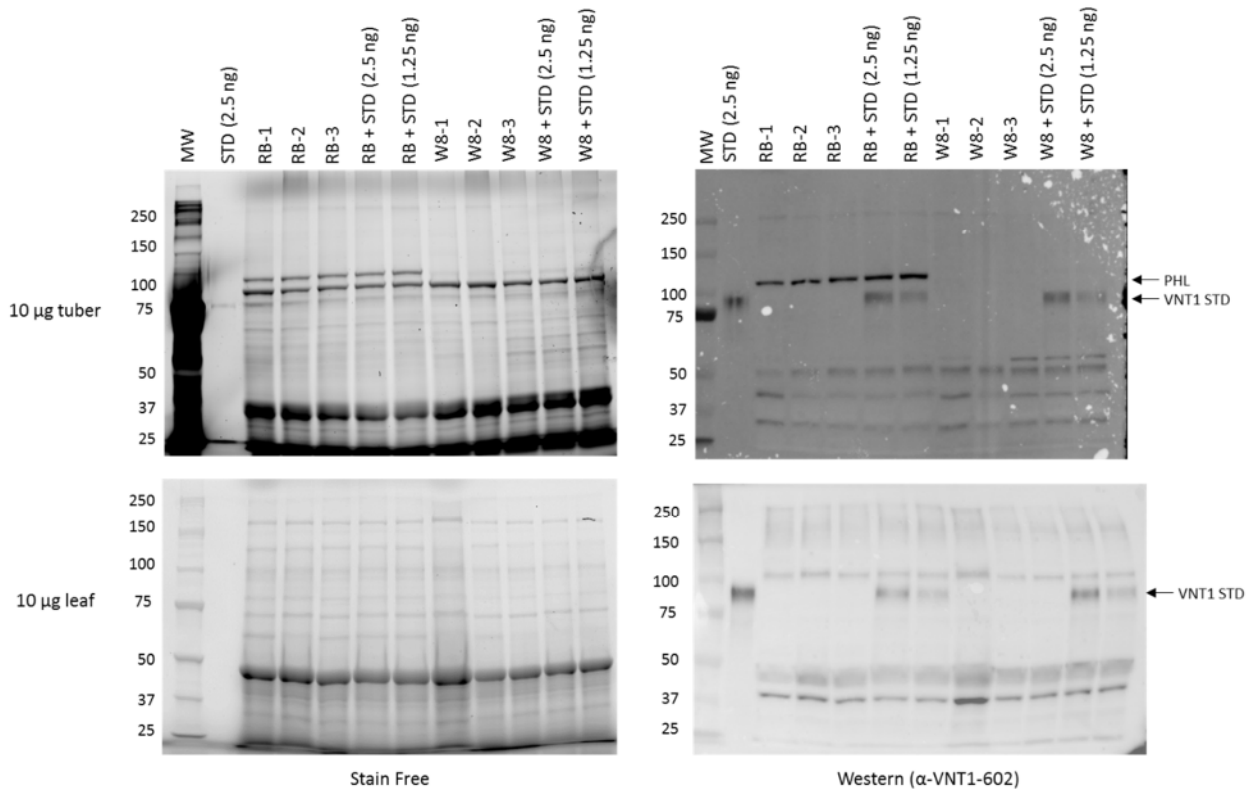


Figure 3. VNT1 was Undetected in W8 by Western Blot
VNT1 protein levels were undetected in three biological replicates of W8 leaf and tuber tissues following western blot analysis using α-VNT1-602 antibody. Russet Burbank (RB) and W8 samples were each spiked with two amounts of VNT1 protein standard (2.5 ng and 1.25 ng). Stain free SDS-PAGE gels indicated equal protein loading in samples (left). Western blot analysis of RB and W8 tissue samples showed the absence of VNT1 protein detection in both tuber and leaf (right). Tuber data are shown on the top panels and leaf data are shown on the bottom panels.

Determining VNT1 Levels in Y9 Samples

The same western blot analysis was performed on Atlantic and Y9 tuber and leaf samples. Extracted proteins from the Atlantic and Y9 tuber and leaf tissues (10 µg) were analyzed by western blot using the α-VNT1-602 antibody. The protein yield and banding patterns were consistent between samples as shown in the stain-free gels (left panels, Figure 4). The only noticeable difference between samples was the decrease in the PHL band, which was also observed in the western blot of tuber lysate (upper right panel, Figure 4). The VNT1 protein standard was included as a positive control, ran alone, and spiked into both Atlantic and Y9 lysates with two different amounts (2.5 ng and 1.25 ng). There were no bands unique to Y9 in the western blots, particularly at or above the VNT1 protein standard (VNT1 STD) (right panels, Figure 4). These data indicate that VNT1 concentrations in Y9 are below the LOQ of 220 ppb in tuber and 450 ppb in leaf.

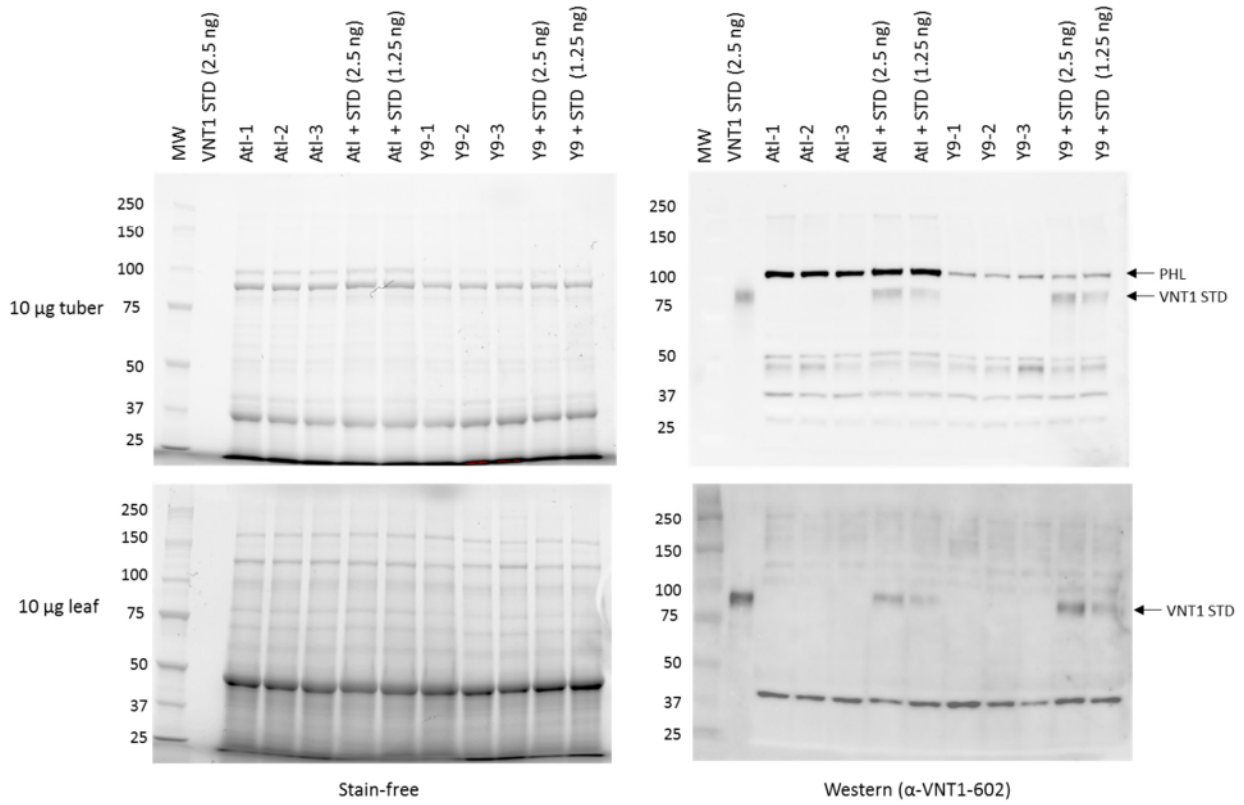


Figure 4. VNT1 was Undetected in Y9 by Western Blot
VNT1 protein levels were determined to be undetected in three biological replicates of Y9 leaf and tuber tissues following western blot analysis using the α-VNT1-602 antibody. Atlantic (Atl) and Y9 were each spiked with two amounts of VNT1 protein standard (2.5 ng and 1.25 ng). Stain free SDS-PAGE gels indicated equal protein loading in samples (left). Western blot analysis of Atl and Y9 tissue samples showed the absence of VNT1 protein detection in both tuber and leaf (right). Tuber data are shown on the top panels and leaf data are shown on the bottom panels.

The W8 and Y9 westerns presented in Figure 3 and Figure 4, respectively, were conducted using 10 µg of protein extracts per lane, consistent with the method used to establish an LOQ of 220 ppb in tuber and 450 ppb in leaf. An additional set of westerns was performed including 4-fold more protein (i.e. 40 µg) for Atlantic and Y9 tuber and leaf samples per lane. VNT1 protein standard (2.5 ng and 1.25 ng) was spiked into 40 µg Atlantic and Y9 samples for comparison. Even at these higher concentrations of total protein, endogenous VNT1 remained undetected in Y9 leaf and tuber samples (Figure 5).

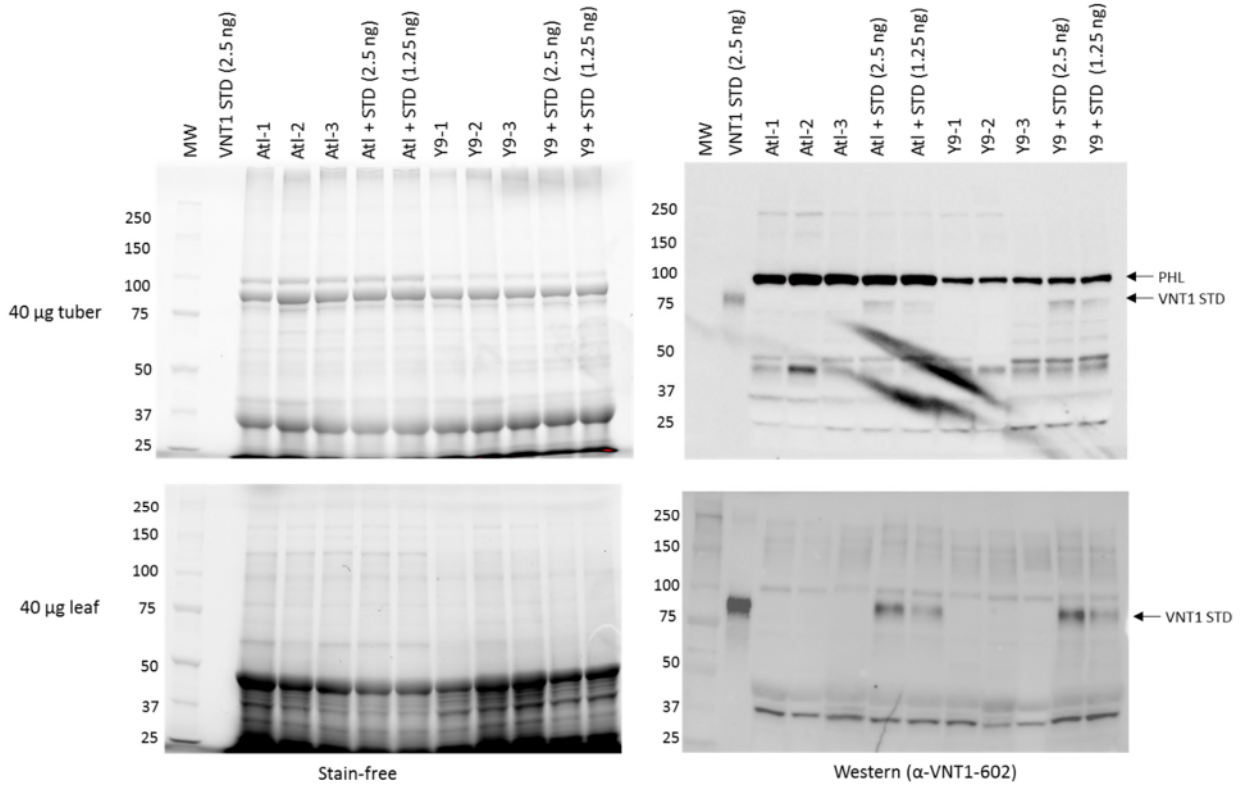


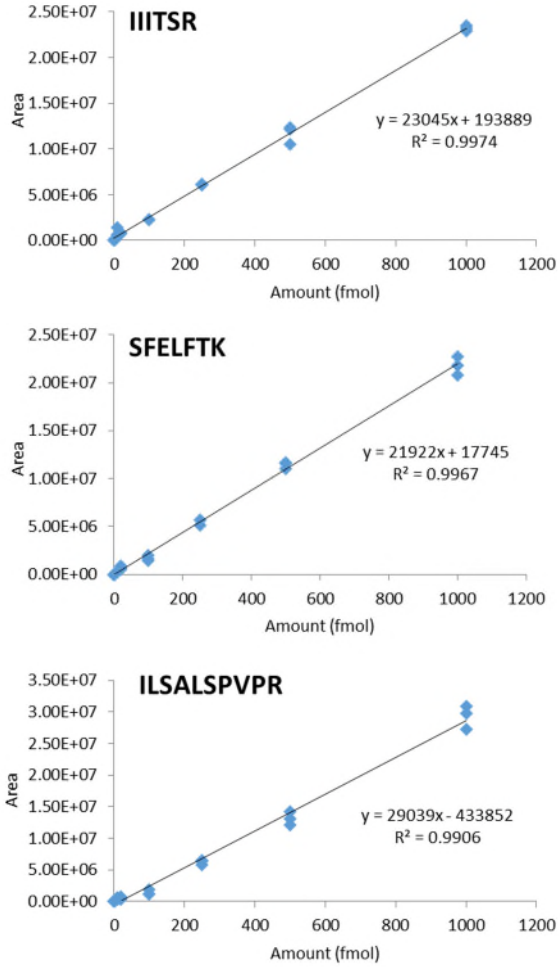
Figure 5. VNT1 is Undetected in 40 µg Y9 Protein Samples using Western Blot Analysis
Forty µg of leaf and tuber proteins were loaded into SDS-PAGE gels for LOQ analysis and VNT1 detection by western blot analysis using α-VNT1-602 antibody. Stain free SDS-PAGE gels indicated equal protein loading in samples (left). Western blot analysis of Atlantic (Atl) and Y9 tissue samples showed the absence of VNT1 protein detection in both tuber and leaf tissues (right). Tuber data are shown on the top panels and leaf data are shown on the bottom panel.

VNT1 Protein Expression Analysis by LC-MS

LC-MS was used in early experiments to evaluate expression of VNT1 in pSIM1678 transformed plants. Initial pSIM1678 transformations were done in Russet Burbank and VNT1 detection experiments, including LC-MS, were conducted on Russet Burbank events. The western blot analysis conducted on pSIM1678 transformed plants confirmed the results from early experiments using LC-MS methods for VNT1 detection and quantitation. For this reason, LC-MS analysis was only carried out on Russet Burbank events and not repeated for Y9.

Determine LOQ for VNT1 using LC-MS

LC-MS/MS analysis was performed on enriched VNT1 protein from *N. benthamiana* following in-gel tryptic digestion. As discussed in Appendix D, multiple peptides were identified that spanned the protein sequence. Three peptides (IIITSR, SFELFTK and ILSALSPVPR) that showed the highest peak intensities were selected as signature peptides for VNT1 detection. Versions of these peptides (IIITSR, SFELFTK, and ILSALSPVPR) were purified and isotopically labeled at the C-terminus. Known amounts of these heavy peptides (1-1000 fmoles) were spiked into 2.0 µg of Russet Burbank leaf protein extract trypsin digests and analyzed using an LC-MRM-MS method to determine the VNT1 limit of quantitation (LOQ) in a Russet Burbank background (Figure 6). The amount (femtomole) of heavy peptide used was linearly proportional to the MS peak intensity of each peptide. All three labeled peptides were detected at the lowest concentration tested (1 fmol), allowing for a conservative estimate of the LOQ (Figure 6). Detection of 1 fmol of VNT1 peptides spiked into Russet Burbank leaf protein extracts equated to an LOQ of 500 ppb (ng of VNT1 per g of leaf tissue). The 500 ppb LOQ is a conservative estimate, as the peak intensity of the 1 fmol samples was higher (0.5-1.0 order of magnitude) than in the unspiked, background samples (0 fmol). The MW of VNT1 is estimated at 102 kDa, so 1 fmol equates to 100 pg of VNT1. BCA analysis determined the concentration of leaf protein extracts as 1.6 mg/mL from 150 mg of leaf tissue, so 2 µg of the trypsin digested extracts corresponds to 190 µg of leaf tissue. As a result, 100 pg of VNT1 in 190 µg of leaf tissue equals 500 ppb.



Peptide	Amount (fmol)	Area
IIITSR	0	1.11E+04
	1	3.53E+04
	10	7.89E+05
	20	7.77E+05
	100	2.27E+06
	250	6.10E+06
	500	1.17E+07
	1000	2.32E+07
SFELFTK	0	8.53E+03
	1	2.81E+04
	10	2.91E+05
	20	6.64E+05
	100	1.71E+06
	250	5.49E+06
	500	1.14E+07
	1000	2.18E+07
ILSALSPVPR	0	6.71E+03
	1	2.70E+04
	10	4.40E+05
	20	5.67E+05
	100	1.46E+06
	250	6.19E+06
	500	1.32E+07
	1000	2.94E+07

Figure 6. VNT1 Limit of Quantitation (LOQ) in Spiked Russet Burbank Leaf Protein Extracts
IIITSR, SFELFTK, and ILSALSPVPR were identified as signature peptides for VNT1 detection and LOQ determination. These peptides were heavy labeled and spiked into 2.0 µg of Russet Burbank leaf tissue. LOQ was determined for each peptide using an LC-MRM-MS method and by measuring the peak intensity (area) at each peptide concentration (fmol). The labeled peptides were all detected at the lowest concentration tested (1 fmol), which equates to an LOQ of 500 ppb.

VNT1 Protein Levels in Potato Samples

Total protein was extracted from leaf tissue of pSIM1278- and pSIM1678-transformed plants (W3 and W8) and *S. venturii*, from which the *Rpi-vnt1* gene was cloned. Three replicates of each sample were analyzed by the UC Davis Proteomics Core facility using an LC-MRM-MS method. Protein lysates from each sample were spiked with heavy peptides (IIITSR, SFELFTK, and ILSALSPVPR) for use as controls. The spiked heavy peptides were detected in each sample, whereas the signature peptides (IIITSR, SFELFTK, and ILSALSPVPR) indicating the presence of endogenous VNT1 were not detected in W3, W8, or *S. venturii* (Figure 7). All endogenous (unlabeled) peptide levels in W3, W8, and *S. venturii* were similar to Russet Burbank, indicating that this detection was low-level background. These data showed that VNT1 is present in plants expressing *Rpi-vnt1* at levels below the LOQ of 500 ppb.

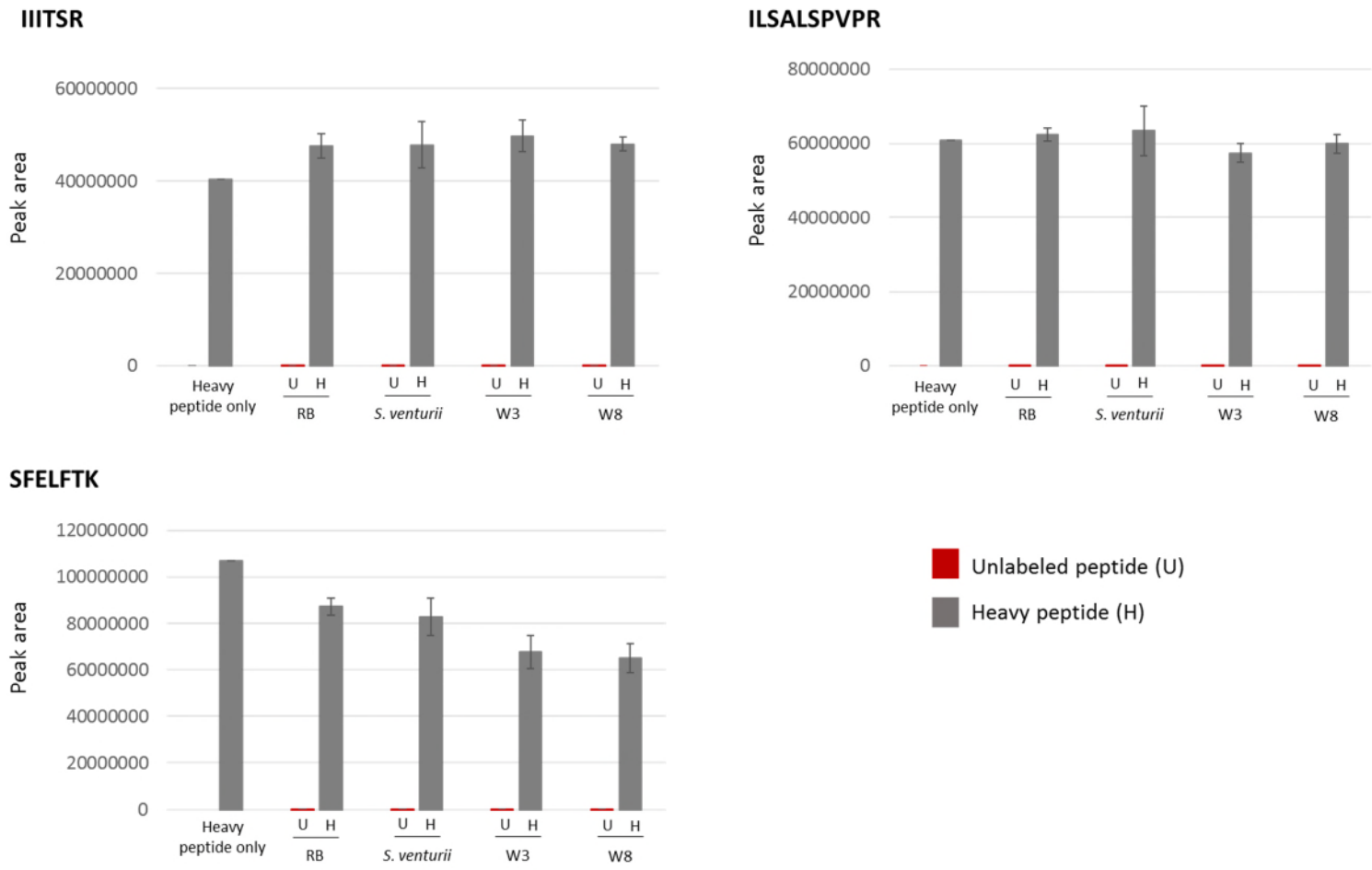


Figure 7. VNT1 was Undetected in W3, W8, and *S. venturii* Leaf Tissue Lysates

Three replicates were used from Russet Burbank (RB), two pSIM1278- and pSIM1678-transformed varieties, W3 and W8, and *S. venturii* to determine if endogenous VNT1 could be detected in leaf tissue samples. LC-MRM-MS methods were used to search for the three signature peptides (IIITSR, SFELFTK, and ILSALSPVPR) in all of the samples. Heavy peptides were spiked into leaf tissue lysates of all the samples to use as controls for detection. Negligible background levels of unlabeled, endogenous peptides (red, U) were detected within the samples, however the heavy peptides (light gray, H) were quantitatively detected in each sample.

The LC-MS analysis performed on Russet Burbank and pSIM1678 transformed events (W3 and W8) corroborate those results found using western blot analysis for VNT1 quantitation and detection of RB and W8. LC-MS analysis indicated an LOQ for VNT1 detection of 500 ppb in leaf material. The western blot analysis established an LOQ of 220 ppb in tuber and 450 ppb in leaf materials for the detection of VNT1. The VNT1 LOQ values are similar between the assays. Since no detectable VNT1 protein was identified in any Russet Burbank or Atlantic pSIM1678-transformed leaf and tuber samples, the levels in these events is conservatively concluded to be below the LOQ of 500 ppb. Additionally, since the western blot results were consistent between W8 and Y9 where VNT1 levels were below the LOQ for each, it is expected that VNT1 detection in Y9 leaf samples would follow the same undetectable pattern as that observed with W8 following LC-MS analysis.

CONCLUSION

The *Rpi-vnt1* gene, which encodes the VNT1 protein and confers resistance to late blight, was introduced into Atlantic to generate Y9. A western blot assay using a VNT1-specific antibody was developed. The western blot assay was able to detect and quantify VNT1 protein with an LOQ of less than 450 ppb in leaf and 220 ppb in tuber tissues. Using this method, VNT1 was not detected in Y9 leaf and tuber field samples, indicating that the amount of VNT1 is below the LOQ. A LC-MS method was developed to detect and quantify VNT1 protein with an LOQ of 500 ppb in leaf tissue and corroborate the western blot assay. A conservative estimate for VNT1 in Y9 tissues was established to be less than 500 ppb.

REFERENCES

Bakker, E., Borm, T., Prins, P., van der Vossen, E., Uenk, G., Arens, M., de Boer, J., van Eck, H., Muskens, M., Vossen, J., et al. (2011). A Genome-Wide Genetic Map of NB-LRR Disease Resistance Loci in Potato. Theoretical and Applied Genetics *123*, 493–508.

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}K 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.

APPENDIX A: VNT1 Standard Quantitation

[CCI]

APPENDIX B: Establishing a Protein Extraction Method

[CCI]

APPENDIX C: Antibody α-VNT1-602 Cross-reacts with PHL

[CCI]

APPENDIX D: Mass Spectrometry Analysis of [CCI] Enriched VNT1 from *N. benthamiana*

[CCI]