*St*PIP1, a PAMP-induced peptide in potato, elicits plant defenses and is associated with disease symptom severity in a compatible interaction with potato virus Y

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Running Title: *St*PIP1 in the potato-PVY compatible interaction

Footnotes:

Title

*St*PIP1, a PAMP-induced peptide in potato, elicits plant defenses and is associated with disease symptom severity in a compatible interaction with potato virus Y

Highlight

We demonstrate the role of *StPIP1*, a gene encoding a predicted small secreted peptide, in the plant anti-viral immunity, and show that it has a role in the determination of tolerance versus sensitivity.

Abstract

The role of small secreted peptides in plant defense responses to viruses has been seldom investigated. Here, we report a role for potato (*Solanum tuberosum*) *PIP1*, a gene predicted to encode a member of the PAMP-induced peptide (PIP) family, in the response of potato to potato virus Y infection (PVY). We show that exogenous application of synthetic *St*PIP1 to potato leaves and nodes increased the production of reactive oxygen species and the expression of plant defense-related genes, showing that *St*PIP1 triggers early defense responses. In support of this hypothesis, transgenic potato plants that constitutively overexpress *StPIP1* had higher levels of leaf callose deposition, and based on measurements of viral RNA titers, were less susceptible to infection by a compatible PVY strain. Interestingly, systemic infection of *StPIP1*-overexpressing lines with PVY resulted in clear rugose mosaic symptoms that were absent or very mild in infected non-transgenic plants. A transcriptomics analysis revealed that marker genes associated with both pattern-triggered immunity and effector-triggered immunity were induced in infected *StPIP1*-overexpressors but not in non-transgenic plants. Together, our results reveal a role for *StPIP1* in eliciting plant defense responses and in regulating plant anti-viral immunity.

Keywords

Viruses, Potato, Pathogen-Associated Molecular Patterns (PAMPs), Pattern-triggered immunity, Peptides, Symptoms

Introduction

Plants have evolved a multi-layer immune system to allow them to deal with the threat of pathogens such as bacteria, fungi, and viruses (Jones and Dangl, 2006; Wang *et al.*, 2019). A first

layer of defense is provided by cell-surface receptors called pattern recognition receptors (PRRs) that detect conserved features of pathogens termed pathogen-associated molecular patterns (PAMPs) in the extracellular space (Boutrot and Zipfel, 2017). Upon detection of PAMPs by PRRs, plant cells initiate immune responses including release of Ca^{2+} ions, reactive oxygen species (ROS), increased expression of pathogen response genes, and deposition of callose at the site of infection, ultimately producing increased resistance to pathogens termed pattern-triggered immunity (PTI) (Jones and Dangl, 2006). Because viruses are obligate intracellular parasites, the extent to which PTI is involved in plant defense against virus has been understated. Recent studies have revealed that virus components can act as PAMPs and trigger PTI-like responses through PRR co-receptors like SERK1 and NIK1 (Gouveia *et al.*, 2017; Niehl *et al.*, 2016; Zvereva *et al.*, 2016).

To overcome PTI, pathogens have evolved proteins called effectors that act to disable the plant innate immunity and allow them for entry into the cell or greater access to host resources. Plants in turn have evolved intracellular receptors, often called R proteins, which detect effectors and initiate effector triggered immunity (ETI) (Jones and Dangl, 2006). Detection of effectors by R proteins triggers an intense immune response, sometimes resulting in programmed cell death, a reaction known as the hypersensitive response (HR) (Valkonen *et al.*, 2017).

Plants have a diverse array of small endogenous peptides, also known as phytocytokines, which are released from pathogen challenged-cells (Gust *et al.*, 2017). These small secreted peptides (SSPs), such as the plant elicitor peptides (PEPs) and PAMP-induced peptides (PIPs), trigger or modulate PTI-like immune responses in neighboring cells, priming them to defend against an oncoming infection. The PIPs in *Arabidopsis*, like other SSPs, are produced as precursor polypeptides (prepropeptides) with N-terminal signal sequences recognized by the secretion pathway. After entering the secretion pathway, the N-terminal signal sequence is removed, and the resulting propeptide further processed into small (~15-25 amino acids) mature peptides. Ultimately, the fully mature peptides are released into the extracellular space where they are perceived, like PAMPs, by PRR-like receptors on neighboring cells (Hou *et al.*, 2014; Matsubayashi, 2018). In Arabidopsis, *AtPIP1* and *AtPIP2* are expressed in response to PAMPs such as flagellin and chitin, and trigger PTI-like immune responses, including ROS and defense gene expression, in perceiving cells (Hou *et al.*, 2014). *AtPIP3* was shown to modulate plant immunity by regulating cross talks between salicylic acid and jasmonic acid signaling pathways (Najafi *et al.*, 2020).

Potato Virus Y (PVY) is the type member of the largest group of RNA plant viruses, the *Potyviridae* (Wylie et al., 2017). Its host range is broad, infecting most solanaceous species

including potato, tomato, peppers, and tobacco, in addition to other plant groups. PVY is listed as one of the top 10 plant viruses in terms of scientific and economic importance (Scholthof *et al.*, 2011). PVY is a single-stranded, positive-sense RNA virus with a genome of 9.7 kb, and exists as a large number of strains, variants, recombinants and isolates. The most commonly found strains in growers' fields are the necrotic strain N and recombinants between the N and O strains, N-Wilga, NTN, and N:O. The O strain occurrence has been declining to low levels in recent years (Funke *et al.*, 2017; Karasev and Gray, 2013).

The cultivated potato, *Solanum tuberosum* L., is the fourth most cultivated staple food crop worldwide (FAOSTAT, 2017). Because of its high level of heterozygosity, potato is propagated vegetatively by using tubers as seeds to ensure genetic identity of the progeny. To maintain low levels of pathogens in potato seed production, seed lots are regularly inspected. Due to its prevalence, PVY is currently the number one reason for seed lots rejection (Karasev and Gray, 2013). The symptoms caused by PVY infection in potato vary depending on the viral strain and host cultivar. Common symptoms include foliar mosaic, rugose mosaic, leaf wrinkling and various necrotic lesions (Lacomme and Jacquot, 2017).

In a previous study, we profiled PVY-induced changes in the transcriptome of potato cultivar Premier Russet (PR), and identified PGSC0003DMG400014879, a gene predicted to encode a PIP family protein, as the most significantly differentially expressed (DE) gene in an incompatible interaction with PVY^{O} (Goyer *et al.*, 2015). This prompted us to investigate the function of this gene in the potato-PVY interaction. In this study, we provide evidence that the gene PGSC0003DMG400014879 encodes a peptide that belongs to the PIP family, and named it *StPIP1.* Transgenic potato plants overexpressing *StPIP1* produced clear rugose mosaic symptoms that were absent or very mild in control plants when infected with a compatible strain, PVY^{NTN}. Our transcriptomics data showed that marker genes of PTI and ETI were induced in infected *StPIP1*-overexpressors but not in non-transgenic plants. This study reveals a function for plant PIP peptides in antiviral immunity.

Materials and methods

Sequence analyses

Full-length genomic, transcript, and polypeptide sequences for PGSC0003DMG400014879 were retrieved from Spud DB

(http://solanaceae.plantbiology.msu.edu/) (Hirsch *et al.*, 2014). Sequence data for *Arabidopsis* genes were retrieved from The Arabidopsis Information Resource (TAIR10) on www.arabidpsis.org (Berardini *et al.*, 2015). The BLAST suite from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find related genes and proteins (Altschul *et al.*, 1990). For putative members of small secreted peptide families in tomato and potato, published peptide sequences of each family (CLV3/CLE, IDA/IDL, CEP, PIP/PIPL) in *Arabidopsis* were used as queries for tBLASTN and BLASTp searches (Supplementary Table S1). Signal peptides were predicted using the programs Phobius (http://phobius.sbc.su.se/) and SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) (Armenteros *et al.*, 2019; Kall *et al.*, 2007). The programs Predotar (https://urgi.versailles.inra.fr/predotar/) (Small *et al.*, 2004), PSORT (http://psort1.hgc.jp/form.html) (Nakai and Horton, 1999), and TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson *et al.*, 2000) were used to predict subcellular localizations. Three-dimensional structure prediction was done with PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley *et al.*, 2015). Cis-regulatory elements were searched in PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al.*, 2002). Multiple sequence alignments were performed with Muscle using default settings (Edgar, 2004) and phylogenetic trees were constructed from those alignments using the maximum likelihood method, 1000 times bootstrapped with Mega7 (https://www.megasoftware.net/) (Kumar *et al.*, 2016).

Plant growth

Potato plants cultivar PR were propagated *in vitro* on solid MS medium (1x MS modified BC potato salts, 2% sucrose, 100 mg/l myo-inositol, 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.1 mg/l thiamin, pH 5.6). After 3-4 weeks, plants were transferred to one-gallon pots filled with soil (four parts potting mix, one part sand) containing slow-release fertilizer (Osmocote Plus) in the greenhouse. Greenhouse temperature conditions were set at 21°C day, 15°C night. Supplemental light was provided by 400-Watt high-pressure sodium lamps to maintain a 14-hour photoperiod. Plants were arranged in a randomized split-block design with six plants per treatment. Treatments included inoculation with two different strains of PVY (O or NTN) and a mock inoculation control. For assessing virus translocation to tubers, three progeny tubers from

each plant were selected, treated with 7 ppm GA3 and incubated for 2-4 weeks at 27°C to break dormancy, and planted to one-gallon pots (three tubers per pot) filled with potting mix.

PVY stocks and inoculation

The PVY strain O isolate used in this study was first identified in a potato tuber from Aberdeen, ID, in 1999 by James Crosslin (USDA/ARS). The PVY NTN HR1 isolate (Genebank ID FJ204166) was donated by Dr. Alexander Karasev (University of Idaho). Mechanical inoculation of potato leaves was done as previously described (Vinchesi *et al.*, 2017).

PVY detection

PVY was detected by reverse transcription (RT)-PCR. Nucleic acids were extracted using a protocol adapted from (Dellaporta *et al.*, 1983). Briefly, three upper leaflets per plant were excised, placed into mesh bag (Agdia®), and pulverized in a buffer containing 100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 500 mM NaCl, and 10 mM 2-mercaptoethanol. Seventy microliters of 10% sodium dodecyl sulfate were added to a 600-µl aliquot of the resulting slurry, mixed, and the sample was incubated at 65˚C for 10 min. To each sample, 200 µl of 5 M acidified potassium acetate (pH 5.7) was added and samples were incubated on ice for 10 min. After centrifugation at 15,900 x g for 10 min, the supernatant was transferred to a new tube. After precipitation with 300 µl cold isopropanol, samples were centrifuged, and the pellet was washed with 70% ethanol, and resuspended in 400 µl deionized water. Nucleic acid extracts were used as templates to synthesize cDNAs with M-MuLV reverse transcriptase using a mixture of random hexamers and oligo$(dT)_{18}$ primers. Resulting cDNAs were used as templates in a multiplex PCR assay as previously described (Lorenzen *et al.*, 2006). Primers sequences are shown inSupplementary Table S2.

Molecular cloning

StPIP1-overexpressing plants - Total RNAs were extracted from leaves from the potato variety PR using the hot phenol method as described previously (Goyer *et al.*, 2015) and treated

with DNase (Ambion® DNA-free™ kit, LifeTechnologies). cDNAs were synthesized by M-MuLV Reverse Transcriptase (New England Biolabs) using an oligo$(dT)_{18}$ primer, and the PGSC0003DMG400014879-encoded cDNA was amplified using PrimeSTAR Max DNA Polymerase (Takara) using the following forward and reverse primers, respectively: 5'-GAATAACCACACAACTCAAC-3' and 5'-AGATTGGTAACAACCATCCA-3'. The 658-bp amplicon was directly cloned into pCR™4Blunt TOPO® vector (ThermoFisher Scientific), and the resulting construct was introduced into One Shot TOP10 *E. coli* cells (ThermoFisher Scientific). Sixteen kanamycin-resistant isolated colonies were then cultured in LB medium supplemented with 50 mg/l kanamycin. Plasmid DNA was extracted from each culture and sent for Sanger sequencing. Sequences alignment showed that PGSC0003DMG400014879 has four alleles encoding three protein isoforms in PR. Clone 1-1, which represents the most dominant allele, was used as template to amplify a 312-bp amplicon using the following forward and reverse primers, respectively: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGAGGGGTCAATT-CATTGTACACATT-3', and 5'-GGGGACCACTTTGTACAAGAAAGCTGG-GTTCATGTCACTATTGATGATGGCTATT-3'. The 312-bp amplicon was then ligated into pDONR™/Zeo vector (ThermoFisher Scientific) using BP clonase following the manufacturer's recommendations, and then subcloned into the pMDC32 plant binary vector (Curtis and Grossniklaus, 2003) by recombination using LR clonase. The final construct was verified by restriction digestion and Sanger sequencing. Primers sequences are shown in Supplementary Table S2.

StPIP1-silencing plants – Artificial micro RNAs (amiRNAs) targeting PGSC0003DMG400014879 were designed using the program WMD3 (http://wmd3.weigelworld.org). The full-length transcript (PGSC0003DMT400038539) was retrieved from SpudDB and used as a target for the WMD3 designer program. The transcript library 'Solanum_tuberosum_v183.mRNA.PUT.fasta' was used to check specificity, with the program set to accept no predicted off-targets. The amiRNA hairpin precursors were produced by overlapping PCR following a procedure recommended by the authors of WMD3 (Ossowski *et al.*, 2008). The first fragment, *a*, was 424-bp and was amplified from the plasmid pRS300 (Addgene) using the following forward and reverse primers, respectively: 5'-CTGCAAGGCGATTAAGTTGGGTAAC-3' (pRS300a) and 5'-GATAGACGGTTGTTACCAATCTATCAAAGAGAATCAATGA-3'. The second fragment, *b*, was 301-bp and amplified from pRS300 using the following forward and reverse primers, respectively: 5'-GATAGATTGGTAACAACCGTCTATCTCTCTTTGTATTCC-3' and 5'-

GCGGATAACAATTTCACACAGGAAACAG-3' (pRS300b). Fragments *a* and *b* were gel purified and a 1:1 ratio of each was used as template to amplify the 701-bp fragment *c* using pRS300a and pRS300b. Fragment *c* was gel purified and the 274-bp fragment *d* was amplified using fragment *c* as template and using the following forward and reverse primers, respectively: pRS300a and 5'-GAAAGATTGCTAACAACCGTTTATCTACATATATATTCCT-3'. Fragment *c* was again used as template to amplify the 451-bp fragment *e* using the following forward and reverse primers, respectively: 5'-GATAACGGTTGTTAGCAATCTTTCACAGGTCGTGATATG-3' and pRS300b. Fragments *e* and *d* were gel purified and a 1:1 ratio of each was used as template to amplify the 705-bp fragment *f* using the following forward and reverse primers, respectively: 5'-CACCCTGCAAGGCGATTAAGTTGGGTAAC-3' and pRS300b. Fragment *f* was cloned into pENTR™/D-TOPO® (ThermoFisher Scientific) using the TOPO cloning reaction following the manufacturer's recommendations. The insert was released by digestion with *Apa*I and *Sac*I restriction enzymes, gel-purified and subsequently ligated into the binary vector pMDC32 previously digested with *Apa*I and *Sac*I restriction enzymes under control of the CaMV 35S promoter. The final construct was named 'pMDC32-PIPmiRNA'. Primers sequences are shown in Supplementary Table S2.

Potato transformation

DNA constructs were introduced into the potato cultivar PR by *Agrobacterium tumefaciens* (strain EHA105)-mediated stable transformation as previously described (Chetty *et al.*, 2015). Briefly, single isolated *A. tumefaciens* colonies containing the transformation vector were grown in 50-ml YEP culture to saturation. A 10-ml aliquot was pelleted, and cells were resuspended in 40 ml of MS medium supplemented with 200 µM acetosyringone to an OD_{600} of 0.8. Potato stem internodes (~5-10-mm long) were incubated for 15 min in the *Agrobacterium* suspension in a 50-ml Falcon tube with gentle shaking. Internodes were then blotted dried on Whatman paper and placed on Petri dishes containing Callus Inducing Medium (CIM) (MS medium supplemented with 0.2 mg/l 1-napthalenic acetic acid, 0.02 mg/l GA3, 2.5 mg/l trans-zeatin riboside) supplemented with 200 µM acetosyringone and overlaid with sterile Whatman filter paper in the dark for two days at room temperature. Internodes were then washed with water supplemented with 250 mg/l cefotaxime, blotted dried, and transferred to Petri dishes containing CIM supplemented with 20

mg/l hygromycin, 250 mg/l cefotaxime, and 200 mg/l carbenicillin. After incubation for two weeks, explants were transferred to Shoot Inducing Medium (SIM) (MS medium supplemented with 0.02 mg/l 1-napthalenic acetic acid, 0.02 mg/l GA3, 2 mg/l trans-zeatin riboside) supplemented with 20 mg/l hygromycin, 250 mg/l cefotaxime, and 200 mg/l carbenicillin. Explants were transferred to new SIM every two weeks. When shoots grew to approximately 1 cm in length (after ~8 weeks on SIM), they were excised and transferred to MS medium supplemented with 20 mg/l hygromycin, 250 mg/l cefotaxime, and 200 mg/l carbenicillin. Plantlets that developed roots under hygromycin selection were then genotyped by PCR.

RT-qPCR

For RT-qPCR from whole leaflets, total RNAs were extracted as described before (Goyer *et al.*, 2015). cDNAs were synthesized as described above except that oligo$(dT)_{18}$ only was used. cDNAs were used as template for quantitative PCR using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent). Primers targeting *StPIP1*, PVY, and reference genes *18S rRNA*, *L2*, and *EF1α*) are shown in Supplementary Table S3.. Details of RT-qPCR conditions are shown in Supplementary Table S5 following the Minimum Information for publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009; Graeber *et al.*, 2011).

For RT-qPCR on potato leaf discs, total RNAs were extracted and cDNAs were synthesized as described before (Moroz *et al.*, 2017). Primers used to measure expression of defense-related genes (*StPR1b, StPR5, StWRKY, StERF3, StPAL1,* and *StJas*) and reference genes (*StUbq* and *StEF1-alpha*) are described in Supplementary Table S4. Details of the workflow according to the MIQE guidelines are shown in Supplementary Table S6.

Calculations were done according to published methods (Schmittgen and Livak, 2008; Taylor *et al.*, 2019). Statistical analyses were done using analysis of variance (ANOVA) or Student's *t*-test from the log transformed normalized expression.

QuantSeq analysis

Total RNAs were extracted from upper leaves (one leaflet from each of three plants) of PR and *StPIP1*-overexpressing (PIP-OE) plants infected or not with PVY^{NTN} (44 days post inoculation

(dpi), 70 days after transplantation) as described before (Goyer *et al.*, 2015). RNAs were then sent to the Core Labs of the Oregon State University Center for Genome Research and Biocomputing for RNA quality control, library preparation and sequencing. RNA quality was checked with an Agilent 2100 bioanalyzer (Plant RNA Nano Chip, Agilent). Libraries were prepared from 500 ng RNA using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina using the manufacturer's recommendations (Lexogen). Library size was verified on an Agilent TapeStation 4200 using High Sensitivity D5000 Screen Tape®, and libraries were quantified by qPCR before sequencing on an Illumina HiSeq3000 (50-bp single end). Read quality was verified using FASTQC. Adapters and poly-A tails were removed from reads using cutadapt. Trimmed reads were then aligned to the potato reference genome (DM_v4.04) using the STAR aligner. Output alignment .bam files were used to calculate the number of reads mapping to exons using HTSeq (Anders *et al.*, 2015) in the "Intersection (nonempty)" mode. Differentially expressed exons were determined from HTSeq count tables using DESeq2 (Love *et al.*, 2014) with parametric fit. Functional enrichment analysis of significantly DE genes was done with g:GOSt in g:Profiler (Raudvere *et al.*, 2019) (https://biit.cs.ut.ee/gprofiler/gost). Genes were considered significantly DE if they had adjusted p-values (q) ≤ 0.05.

Measurements of second messengers Ca^{2+} and ROS

To measure cytosolic Ca^{2+} concentration, aequorin-based luminescence assay was performed using aequorin-expressing transgenic potato cultivar Désirée. Procedures of reconstitution, luminescence measurement, and data analysis and normalization were described in a previous publication (Moroz and Tanaka, 2020). Leaf discs (5-mm diameter) were harvested from 5-6-week-old plants and used for the assay. For ROS measurement, luminol-based chemiluminescence assay was performed as described previously (Moroz and Tanaka, 2020). Leaf discs (5-mm diameter) and nodes (5-mm long) were harvested from 5-week-old plants and used for the assay. Results were expressed as relative light units (RLUs/tissue) after subtraction of the data at time 0 from those at each time point of the measurement.

Callose analysis

Callose analysis was as previously described (Adam and Somerville, 1996; Gomez-Gomez *et al.*, 1999), with minor modifications. Potato leaflets (~10 weeks after transfer from tissue culture to soil) were placed in a 3:1 (v/v) solution of ethanol and lactophenol (1:1:1:1 v/v of phenol: glycerol: lactic acid: water) and left stationary for one week. The cleared leaflets were then incubated sequentially in 50% ethanol overnight, 67 mM K_2HPO_4 (pH 12) for one hour, and 0.01% aniline blue in 67 mM K_2HPO_4 (pH 12) for one hour. The midvein of each potato leaflet was then removed and half of the leaflet mounted in 70% glycerol, K_2HPO_4 (pH 12) on a glass microscope slide. Callose deposits were detected by UV epifluorescence using a Leica MZFLIII stereomicroscope. The entire half leaflet was examined for callose deposits and 2-3 representative pictures were taken from six leaflets per genotype. Callose spots were counted in a 1-mm^2 area in the center of each picture. Graphed data is the average callose spots in the 1-mm^2 area from 14 pictures per genotype and error bars represent the standard error of the data.

Results

Bioinformatics analyses predict that PGSC0003DMG400014879 belongs to the family of PAMP-induced-peptides (PIP)

In a previous study, we identified PGSC0003DMG400014879 as the most highly repressed gene in the cultivar PR in response to PVY^O inoculation (Goyer *et al.*, 2015). This gene is annotated as an ATPase Binding Cassette (ABC) transporter family protein in the potato genomics resource database Spud DB. However, ABC transporters are made of four major subunits with two transmembrane hydrophobic domains and two nucleotide binding domains and contain the amino acid signature sequence [LIVMFY]S[SG]GX3[RKA][LIVMYA]X[LIVFM] as consensus (Kang *et al.*, 2011). In contrast, the protein predicted to be encoded by PGSC0003DMG400014879 does not contain the canonical signature sequence. Furthermore, three-dimensional structure prediction analysis using PHYRE2 indicated no secondary structure aside from an alpha helix in the predicted N-terminal signal peptide region (see below) (Supplementary Fig. S1). These observations indicated that the PGSC0003DMG400014879 gene is not correctly annotated, possibly due to similarities between the C-terminal part of the PGSC0003DMG400014879-encoded protein and

329 protein sequences of P-loop NTPase superfamily members, which include ABC transporters
330 (Pathak *et al.*, 2014).

331 A BLASTp search of non-redundant protein sequences using the predicted protein product
332 of PGSC0003DMG400014879 as the query sequence resulted in many matches with annotations
333 such as 'hypothetical' or 'uncharacterized'. Eleven hits were from the Solanaceae (E-values $\leq 10^{-20}$, coverage $\geq$ 89%, identity $\geq$ 55%) and were often predicted to be small (<100 amino acids)
335 polypeptides, while there were no significant matches with ABC transporter-annotated sequences.
336 One hit (47% identity, 61% similarity 97% coverage) was annotated as "precursor of CEP16-like"
337 (from *Hevea brasiliensis,* sequence ID: XP_021642728.1). CEPs (C-Terminally Encoded Peptides)
338 are a class of secreted peptides (Roberts *et al.*, 2013). These results suggested that
339 PGSC0003DMG400014879 may belong to a family of genes encoding small secreted peptides. To
340 confirm this hypothesis, we performed phylogenetic analyses with amino acid sequences from
341 small secreted peptides including CLAVATA3 (CLV3/CLE) (Yamaguchi *et al.*, 2016), CEP
342 (Roberts *et al.*, 2013), INFLORESCENCE DEFICIENT IN ABSCISSION (IDA)/IDA-Like (IDL)
343 (Vie *et al.*, 2015), and PIP/PIP-Like (PIPL) (Najafi *et al.*, 2020; Vie *et al.*, 2015) families from
344 Arabidopsis and tomato, two nearby paralogs of PGSC0003DMG400014879 on chromosome 3,
345 PGSC0003DMG400014880 and PGSC0003DMG400014833, and one gene,
346 PGSC0003DMG400024991, on chromosome 2 with 63% similarity (Fig. 1). The encoded-potato
347 peptides grouped within the PIP and CEP branches, most closely to *At*PIP2 and *At*PIP3. Based on
348 these results, we named PGSC0003DMG400014879, PGSC0003DMG400014880,
349 PGSC0003DMG400014833, and PGSC0003DMG400024991 *StPIP1, StPIP2, StPIP3, and*
350 *StPIP4*, respectively.

351 A multiple sequence alignment highlights the similarity and identity between the Arabidopsis
352 PIPs and the potato proteins (Fig. 2). Particularly conserved are the 'RPL' motif defining the
353 predicted signal peptide cleavage point (see below), and the 'GPS(P)xGxGH' motif within the
354 propeptides (Hou *et al.*, 2014). The propeptides of *St*PIP1, 2, and 3 have two conserved
355 'GPS(P)xGxGH' motifs, while *At*PIP1 and *St*PIP4 have only one (Hou *et al.*, 2014; Najafi *et al.*,
356 2020; Vie *et al.*, 2015).

357 In agreement with a functional prediction as a secreted peptide, the programs Predotar,
358 PSORT, and TargetP predicted a subcellular localization in the 'endoplasmic reticulum' (99%
359 probability), 'outside' (74% probability), or the 'secretory pathway' (98% probability),
360 respectively. The programs Phobius and SignalP5.0 predicted that the N-terminal signal peptide is

cleaved between the 24th and 25th residues at the SEARP motif between the alanine and the arginine (Supplementary Fig. S2).

We previously showed that *StPIP1* transcripts are present at low levels in PR leaves (Goyer *et al.*, 2015). To find out whether *StPIPs* are expressed in other potato tissues, we searched gene expression data in the Expression Atlas (https://www.ebi.ac.uk/gxa/home) (Papatheodorou *et al.*, 2020). Only *StPIP1* and *StPIP4* had detectable expression levels. Both genes are expressed in the petiole (Transcript Per Million (TPM) values of 1 and 0.6, respectively), while *StPIP1* is also expressed in the shoot apex (TPM=0.7). In addition, searches for potential stimuli in the available data sets showed that *StPIP1, StPIP2,* and *StPIP4* are all induced in response to *Phytophthora infestans* (log_2 fold changes of 1.8, 2.3, and 2.4, respectively) in a Russet Burbank (RB) line expressing the *Phytophthora infestans* resistance gene *Rpi-blb1* (Gao et al., 2013).

To find further clues as to its function, we searched for cis-regulatory elements in the 1,000-bp region upstream of the start codon of *StPIP1* and found several (biotic) stress responsive cis-regulatory elements (Supplementary Table S7), indicating a possible function of *StPIP1* in (biotic) stress response.

Together, sequence analyses indicated that the gene PGSC0003DMG400014879 encodes a putative PIP family member and is expressed in response to biotic stresses.

Exogenous application of *St*PIP1 induces ROS production and expression of defense-related genes in potato

To test for possible bioactivity of the *St*PIP1-derived peptides, we measured changes in the early stages of defense responses to pathogens, which usually involve, amongst others, increases in Ca^{2+} cytosolic concentrations, increases in ROS production, and changes in expression of plant immunity-associated genes (Yu *et al.*, 2017), upon exogenous application of chemically-synthesized *St*PIP1. Since we did not know the exact composition of possible final mature *St*PIP1 peptides, we used four chemically-synthesized variants of the *St*PIP1 propeptide: StPIP1_long that contains both of the conserved 'GPSPxGxGH' motifs and has the first proline of the 'GPSP' motif hydroxylated (hydroxylation of the first proline of the 'GPSP' motif has been shown to increase peptide activities in Arabidopsis (Hou *et al.*, 2014)); StPIP1_short that contains only the second 'GPSPxGxGH' motif and has the first proline of the motif hydroxylated; StPIP1_short_NoHY

which is identical to StPIP1_short but has no hydroxylated proline; and StPIP1_short_NoC which is identical to StPIP1_short but has the last eight C-terminal amino acids deleted (Supplementary Table S8).

First, we measured changes in cytosolic Ca^{2+} concentration in leaves of 5-6-week-old aequorin-expressing potato cultivar Désirée plants in response to exogenous treatment with the synthetic *St*PIP1-derived peptides. None of the peptides stimulated cytosolic Ca^{2+} transient, whereas a known elicitor peptide, *St*Systemin, triggered increased cytosolic Ca^{2+} 5-7 min after peptide application (Supplementary Fig. S3), showing a lack of function of *St*PIP1 in Ca^{2+} signaling. Second, we measured apoplastic ROS production in nodes and leaves of potato cultivars RB, which is susceptible to all PVY strains, and PR. In nodes, all peptides tested induced ROS production in RB except StPIP1_short, while StPIP1_short_NoC and StPIP1_short_NoHY induced ROS in PR (Fig. 3A and B, Supplementary Fig. S4). Similar to the Ca^{2+} measurements in leaves, none of the peptides stimulated ROS production in leaves, except for StPIP1_short_NoHY in PR(Supplementary Fig. S4). Overall, the variant StPIP1_short_NoHY elicited the highest levels of ROS production. Last, we used RT-qPCR to measure changes in the abundance of mRNA transcripts derived from a selection of known defense-related marker genes in response to peptide treatment (Fig. 4). Overall, if considering only transcripts with a > 2-fold change in abundance, RB was more responsive to all peptide treatments than PR (Fig. 4). In RB, levels of all transcripts significantly increased in response to all *St*PIP1-derived peptides (Fig. 4A), and the magnitude of response was comparable to responses induced by *St*Systemin (Supplementary Fig. S5), except *St*ERF3 and *St*Jas that were more weakly induced by *St*PIP1 peptides. In PR, StPIP1_short_NoC induced the expression of *StPR1b* by at least 4-fold (Fig. 4B). These results show that *St*PIP1 peptide variants are able to induce the expression of defense-related genes in potatoes.

Overexpressors of *StPIP1* show differences of symptoms in compatible reactions with PVY

To further investigate the role of *StPIP1* in the defense response to PVY, we generated transgenic PR potato plants that either overexpress or silence the expression of *StPIP1*. We identified three independent *StPIP1*-overexpressing lines, PIP-OE1, PIP-OE8, and PIP-OE14 that have different increased levels of expression of *StPIP1* compared to control PR, as determined by RT-qPCR (Supplementary Fig. S6)). When grown *in vitro*, PIP-OE1 and PIP-OE14 plantlets were

shorter than control PR and had short internodes and relatively small leaves (Supplementary Fig. S7). However, this phenotype was not apparent when plants were grown in soil in a greenhouse (Supplementary Fig. S7). We also identified three independent artificial microRNA lines Pami5.2, Pami8, and Pami9 that had lower transcript levels as determined by RT-qPCR (Supplementary Fig. S8). Under standard greenhouse conditions, lines Pami5.2 and Pami9 showed some novel phenotypic characteristics, such as mild chlorosis and leaf wrinkling in the upper leaves. Line Pami8 showed a severe developmental phenotype characterized by stunting, a prostrate growth habit, and severe wrinkling of the leaves when grown *in vitro* or in a greenhouse (Supplementary Fig. S8).

We then inoculated *StPIP1*-overexpressing and -silenced plants with PVY^{O} (incompatible interaction) or PVY^{NTN} (compatible interaction), and monitored the rate (i.e. number of plants showing localized necrotic lesions) and onset (i.e. time of first appearance of localized necrotic lesions) of HR on the inoculated leaves. We did two repeated experiments with *StPIP1*-overexpressing lines, and one experiment with *StPIP1*-amiRNA lines. As expected, in all three experiments, most or all of the non-transgenic PR plants developed localized round necrosis characteristic of a HR on leaves inoculated with PVY^{O}, but no HR-like symptoms were observed on PR leaves inoculated with PVY^{NTN} (Supplementary Fig. S9 and Supplementary Tables S9-S11). Mean time for onset of HR varied between experiments from ~9 to ~20 days after inoculation (Supplementary Tables S9-S11). However, in *StPIP1*-overexpressing and -silencing lines, the rate and onset of HR caused by PVY^{O} were similar to those in untransformed PR, the only significant difference being a delay in the onset of HR in Pami5.2 (Supplementary Tables S9-S11). These results showed that modulating the expression of *StPIP1* had no or little effect on rate and onset of HR, indicating that *StPIP1* may not play an important role in PVY-induced HR. It is noteworthy that the HR in PR seemed to be mild because the initial localized necrosis usually did not expand from the initial point of appearance and did not lead to leaf drop as would be observed for a robust HR.

Next, we used RT-PCR to assess PVY systemic infection. We tested systemic leaves from inoculated plants (i.e. in-season infection) and leaves from tuber progeny (i.e. seedborne infection) (Supplementary Tables S9-S11 and Supplementary Fig. S10-S12). In two of three trials (Supplementary Tables S10 & S11 and Supplementary Fig. S11 & S12), we could not detect the virus in any of the PR plants inoculated with PVY^{O}. In the first trial (Supplementary Table S9 and Supplementary Fig. S10), although all PR plants inoculated with PVY^{O} tested positive in in-season

leaves, only half of the progeny plants tested positive. Together, these results indicate a certain degree of resistance of PR to PVY^{O}, consistent with the mild HR observed. However, there was no significant difference in infection rates between PR and either *StPIP1*-overexpressing or -silenced lines in response to PVY^{O} (Supplementary Tables S9-S11 and Supplementary Fig. S10-S12). In the case of PVY^{NTN}, in two of the trials (Supplementary Tables S9 & S11 and Supplementary Fig. S10 & S12), all PR plants tested positive for the virus in both in-season leaves and tuber progeny, consistent with a lack of resistance of PR to this strain. In the second trial, only two out of six plants inoculated with PVY^{NTN} tested positive for the virus in both in-season leaves and tuber progeny (Supplementary Table S10 and Supplementary Fig. S11), which may be due to technical failure, but the infection rate was still higher than in plants inoculated with PVY^{O} (i.e. zero out of six plants tested positive). This may explain the significant differences observed between PR and overexpressing lines in that trial (Supplementary Table S10 and Supplementary Fig. S11). Otherwise, there was no significant difference in infection rates between PR and either overexpressing or silencing lines (Supplementary Tables S9 & S11 and and Supplementary Fig. S10 & S12). These results showed that modulating the expression of *StPIP1* had no effect on rate of systemic infection with either PVY^{O} or PVY^{NTN} strains.

Finally, in PVY-inoculated PR, *StPIP1*-overexpressing and *StPIP1*-silenced lines, we monitored the development of systemic symptoms on systemic leaves. In all experiments, there were no symptoms or very mild mosaic symptoms observed on non-inoculated leaves in either transgenic or control PR plants that were inoculated with PVY^{O} (Supplementary Tables S9-S11). Likewise, PR plants inoculated with PVY^{NTN} did not show symptoms or produced only mild symptoms in three out of six plants at >50 dpi in one of the trials (Supplementary Table S9). In contrast, PIP-OE1 and PIP-OE14 (and PIP-OE8 in our first trial) inoculated with PVY^{NTN} produced clearly visible rugose mosaic symptoms starting as early as ~30 dpi (Fig. 5, Supplementary Tables S9-S10). This observation was consistent throughout experiments. We hypothesized that the strong phenotypic reaction of *StPIP1*-overexpressing plants infected with PVY^{NTN} is due to higher virus amounts in leaf tissues. To test this hypothesis, we measured the amount of viral RNA relative to two reference genes, *L2* and *EF1α*, by RT-qPCR in leaves of PR and PIP-OE1 infected with PVY^{NTN} 44 days after inoculation. The relative amount of viral RNA was over two-fold lower in leaves of PIP-OE1 compared to that in leaves of PR (Fig. 6, Supplementary Fig. S13), and this difference was statistically significant ($p < 0.05$), rejecting our initial hypothesis that rugose mosaic symptoms are due to higher viral load. Instead, our results indicated that overexpression of *StPIP1*

decreased the viral load during the compatible interaction with PVY^{NTN}, suggesting that the associated rugose mosaic symptoms may be due to an increased plant defense response.

Infection with PVY^{NTN} induces major changes in expression of plant defense response genes in *StPIP1*-overexpressing lines

To test our hypothesis that foliar symptoms in *StPIP1*-overexpressing lines infected with PVY^{NTN} were due to changes in plant defense response, we analyzed changes in gene expression at the transcriptome level in systemic leaves infected with PVY^{NTN} in PR and PIP-OE1 at 44 dpi using QuantSeq (Moll *et al.*, 2014). Four comparisons were made: mock-inoculated PR versus mock-inoculated PIP-OE1; PVY^{NTN}-infected PR versus mock-inoculated PR; PVY^{NTN}-infected PIP-OE1 versus mock-inoculated PIP-OE1; and PVY^{NTN}-infected PR versus PVY^{NTN}-infected PIP-OE1 (Fig. 7, Supplementary Tables S12-S15). When PVY^{NTN}-infected PR was compared to mock-inoculated PR, only six genes were differentially expressed (DE) ($q \leq 0.05$) (Fig. 7A, Supplementary Table S12), indicating that PVY^{NTN} infection had little effect on the overall transcriptome of PR in systemic leaves. When mock-inoculated PIP-OE1 was compared to mock-inoculated PR, 92 genes were DE besides *StPIP1* itself (Fig. 7B, Supplementary Table S13). This indicates that the overexpression of *StPIP1* had mild effect on the overall transcriptome of PR under normal growth conditions. Strikingly, when comparing PVY^{NTN}-infected versus mock-treated-PIP-OE1 plants, 3,500 genes were DE (Fig. 7C, Supplementary Table S14). Likewise, when comparing PVY^{NTN}-infected PIP-OE1 versus PVY^{NTN}-infected PR plants, 1,921 genes were DE (Fig. 7D, Supplementary Table S15). Amongst genes DE with a $q \leq 0.05$ and a $|\log2(\text{FoldChange})|$ cutoff ≥ 2, about half (47%) were common between the two comparisons, while ~32% were specific to the comparison of PVY^{NTN}-infected and mock-inoculated PIP-OE1, and 20% were specific to the comparison of PVY^{NTN}-infected PIP-OE1 versus PVY^{NTN}-infected PR (Fig. 7E).

We then used the statistical tool g:GOSt from g:Profiler to perform functional profiling of the significantly DE ($q \leq 0.05$, $|\log_2(\text{FoldChange})| \geq 2$) genes in PVY^{NTN}-inoculated PIP-OE1 compared to mock PIP-OE1 or PVY^{NTN}-inoculated PR (Supplementary Fig. S14 and S15). Several significantly-enriched terms ($p < 0.05$), common to both comparisons, were clearly associated with plant defense responses (e.g. 'Plant pathogen interaction', 'Systemic acquired resistance', 'Chitinase activity') and signaling (e.g. 'Protein phosphorylation', 'Calcium ion binding', 'Regulation of DNA binding factors activity'). Genes associated with pathogen responses are listed

517 in Tables 1 and 2. In addition to genes identified by gProfiler, many other genes were found to have
518 annotations related to plant defense responses such as several WRKY transcription factors, Hsr203J
519 (an HR marker gene), glucanases, and Hcr2-0A-annotated resistance genes (Supplementary Tables
520 S14 and S15). Interestingly, *StPIP2* (PGSC0003DMT400038540), a paralog of *StPIP1*, was also
521 induced in both comparisons.

522 Together, these results show that PVY^{NTN} triggers the expression of defense-related genes
523 in *StPIP1*-overexpressing lines, responses that were absent in PR control. Further, the DE defense-
524 related genes likely account for the stark difference in symptom presentation between PR and
525 *StPIP1*-overexpressing lines.

526

527 Callose deposition is higher in *StPIP1*-overexpressing plants

528 Callose deposition at the cell wall is a defense response that restricts pathogen penetration
529 through the cell wall and movement through plasmodesmata (Ellinger *et al.*, 2013; Iglesias and
530 Meins, 2000). To further assess the status of plant defense response in *StPIP1*-overexpressing
531 plants, we analyzed callose deposition in systemic leaves in virus-free and fully infected plants 44
532 dpi with PVY^{NTN}. Using aniline blue staining to detect callose in leaf tissue, we observed a
533 significant increased amount of callose in virus-free PIP-OE1 compared to virus-free PR leaves
534 (~4-fold) (Fig. 8). Leaves of PVY-infected PIP-OE1 plants also had higher levels of callose
535 compared to leaves of PVY-infected PR, although it was not statistically significant (Fig. 8). These
536 results revealed at the cellular level that *StPIP1*-overexpressing plants are in a primed state with
537 enhanced defense response.

538

539 Discussion

540 We report here evidence that the potato gene *StPIP1* is involved in the antiviral defense
541 response against PVY in potato, and provide evidence that a peptide encoded by this gene can elicit
542 plant defenses. We provide several lines of evidence for its function: (i) phylogenetic and amino-
543 acid sequence analyses show that the *St*PIP1 clusters with PIP family members from Arabidopsis
544 and that the encoded protein contains the signature motif GPS(P)xGxGH along with a putative
545 transit peptide for targeting to the apoplast; (ii) the promoter of *StPIP1* contains several putative
546 cis-regulatory elements that are involved in biotic stress response; (iii) exogenous application of

synthetic *St*PIP1-derived peptides triggered ROS accumulation and increased expression of plant immunity marker genes; (iv) plants that overexpress *St*PIP1 showed phenotypic reaction (rugose mosaic), major changes in the expression of genes related to plant immunity response, higher levels of callose deposition, and had lower viral titer when systemically infected with a compatible strain of PVY.

Despite evidence of an increased plant defense response in *StPIP1*-overexpressing plants infected with PVY^{NTN}, overexpression of *StPIP1* did not fully protect the plants from systemic infection, but rather decreased the virus titer. Our results are similar with those in Arabidopsis where overexpression of *AtPIP1* increased resistance to *P. syringae* but did not stop infection (Hou *et al.*, 2014; Najafi *et al.*, 2020). This can be explained by the relatively slow and weak response mediated by *St*PIP1. Indeed, increases in ROS production after exogenous application of *St*PIP1 were small and delayed relative to those observed with *St*Systemin, and few defense genes responded to *St*PIP1 treatment in cultivar PR. Although *StPIP1*-overexpressing plants had higher callose deposition even before encountering the virus, which seems to indicate that part of the plant defense system was already primed, the virus was still capable of overcoming those defenses and spread systemically. In agreement with our observation, a recent report has also shown that callose deposition is not a guarantee of virus restriction (Lukan *et al.*, 2018). Once the virus went systemic, *StPIP1*-overexpressing plants were able to increase the expression of a large number of plant defense genes as shown by QuantSeq, and able to keep the virus titer at lower level than in non-transgenic plants. The strong foliar phenotypic reaction (i.e. rugose mosaic) in PVY^{NTN}-infected *StPIP1*-overexpressing plants may be due to an excess of energy devoted to plant defense responses to the detriment of the overall plant fitness. In other words, the overexpression of *StPIP1* broke the tolerance of PR, a "Typhoid Mary" cultivar that displays little to no symptoms while still developing a systemic infection, to PVY^{NTN}, and turned it into a sensitive cultivar whose defense response is too weak to stop virus multiplication and/or movement completely.

While *StPIP1*-overexpressing lines showed severe symptoms (i.e. rugose mosaic) that were quasi-absent in PR when infected with a compatible strain, PVY^{NTN}, they showed no such difference when infected with PVY^{O}, an incompatible strain that sometimes is able to overcome HR and become systemic. A possible explanation is that the PVY^{O}-triggered ETI response overshadows the *St*PIP1-mediated PTI response. Testing the effect of overexpressing *StPIP1* against compatible strains of PVY in a cultivar lacking resistance genes (e.g. Russet Burbank) would help to validate this hypothesis. If confirmed, a cross-talk between resistance responses (i.e.

579 ETI and PTI) may explain why *StPIP1* is transiently downregulated in the early stages of the
580 incompatible PR-PVY^{O} interaction, but not in the compatible PR-PVY^{NTN} interaction (Goyer *et al.*,
581 2015). In this context, *StPIP1* may be a target of a strain-specific PVY effector that triggers ETI,
582 such as HCPro (Chowdury *et al.*, 2019), in a similar way to the capsid protein of Plum pox virus,
583 an avirulence factor that triggers ETI but also suppresses PTI (Nicaise and Candresse, 2017).
584 Another plausible explanation is that virus strain-specific features (e.g. RNA and/or protein
585 sequence compositions) determine recognition by and activation of the *St*PIP1-mediated response.
586 Future studies should focus on identifying these important features and the mechanism of
587 recognition.

588 Receptors for SGP-rich peptides are typically leucine-rich repeat-containing receptor-like
589 kinases (LRR-RLK) (Stenvik *et al.*, 2008; Yamaguchi *et al.*, 2010; Yamaguchi *et al.*, 2006). In
590 Arabidopsis, *At*RLK7 is the receptor for *At*PIP1 and *At*PIP2 (Hou *et al.*, 2014). Hou *et al* (2014)
591 initially identified RLK7 as a promising receptor candidate for *At*PIP1 because it was one of the
592 few class XI LRR-RLKs that were induced in response to pathogen infection or PAMP elicitation.
593 To identify candidate receptors of *St*PIP1, we searched the QuantSeq data for LRR-RLKs that were
594 induced in PVY^{NTN}-inoculated PIP-OE1 compared to either mock-inoculated PIP-OE1 or PVY^{NTN}-
595 inoculated PR. The expression of the closest homolog of *At*RLK7 in potato,
596 PGSC0003DMG400004966 (transcript ID PGSC0003DMT400012744), was not induced.
597 However, six genes encoding putative LRR-RLKs, based on the presence of a LRR domain, a
598 single pass transmembrane domain, and an intracellular Ser/Thr Kinase domain (Shiu and Bleecker,
599 2001), were found to be significantly induced in PVY^{NTN}-inoculated PIP-OE1 (Supplementary
600 Table S16). Two of them, PGSC0003DMG400011989 and PGSC0003DMG400027586, belongs
601 to class XI LRR-RLKs, are approximately 50% similar (> 95% coverage) to RLK7 (Pitorre *et al.*,
602 2010) as well as the *At*PEP1 receptors *At*PEPR1/2 (Yamaguchi *et al.*, 2010; Yamaguchi *et al.*,
603 2006), and are expressed in leaf and petiole like *StPIP1* (as well as shoot apex and tuber in the case
604 of PGSC0003DMG400027586) (https://www.ebi.ac.uk/gxa/home), making them attractive
605 candidate receptors for *St*PIP1. Future studies are warranted to assess if these genes encode *St*PIP1
606 receptors. In addition, because PIP1-RLK7-induced responses are partially dependent on BAK1 in
607 Arabidopsis (Hou *et al.*, 2014), it would be interesting to investigate whether BAK1 orthologs in
608 potato are involved in *St*PIP1 signaling.

609

610

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Amino acid sequences of small secreted peptides from Arabidopsis, tomato, and potato.

Table S2. Primers used for cloning, genotyping, and detection of PVY by RT-PCR.

Table S3. Primers used for RT-qPCR for determination of *StPIP1* gene expression and PVY levels.

Table S4. Primers used for RT-qPCR on defense genes from potato leaf discs.

Table S5. MIQE corresponding to Table S3.

Table S6. MIQE corresponding to Table S4.

Table S7. Cis-acting regulatory elements in the 1,000-bp promoter region of *StPIP1*.

Table S8. Sequences of *St*PIP1 propeptides used in this study.

Table S9. Rate and onset of hypersensitive response, symptom presentation, and PVY infection in PR and *StPIP1*-overexpressing lines in Experiment 1.

Table S10. Rate and onset of hypersensitive response, symptom presentation, and PVY infection in PR and *StPIP1*-overexpressing lines in Experiment 2.

Table S11. Rate and onset of hypersensitive response, symptom presentation, and PVY infection in PR and *StPIP1* artificial microRNA lines in Experiment 3.

Table S12. List of DE genes in systemic leaves of PVY^{NTN}-infected versus mock Premier Russet 44 days after inoculation as determined by QuantSeq.

Table S13. List of DE genes in systemic leaves of non-infected (mock) PIP-OE1 versus Premier Russet 44 days after treatment as determined by QuantSeq.

Table S14. List of DE genes in systemic leaves of PVY^{NTN}-infected- versus mock-PIP-OE1 44 days after inoculation as determined by QuantSeq.

Table S15. List of DE genes in systemic leaves of PVY^{NTN}-infected-PIP-OE1 versus PVY^{NTN}-infected-Premier Russet 44 days after inoculation as determined by QuantSeq.

Table S16. Leucine-Rich Repeat domain containing genes induced in PIP-OE1 infected with PVY^{NTN} compared to both mock-inoculated PIP-OE1 and PVY^{NTN}-inoculated Premier Russet.

Fig. S1. PHYRE structural model prediction of *St*PIP1.

Fig. S2. SignalP-5.0 prediction of signal peptide and cleavage for *St*PIP1.

Fig. S3. Cytosolic Ca^{2+} concentration in leaves of cultivar Désiree after application of variants of *St*PIP1 and *St*Systemin.

Fig. S4. ROS production in potato leaves and nodes in response to *St*PIP1 and its variants.

Fig. S5. Expression of defense-related genes in leaves in response to StPIP1 and its variants.

Fig. S6. *StPIP1* gene expression increase in *StPIP1*-overexpressing lines as determined by RT-qPCR. Data are means ± SE (n=6).

Fig. S7. Growth phenotype of *in vitro*-grown *StPIP1*-overexpressing line PIP-OE1 compared to control Premier Russet plantlets.

Fig. S8. Characterization of artificial microRNA lines silencing *StPIP* expression.

Fig. S9. Hypersensitive response (HR) on the adaxial (upper row) or abaxial (lower row) sides of PVY-inoculated leaves of potato cultivar Premier Russet.

Fig. S10. PVY testing by RT-PCR of leaves from in-season infected plants (A) and seedborne infected plants (B-F) for Experiment 1.

Fig. S11. PVY testing by RT-PCR of leaves from in-season infected plants (A-C) and seedborne infected plants (D-F) for Experiment 2.

Fig. S12. PVY testing by RT-PCR of leaves from in-season infected plants (A-C) in the *StPIP1* artificial microRNA (Pami) Experiment.

Fig. S13. Quantification of PVY relative to the reference gene *EF1α* in systemically infected Premier Russet and PIP-OE1 plants.

Fig. S14. Manhattan plot illustrating the enrichment analysis based on gene ontology from the comparison between PVY^{NTN}-infected- versus mock-PIP-OE1.

Fig. S15. Manhattan plot illustrating the enrichment analysis based on gene ontology from the comparison between PVY^{NTN}-infected PIP-OE1 *vs*. PVY^{NTN}-infected Premier Russet.

Data availability

Raw Illumina sequencing data are available at the NCBI Sequence Read Archives under the accession PRJNA669287.

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Author contributions

Conceptualization: MMC, AG; Formal Analysis: MMC, NM, KT, CJR, LT, AG; Funding acquisition: MMC, KT, JCA, AG; Investigation: MMC, NM, CJR, AG; Project Administration: AG; Supervision: KT, JCA, AG; Visualization: MMC, NM, CJR, AG; Writing – Original Draft Preparation: MMC, AG; Writing – Review & Editing: MMC, KT, JCA, AMR, AG.

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Table 1. Selected DE genes with immune-related GO enriched terms in PVY^{NTN}-inoculated PIP-OE1 compared to PVY^{NTN}-inoculated PR.

Transcript ID	log2(FC)	StdErr	P-adj	Gene Annotation[a]	GO Terms
PGSC0003DMT400037745[c]	3.470	0.541	$9.76x10^{-9}$	Conserved gene of unknown function[b]	
PGSC0003DMT400037744	3.208	0.586	$1.99x10^{-6}$	Conserved gene of unknown function[b]	Regulation of systemic acquired resistance
PGSC0003DMT400041025[c]	2.691	0.606	$2.45x10^{-4}$	Conserved gene of unknown function[b]	(GO:0010112)
PGSC0003DMT400014779	2.492	0.606	$8.59x10^{-4}$	NPR1/NIM1-interacting protein NIMIN2c	
PGSC0003DMT400046345	3.210	0.348	$7.95x10^{-18}$	Cyclic nucleotide-gated calmodulin-binding ion channel	
PGSC0003DMT400059272	2.964	0.372	$2.77x10^{-13}$	Calcium-binding protein	
PGSC0003DMT400027849	2.845	0.407	$2.56x10^{-10}$	Ethylene responsive transcription factor ERF4	
PGSC0003DMT400074364	2.774	0.490	$7.17x10^{-7}$	SGT1 (suppressor of the G2 allele of *skp1*)	
PGSC0003DMT400083031[c]	2.616	0.347	$5.75x10^{-12}$	Enhanced disease susceptibility 1 protein (EDS1)	Plant-pathogen interaction (KEGG:04626)
PGSC0003DMT400025744[c]	2.583	0.185	$3.32x10^{-41}$	Endoplasmin homolog	
PGSC0003DMT400021079	2.541	0.270	$1.75x10^{-18}$	Calcium-binding allergen Ole e	
PGSC0003DMT400041342	2.151	0.339	$1.54x10^{-8}$	Calmodulin	
PGSC0003DMT400024594	2.148	0.208	$2.94x10^{-22}$	Heat shock protein 83	
PGSC0003DMT400003364	2.531	0.388	$5.01x10^{-9}$	Calcium ion binding protein	
PGSC0003DMT400053338[c]	2.478	0.462	$3.42x10^{-6}$	Serine-threonine protein kinase, plant-type	
PGSC0003DMT400070945	2.357	0.409	$4.16x10^{-7}$	Serine-threonine protein kinase, plant-type	Plant-pathogen interaction (KEGG:04626),
PGSC0003DMT400083727	2.347	0.583	$1.20x10^{3}$	Calmodulin	MAPK signaling pathway (KEGG:04016)
PGSC0003DMT400044026	2.272	0.632	$5.14x10^{-3}$	Calcium-binding EF hand family protein	
PGSC0003DMT400061478[c]	2.266	0.503	$1.92x10^{-4}$	Cytoplasmic small heat shock protein class I	
PGSC0003DMT400044379[c]	2.128	0.328	$6.67x10^{-9}$	Transcription factor TSRF1	MAPK signaling pathway (KEGG:04016)
PGSC0003DMT400034487[c]	2.085	0.601	$7.78x10^{-3}$	ERF transcription factor 5	
PGSC0003DMT400003888	2.756	0.589	$9.26x10^{-5}$	Chitinase 134	MAPK signaling pathway (KEGG:04016), Chitinase activity (GO:0004568)
PGSC0003DMT400003877	2.663	0.277	$2.02x10^{-19}$	Class II chitinase	Chitinase activity (GO:0004568)
PGSC0003DMT400022352	2.652	0.325	$6.31x10^{-14}$	Endochitinase (Chitinase)	

PGSC0003DMT400069033	2.011	0.280	7.05×10^{-11}	Endochitinase 2

[a] Annotations come from the Potato Genome Sequencing Consortium (PGSC; http://solanaceae.plantbiology.msu.edu/).

[b] Although PGSC does not list an annotation for these genes, BLAST analysis shows that they code for proteins with sequence similarity to NPR1/NIM1-Interacting (NIMIN) proteins in Arabidopsis (Weigel et al, 2001) and tobacco (Zwicker et al, 2007).

[c] Genes which are DE in this specific comparison.

Table 2. Selected DE genes with immune-related GO enriched terms in PVY^{NTN}-inoculated PIP-OE1 compared to mock-inoculated PIP-OE1.

Transcript ID	log2(FC)	StdErr	P-adj	Gene Annotation[a]	Go Terms
PGSC0003DMT400037744	4.026	0.549	1.79x10-^{11}	Conserved gene of unknown function[b]	
PGSC0003DMT400014779	3.66	0.472	7.54x10^{-13}	NPR1/NIM1-interacting protein NIMIN2c	Systemic acquired resistance (GO:0009627),
PGSC0003DMT400059031[c]	3.610	0.471	1.67x10^{-12}	Aspartate aminotransferase	Defense response, incompatible interaction
PGSC0003DMT400051169[c]	2.390	0.637	0.002	Phytoalexin-deficient 4-2 protein	(GO:0009814)
PGSC0003DMT400055847[c]	-2.088	0.654	0.010	Lipid binding protein	
PGSC0003DMT400063776[c]	2.257	0.505	1.27x10^{-4}	Calmodulin-binding protein	Systemic acquired resistance (GO:0009627), Defense response, incompatible interaction (GO:0009814), Calmodulin binding (GO:0005516)
PGSC0003DMT400074364	3.329	0.516	5.87x10^{-9}	SGT1 (suppressor of the G2 allele of *skp1*)	Plant-pathogen interaction (KEGG:04626)
PGSC0003DMT400013094[c]	3.164	0.629	1.05x10^{-5}	PR1 protein	
PGSC0003DMT400083027[c]	2.837	0.418	6.91x10^{-10}	Enhanced disease susceptibility 1 protein (EDS1)	
PGSC0003DMT400027849	2.624	0.419	1.65x10^{-8}	Ethylene responsive transcription factor ERF4	
PGSC0003DMT400059272	3.167	0.338	1.49x10^{-18}	Calcium-binding protein	Plant-pathogen interaction (KEGG:04626), Calcium ion binding (GO:0005509)
PGSC0003DMT400003364	3.142	0.357	2.42x10^{-16}	Calcium ion binding protein	
PGSC0003DMT400021079	2.731	0.303	3.15x10^{-17}	Calcium-binding allergen Ole e	
PGSC0003DMT400044026	2.403	0.655	0.002	Calcium-binding EF hand family protein	
PGSC0003DMT400052233[c]	2.276	0.652	0.004	Polcalcin Jun o	
PGSC0003DMT400041342	2.173	0.327	1.79x10^{-9}	Calmodulin	
PGSC0003DMT400083727	2.03	0.5777	0.004	Calmodulin	
PGSC0003DMT400046345	3.117	0.509	3.92x10^{-8}	Cyclic nucleotide-gated calmodulin-binding ion channel	Plant-pathogen interaction (KEGG:04626), Integral component of membrane (GO:0016021)
PGSC0003DMT400070945	2.250	0.430	4.24x10^{-6}	Serine-threonine protein kinase, plant-type	
PGSC0003DMT400024594	3.127	0.481	4.12x10^{-9}	Heat shock protein 83	
PGSC0003DMT400025743[c]	2.631	0.232	4.00x10^{-27}	Endoplasmin homolog	

PGSC0003DMT400037335[c]	2.177	0.192	4.00×10^{-27}	Heat shock cognate protein 80	Plant-pathogen interaction (KEGG:04626), Protein processing in endoplasmic reticulum (KEGG:04141)
PGSC0003DMT400003877	2.986	0.399	5.87×10^{-12}	Class II chitinase	Chitinase activity (GO:0004568)
PGSC0003DMT400022352	2.715	0.230	3.07×10^{-29}	Endochitinase (Chitinase)	
PGSC0003DMT400069033	2.201	0.210	3.80×10^{-23}	Endochitinase 2	
PGSC0003DMT400003888	2.004	0.559	0.003	Chitinase 134	

[a] Annotations come from the Potato Genome Sequencing Consortium (PGSC; http://solanaceae.plantbiology.msu.edu/).

[b] Although PGSC does not list an annotation for this gene, BLAST analysis shows that it codes for a protein with sequence similarity to NPR1/NIM1-Interacting (NIMIN) proteins in Arabidopsis (Weigel et al, 2001) and tobacco (Zwicker et al, 2007).

[c] Genes which are DE in this specific comparison.

Figure Legends

Fig. 1. Maximum-Likelihood phylogenetic tree of the small secreted peptide families CLV3/CLE, CEP, IDA/IDL, and PIP/PIPL. The sequences of CLV3/CLE, CEP, IDA/IDL, and PIP/PIPL proteins from *Arabidopsis* were retrieved from Goad et al (2016) (CLV3/CLE), Roberts et al (2013) (CEP), and Vie et al (2015) (IDA/IDL, PIP/PIPL). Sequences for 10 CLE peptides from tomato (IDs starting with 'Sl') were retrieved from Zhang et al (2014). Sequences of four previously uncharacterized paralogous secreted peptides from potato (StPIP1, StPIP2, StPIP3, and StPIP4) were retrieved from SpudDB (solanaceae.plantbiology.mu.edu). Genes encoding StPIP1, StPIP2, and StPIP3 are paralogs located on chromosome 3 (PGSC0003DMG400014879 (StPIP1), PGSC0003DMG400014880 (StPIP2), and PGSC0003DMG400014874 (StPIP3). Gene names ending with an asterisk are putative members of small secreted peptide families from tomato and potato that were found using the BLAST suite from NCBI. The tree was constructed using MEGA7 with 1000 bootstraps. A few CEP-annotated peptides grouped with PIP or IDL/IDA families. **Fig. 2.** Multiple sequence alignment comparing the PIP prepropeptides of Arabidopsis (AtPIP1-3) to those of potato (StPIP1-4). Genes encoding StPIP1, StPIP2, and StPIP3 are paralogs located on chromosome 3 (PGSC0003DMG400014879 (*StPIP1*), PGSC0003DMG400014880 (*StPIP2*), and PGSC0003DMG400014874 (*StPIP3*). The dotted line indicates the predicted signal peptide cleavage motif and solid lines indicate the core PIP motifs, 'GPSP' and 'GxGH', that indicate the C-terminal end of the mature PIP peptides. The alignment was done with ClustalW and shading was done with BoxShade.

Fig. 3. ROS production in potato leaves and nodes in response to StPIP1 and its variants. Potato plants used are as follows: A, nodes of Russet Burbank (RB); B, nodes of Premier Russet (PR). All peptides were added at the final concentration of 1 µM. RLU is presented as a result of subtraction of RLU_0 (at time 0) from RLU_t (at each time point of the measurement). Line graphs are shown as mean values ± SE (n = 8).

Fig. 4. Expression of defense-related genes in leaves in response to StPIP1 and its variants. Potato plants used are as follows: A, Russet Burbank (RB); B, Premier Russet (PR). Leaf discs were treated for 30 min with the indicated peptides at the final concentration of 1 µM. The expression level of potato defense-related genes were monitored by RT-qPCR. Data are shown as normalized fold expression compared to mock control ($2^{-\Delta\Delta Ct}$). Two reference genes, *StEF1α* and *StUbq*, were

used for normalization. Histogram bars are mean values ± SE. Unpaired Student's *t*-test from log transformed values was used for statistical analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).Note the difference of scale between the two graphs.

Fig. 5. Symptoms of StPIP1-overexpressing lines compared to Premier Russet (PR) upon inoculation with PVY^{NTN}. Symptoms including rugose mosaic, stunting, and chlorosis were clearly visible in StPIP1 overexpressors infected with PVY^{NTN}. A) Mock-and PVY^{NTN}-inoculated PR and StPIP1-overexpressing line #1 (PIP-OE1) 44 days after inoculation. B) Close up images of canopy leaves of mock-and PVY^{NTN}-inoculated PR and PIP-OE1 47 days after inoculation. C) Three independent StPIP1-overexpressing lines (PIP-OE14, PIP-OE8, and PIP-OE1) infected with PVY^{NTN} showing clearly visible symptoms compared to a relatively asymptomatic PR control 69 days post inoculation.

Fig. 6. Quantification of PVY in systemically infected Premier Russet and PIP-OE1 plants. Leaf samples were harvested 44 days after inoculation with PVY^{NTN}. PVY titer was determined by RT-qPCR relative to the reference gene L2. Data are means ± SE (n=4 for Premier Russet, n=5 for PIP-OE1). Asterisk indicate a significant difference ($p<0.05$) as determined by student *t*-test.

Fig. 7. QuantSeq Volcano plots (A-D) and Venn diagram (E) of differentially expressed (DE) genes in PR and the StPIP1overexpressor PIP-OE1 44 days after inoculation with PVY^{NTN}. Data points in red are significantly DE with adjusted p-values (q) ≤ 0.1 (default for DEseq2), however we use the threshold of $q \leq 0.05$ to define significantly DE genes. A) Comparison between PVY^{NTN}-treated and mock-treated PR. The plot represents each gene with a dot. B) Comparison between mock-inoculated PIP-OE1 and PR. C) Comparison between PVY^{NTN}-treated and mock-treated PIP-OE1. D) Comparison between PVY^{NTN}-inoculated PIP-OE1 and PR. Arrows show the overexpressed StPIP1 in B and D. Note the change of scale on the y-axis between plots. E) Venn diagram showing the number of common and unique DE genes ($|\log_2(FC)| \geq 2$; $q \leq 0.05$) between (C) and (D) comparisons.

Fig. 8. Callose spots in virus-free (mock) and PVY^{NTN}-infected systemic leaves of *StPIP1*-overexpressing PIP-OE1 line and Premier Russet. Data is the average callose spots in the 1-mm^2 area from 14 pictures per genotype and error bars represent the standard error of the data. Shared letters indicate that there was no significant difference as determined by ANOVA (p=0.05).