Identification of differentially expressed genes in tolerant and susceptible potato cultivars in response to *Spongospora subterranea* f. sp. *subterranea* tuber infection

M. Lekota, N. Muzhinji and J. E. Waals*

Department of Plant and Soil Sciences, University of Pretoria, Private Bag X20, Hatfield, Pretoria 0028, South Africa.
***E-mail**: jacquie.vanderwaals@up.ac.za; Tel.: +27 (0)12 420 4065

Gene expression in potato cultivars

Abstract

Powdery scab caused by *Spongospora subterranea* f. sp. *subterranea* (Sss) has recently become one of the most devastating potato diseases of economic importance in South Africa. The use of resistant cultivars has long been considered the most effective and sustainable strategy to manage the pathogen. However, little is known about the molecular mechanisms underlying resistance of potato tubers to Sss. Using RNA-sequencing (RNA-seq), 2058 differentially expressed genes (DEGs) were identified from two potato cultivars (tolerant and susceptible) in response to Sss infection. Analysis of the expression patterns of ten selected defense-response genes was carried out at two different stages of tuber growth using RT-qPCR to validate the RNA-seq data. Several defense related genes showed contrasting expression patterns between the tolerant and susceptible cultivars, including marker genes involved in the salicylic acid hormonal response pathway (*StMRNA*, *StUDP* and *StWRKY6*). Induction of six defense related genes (*StWRKY6*, *StTOSB*, *StSN2*, *StLOX*, *StUDP* and *StSN1*) persisted until harvest of the tubers, while three other genes (*StNBS, StMRNA* and *StPRF*) were highly up-regulated during the initial stages of disease development. The results of this preliminary study suggested that the tolerant potato cultivar employs quantitative resistance and salicylic acid pathway hormonal responses against tuber infection by Sss. The identified genes have the potential to be used in the development of molecular markers for selection of powdery scab resistant potato lines in marker assisted breeding programs.

Keywords: Differential gene expression, potato, powdery scab, RT-qPCR, RNA-sequencing, *Spongospora subterranea* f. sp. *subterranea*

Introduction

Potato (*Solanum tuberosum* L.) production is constrained by a number of diseases including powdery scab caused by *Spongospora subterranea* f. sp. s*ubterranea* (Sss). The disease has been associated with devastating losses resulting from reduced yields, low quality and poor marketability of potato tubers (Nitzan *et al.,* 2008)

Currently, there are no effective methods for the control of powdery scab disease of potatoes in South Africa (van der Waals, 2018). A limited number of chemicals, including fluazinam and flusulfamide, are registered in some countries for control of Sss (Falloon, 2008). However, the use of chemicals is not effective in controlling the pathogen, therefore integrated pest management strategies such as the use of certified disease-free seed tubers (Tegg *et al*., 2015) and planting in uninfested soil (Falloon *et al.,* 2003) are recommended to minimize yield losses. Until now, most studies on the potato-Sss pathosystem have focussed on the symptoms (Merz & Fallon, 2008), occurrence (Kim *et al.,* 2003), epidemiology (Bittara *et al.,* 2016), lifecycle (Merz, 1997), chemical control (Simango & van der Waals, 2017) and biological control of the disease (Nakayama, 2017).

The use of potato cultivars resistant to Sss is considered as an effective, durable and environmentally friendly approach to manage the disease (Merz & Falloon*,* 2008). Potato cultivars exhibit differential response to Sss tuber infection from highly resistant to highly susceptible, suggesting that resistance to powdery scab is quantitatively inherited (Merz *et al.,* 2004). Nevertheless, the genetic and molecular mechanisms of powdery scab resistance in potato tubers have not yet been fully elucidated. Plant defense to pathogens requires a complex interaction between the host and the pathogen that involves the up- or down-regulation of genes (Berger *et al.,* 2007).

Identification of the differentially expressed genes (DEGs) involved in potato tuber defense to Sss is important in understanding molecular mechanisms related to resistance. This will serve as a valuable potential resource for potato breeders in understanding the dynamic interactions between potato and the pathogen. Transcriptomic analysis during the interaction between plants and pathogens is commonly used to provide new insights into the underlying molecular mechanisms of plant resistance. Transcriptome comparisons between resistant and susceptible varieties have been used to elucidate gene expression profiling of potato in response to infection by *Phytophthora infestans* (Gao *et al.,* 2013), *Streptomyces turgidiscabies* (Dees *et al.,* 2016), PVY (Goyer *et al.,* 2015) and *Pectobacterium carotovorum* subsp. *brasiliense* (Kwenda *et al.,* 2016). Many genes were found associated with resistance signal transduction

and defense mechanisms in potatoes. For example, WRKY transcription factors were significantly up-regulated in potatoes after *Phytophthora infestans* infection (Gao *et al.,* 2013).

In order to gain insight into the transcriptional reprogramming that occurs during the compatible/incompatible interaction of potatoes with Sss, discovery of genes by RNA-seq and gene expression profiling by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) were carried out on two potato cultivars with variable susceptibility to powdery scab at two different time points of potato plant growth. Results from this study will improve the understanding of potato tuber resistance to Sss and aid in defining future potato breeding programs.

Materials and methods

Plant material and inoculation

Two potato cultivars, namely Innovator (tolerant) and Vanderplank (susceptible) as determined in a previous study (authors' unpublished data) were selected for this greenhouse experiment. Briefly, 12 potato cultivars commonly grown in South Africa were evaluated in three greenhouse trials for their susceptibility to Sss root and tuber infection. Visual assessments, microscopic examination and qPCR were used to evaluate susceptibility of cultivars to Sss potato diseases namely; powdery scab, root infection and root galls. Cultivars were ranked according to average powdery scab, root infection and root gall disease indices for the three greenhouse trials. Innovator, a processing cultivar used for the French fry market, has russet skin with yellow fresh, and was the most tolerant cultivar to powdery scab, as confirmed by the AHDB (2019). Vanderplank is an all-purpose cultivar with white skin and flesh (Potato Seed Production, 2019), and was the most susceptible to powdery scab. Both cultivars are open pollinated with short to medium growing periods.

Two pot trials for gene expression were planted in 2017 in a temperature-regulated greenhouse at the University of Pretoria. A total of twenty-four sprouted mini-tubers per cultivar were individually planted in plastic pots (13.5 cm height x 15 cm diameter) filled with pasteurised sandy-loam soil and grown in a greenhouse maintained at 22 ± 2 °C with a 16-hr photoperiod. Plants were watered every second day with 200 ml of sterile distilled water for maintenance of moist soil conditions, favourable for tuber infection by Sss. Seven days after planting, 12 plants of each cultivar were inoculated with 4 g of Sss inoculum suspended in 50 ml of distilled water, equivalent to 5 x 10^4 sporeballs per pot, determined using a haemocytometer. The remaining 12 plants per cultivar were inoculated with sterile distilled

water and served as the un-inoculated control treatment. The experiment was set up with three biological replications of the two treatments (inoculated and un-inoculated) for each cultivar. To determine suitable time points for RNA isolations, extra pots were planted to monitor tuber initiation and development on the selected cultivars. Tuber initiation on both cultivars started five weeks after emergence (WAE). Diriwachter and Parbery (1991) indicated that tubers are susceptible to Sss only during the first 14-20 days of tuber initiation; hence 7 WAE was selected as the early stage of tuber development.

Tuber sampling

Twelve plants per treatment (inoculated and non-inoculated) for both cultivars, Innovator and Vanderplank were destructively sampled at 7 and at 15 WAE for powdery scab disease assessment and RNA extractions. Seven tubers from two plants of each replication were randomly selected, washed and scored for powdery scab severity according to a standard disease assessment key derived by Falloon *et al.*, (1995), where 0 was no visible disease symptoms and 10 was when tuber surface was completely covered with tuber lesions. Powdery scab disease indices for the two cultivars were calculated as disease index = disease incidence x disease severity (Merz *et al.*, 2012). For gene expression analysis, whole potato tubers were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction (diseased and symptomless tubers from two plants were pooled for each of the three biological replicates).

RNA sample preparation and quality check

For total RNA extraction, 100 mg of homogenized tuber samples from two plants of each treatment were ground into a fine powder. All RNA extractions were performed in three biological replications. RNA was extracted using a Direct Zol™ RNA extraction kit (Zymo Research, USA) together with Tri-Reagent® (Sigma-Aldrich, Germany) following the manufacturer's instructions. Contaminating DNA was removed from the samples using Invitrogen™ Ambion™ TURBO DNA-free kit, according to the manufacturer's instructions. The yield and purity of RNA was evaluated by measurement of absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Thermo Scientific Technologies). RNA integrity of the samples was assessed using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).

Preparation of cDNA libraries and Illumina Sequencing

Construction of cDNA libraries and sequencing was carried out at the Beijing Genomics Institute (BGI-Shenzhen, China; http://www.genomics.cn/en/index). Aliquots of high quality total RNA (5ug, 100 ng/µl) from the three biological replicates for each treatment combination were sent to BGI and were used for poly(A) + mRNA isolation and preparation of cDNA libraries using the TruSeq RNA sample Prep Kit v2 (Illumina, San Diego, CA, USA) following manufacturer's instructions. The quality of the libraries was re-checked and quantified using an Agilent BioAnalyzer 2100 system and qPCR. Finally, the cDNA libraries were sequenced with an Illumina Hi Seq 2000 sequencer generating 90 bp paired-end reads

Quality validation, read mapping and functional enrichment analysis

Quality of individual sequences was evaluated using TrimGalore (Cutadapt and FastQC) tool (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) analysis. High quality sequence reads were aligned and mapped to the potato reference genome sequence (*Solanum tuberosum* Group Phureja DM v4.04) (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml), using Hisat2 v2.1 spliced aligner. Transcript assembly and quantification of mapped reads (counting reads mapped against exons), were performed using StringTie v1.3.3 and featureCounts (http://subread.sourceforge.net/), respectively. Generated read counts for each sample were used for post mapping quality check prior to performing differential expression analysis. The percentage of uniquely mapped reads ranged from 73 to 85%. A False Discovery Rate (FDR) threshold of 10% and an absolute log 2-fold change > 1 were used to determine differentially expressed genes. G-fold (v1.1.4) analysis tool was used to perform differential expression (DE) analysis. Functional enrichment analysis of differentially expressed genes obtained from each comparison (direct pairwise comparison between cultivars Innovator and Vanderplank or cultivar specific comparisons of inoculated samples and un-inoculated controls) was performed using g: Profiler web Server (Reimand *et al.*, 2016) Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/) was used to make an illustration of the differentially expressed transcripts displayed in Venn diagrams (Oliveros, 2007-2015).

RT-qPCR gene expression analysis

Ten randomly selected DEGs were analysed by RT-qPCR for gene expression profiling. The expression levels were monitored in potato tubers of tolerant (Innovator) and susceptible (Vanderplank) cultivars, with the two treatments (inoculated and un-inoculated) at the two

different timed harvest points, 7 WAE and 15 WAE. All RNA extractions were performed in three biological replicates and each cDNA sample was analyzed three times (three technical replications) per treatment (24 samples). First-strand cDNA was synthesized from 1 µg of total RNA using Superscript™ III First-Strand cDNA Synthesis ImProm-II™ Reverse Transcriptase kit according to the manufacturer's protocol (Promega, USA). Gene expression analysis was performed by RT-qPCR, using a SYBR green dye system, according to Minimum Information for Publication of Quantitative qPCR Experiments (MIQE) guidelines (Bustin *et al.,* 2009). Specific primer sets were designed using an online primer design software Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) from the specific sequence of *Solanum tuberosum* Group Phureja DM v4.04 (http://*solanaceae*.plantbiology.msu.edu/pgsc_download.shtml). The primer pairs were chosen and validated *in-silico* using primer BLAST specific analysis (http://www.ncbi.nlm.nih.gov/Blast.cgi) and according to RT-qPCR efficiency calculation. Primer sequences as well as expected amplicon product sizes are listed in Table 1.

The RT-qPCR reactions were performed in a CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). For RT-qPCR, 2 µl of cDNA was added to 10 µl iTaq Universal SYBR® Green supermix (Bio-Rad, USA) and primers at a concentration of 0.5 µm under the following conditions: an initial activation cycle (10 min at 95 °C), followed by 40 cycles of denaturation, annealing and polymerization (15 sec at 95 °C, 60 sec at 60 °C). PCR amplification was conducted in a total volume of 20 µl, with 1 µl of diluted (1:20) cDNA, 50 nmol l^{-1} primers and 10 µl of SYBR® Green Supermix. To determine the specificity of the amplicons, melting curve analysis was performed over the temperature range of 60 to 95 °C. The RT-qPCR efficiency (E) of each primer pair was determined using standard curves generated according to the equation $E = 10^{-1}$/slope of five triplicate cDNA pool dilutions (undiluted, 0.25, 0.0625, 0.015, and 0.003). Standard curves were used to validate primer pairs. All standard curves had a PCR efficiency of between 90 - 110% with a R^2 value higher than 0.9. The comparative $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001) was used to evaluate the relative expression level of each gene. The samples were normalized to β-tubulin and elongation factor1-α (*EF1-α*) as the reference genes and the un-inoculated samples were used as calibrators (Nicot *et al*., 2005). Each sample was run in three technical replications. In the amplification process, the detection of fluorescence was carried out during the combined annealing step. Melting curve analysis was performed to verify amplification of the specific target DNA. The melting curves step started with slow heating at 60 °C with a rate of 1 °C up to 90 °C and each step followed as per the manufacturer's instructions.

Table 1 Primer information for reverse transcriptase quantitative polymerase chain reaction validation of RNA sequencing data and gene expression analysis of potato tubers

Gene name	Gene description	Accession number	Primer sequence (5'-3')	Expected amplicon size (bp)	PCR efficiency (%)[a]
StSN2	Snakin-2 protein	NW_006239862.1	F-TGCATGCCAAAACTCATCAT/ R-GATCGACGGCTTCAATCACT	162	85
StSN1	Snakin-1 protein	NW_006240278.1	F-TGCAAGCAAGTGATTGAAGC/ R-AAGCCAGGAAAACGAGAGGT	190	91
StTOSB	Tospovirus resistance protein B	NW_006239163.1	F-CACCTTTGCGACATCTCTGA/ R-CTCAGTTTCCCACGGTTTGT	203	96
StNBS	NBS-LRR protein	NW_006239040.1	F-CAACTTCAGCAGATCGTCCA/ R-GGATTGCCTCTTGTGGTTGT	235	97
StDEF	Defensin J1-2	NW_006239640.1	TCCAATATGGCTGGCTTTTC/ R-AGTGTCTGGTGCAGAAGCAA	226	110
StLOX	Lipoxygenase	NW_006238985.1	F-CAAATCGCCCCACAGTAAGT/ R-CAAATTTGTCGAAAGCAGCA	243	89
StMRNA	MRNA, 1346 bp sequence	NW_006239280.1	F-AGTCGAACAAATGGCGTCTT/ R-CCGCATGGTGTTTCTCTACA	242	118
StUDP	UDP-glucoseglucosyl transferase	NW_006239431.1	F-TGGTGCAATCAGCTCAAGAC/ R-TAGGCCTCACACCAATCTCC	184	112
StWRKY6	WRKY transcription factor 6	NW_006238949.1	F-GGGTTAATTCGTGGTCGAGA/ R-TAATCTTCCGGCGACTTGAC	203	114
StPRF	PRF	NW_006239027.1	F-TCATTTGCTGAGTGCTGGAC/ R-AGCCACTTTGGAAGCTGAAA	218	107
ef1α[b]	Elongation factor 1-α (ef1α)	NW_006238970.1	F-ATTGGAAACGGATATGCTCCA/ R-TCCTTACCTGAACGCCTGTCA	101	86
β-tubulin[b]	β-tubulin	NW_006238934.1	F-ATGTTCAGGCGCAAGGCTT/ R-TCTGCAACCGGGTCATTCAT	101	101

[a]PCR amplification efficiencies and regression coefficients for the standard curves are reported for each primer pair.

[b]Reference genes.

Statistical analysis

Data were analyzed with the General Linear Model (GLM) procedure in the Statistical Analysis System computer program (SAS 9.4; SAS Institute, 2006, Cary, NC). Mean values were separated using the Least Significant Difference (LSD) test at 5% to check whether RT-qPCR results were statistically different when comparing inoculated to un-inoculated samples and the difference between the two cultivars.

Results

Powdery scab diseas on potato tubers

Plants inoculated with Sss inoculum resulted in the development of powdery scab lesions in the susceptible cultivar Vanderplank, while there were no visible powdery scab lesions on the tolerant cultivar Innovator at both time points (Figure 1). This confirmed that Innovator maintained its tolerance to Sss tuber infection. Moreover, no symptoms were observed on the un-inoculated controls of both cultivars at the two time points.

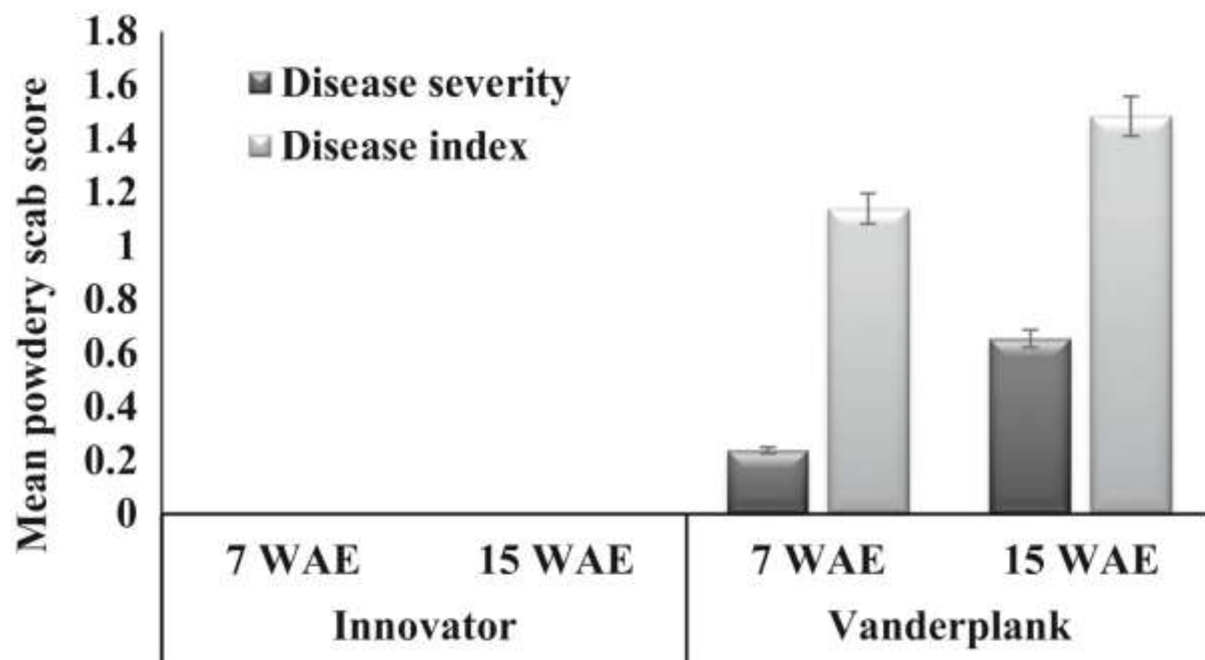

Figure 1. Mean powdery scab disease incidence, severity and index of two potato cultivars evaluated for *Spongospora subterranea* f. sp. *subterranea* tuber infection in two greenhouse pot trials. Values are log_{10} transformed means of the three biological replicates for each cultivar. Bars represent the standard error.

Identification and functional classification of differentially expressed genes

A total of 2058 DEGs were identified in both the tolerant and susceptible cultivars; of these, 1076 and 954 DEGs related to growth and / or development of the plant were found in Innovator and Vanderplank cultivars, respectively (Supplementary Tables 1 and 2). In addition, 28 DEGs were present in both cultivars at 7 weeks after emergence (WAE). The heat map

created to highlight the relationship and clustering between expressed genes in the inoculated and un-inoculated samples showed a clear difference between the samples (Figure 2). The Venn diagrams constructed showed the distribution of expressed genes in both cultivars (Figures 3A and B). A total of 61 % of the genes were up-regulated (Supplementary Table 3), while only 39 % of the genes were down-regulated in both cultivars (Supplementary Table 4).

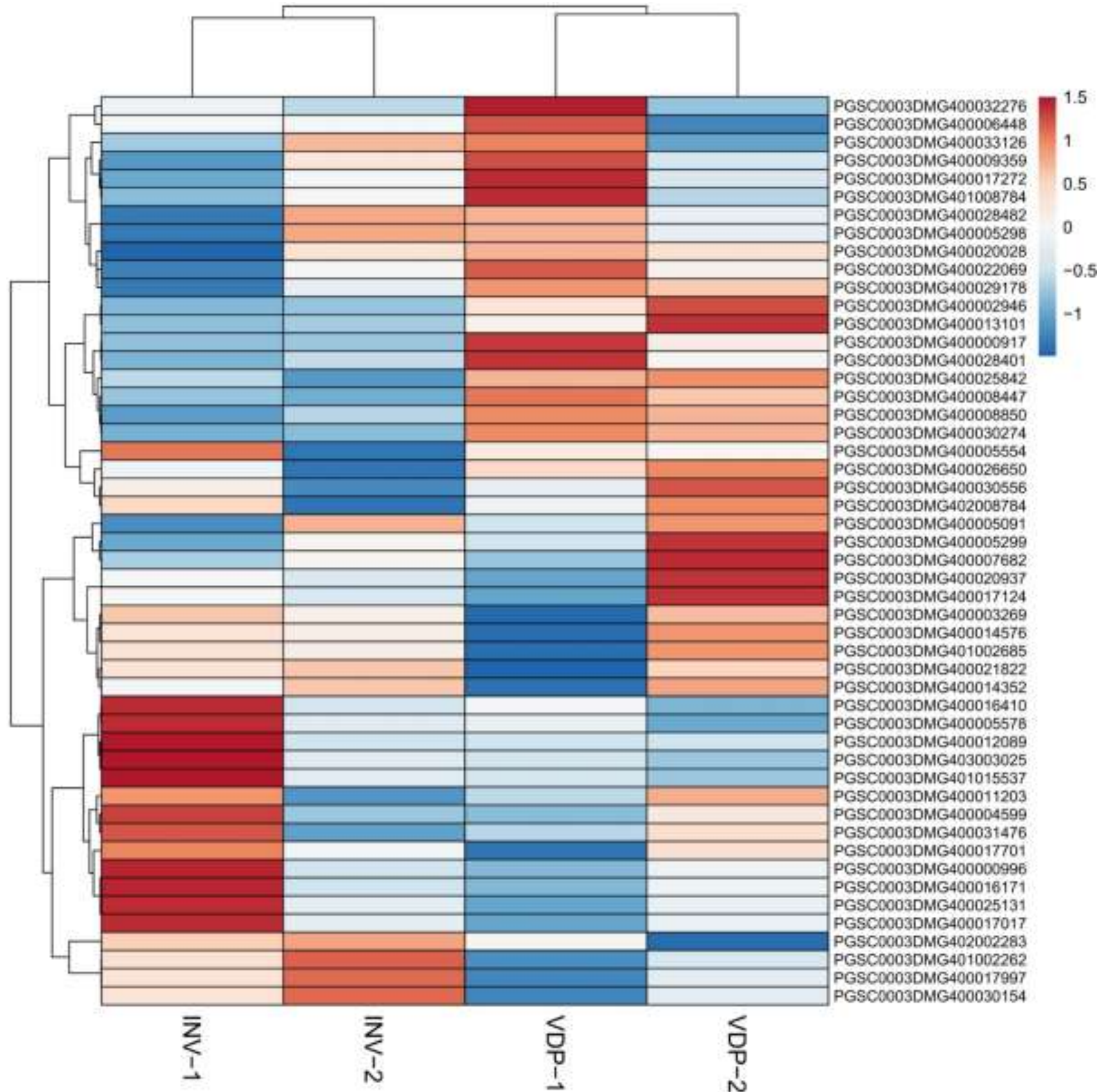

Figure 2. Heat map showing expression profiles of differentially expressed genes in response to *Spongospora subterranea* f. sp. *subterranea* infection between the two cultivars, INV-1 (inoculated Innovator), INV-2 (uninoculated Innovator) and VDP-1 (inoculated Vanderplank), VDP-2 (uninoculated Vanderplank). The up-regulated genes are shown in red while the down-regulated genes are shown in blue.

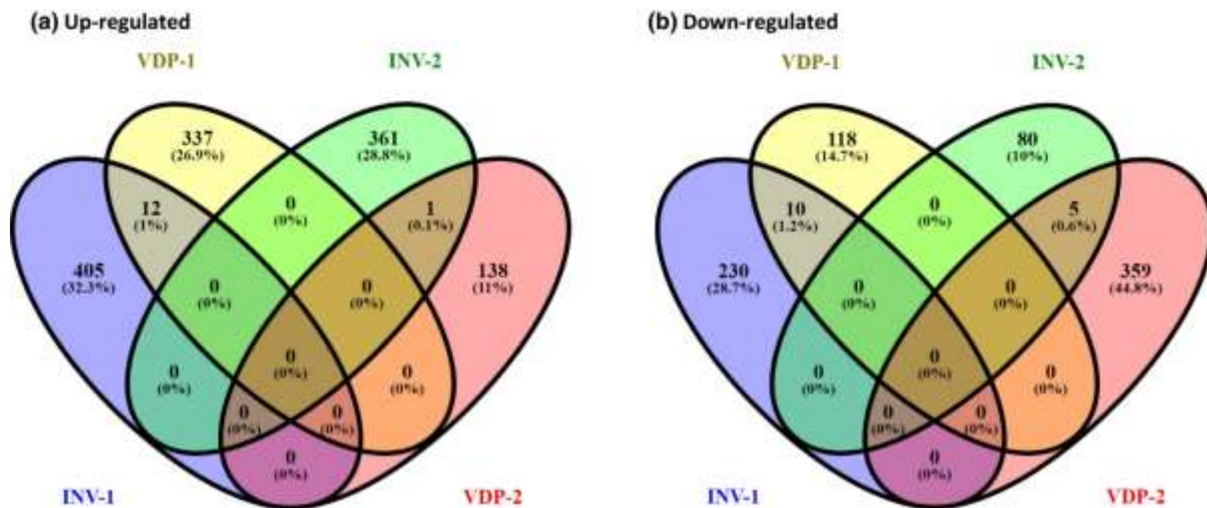

Figure 3. Venn diagrams illustrating the differentially expressed genes in the treatments. (a) Up-regulated genes and (b) down-regulated genes in Innovator (tolerant) and Vanderplank (susceptible) cultivars inoculated with *Spongospora subterranea* f. sp. *subterranea* in comparison to uninoculated control plants. INV = Innovator; VDP = Vanderplank; 1 = inoculated with *Spongospora subterranea* f. sp. *subterranea*; 2 = uninoculated control.

To identify the potential relationships between the gene expression patterns and their biological functions, the DEGs were classified into functional categories (Figure 4) according to their putative function obtained using Gene Ontology (GO) in g: Profiler webserver (http://biit.cs.ut.ee/gprofiler/). Candidate genes were assessed for their involvement in potato defense responses based on differential expression in the two cultivars. Genes were grouped into the following functional groups: transport, defense response, metabolic processes, response to stress, reduction processes and gene regulation. All six functional groups were present in both the Innovator and Vanderplank cultivars. Among the 1076 DEGs specific to the tolerant cultivar, GO enrichment analyses revealed that 129 DEGs were overrepresented in the gene regulation biological process category, followed by genes belonging to the metabolic process category (). Enrichment analysis showed a comparable response of the two cultivars to Sss infection. A significant increase in up-regulation of defense related genes was observed in plants inoculated with Sss than in the un-inoculated control plants.

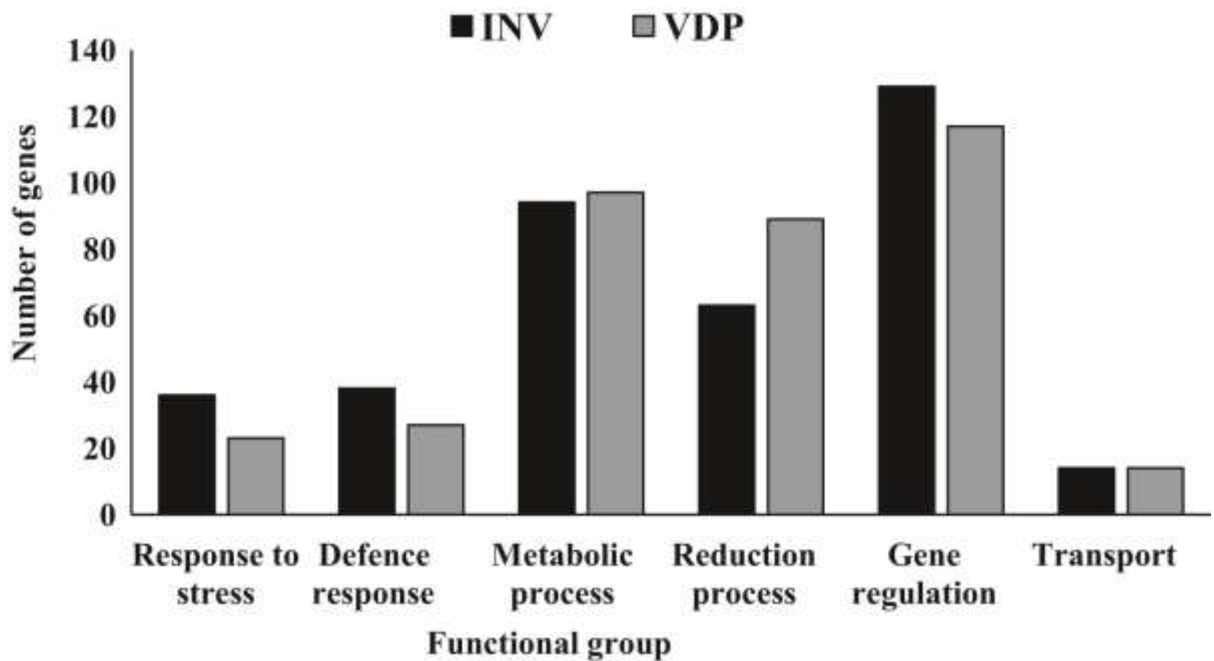

Figure 4. Gene ontology (GO) categories of differentially expressed genes showing functional classification of expressed genes derived from each cultivar (INV = Innovator; VDP = Vanderplank).

Analysis of differentially expressed defense related genes using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

For confirmation of RNA-seq results and gene expression analysis, 10 genes (*StWRKY6*, *StSN2*, *StLOX*, *StTOSB, StNBS*, *StMRNA*, *StDEF, StUDP, StPRF* and *StSN1*) were analyzed in inoculated and un-inoculated plants of two potato cultivars differing in Sss susceptibility, using RT-qPCR. The RT-qPCR provided a detailed picture of how changes occurred in gene expression levels of the selected genes at 7 WAE and 15 WAE in the tolerant and susceptible cultivars. The RT-qPCR results were in agreement with the RNA-seq expression patterns in the tolerant and susceptible cultivars (Figures 5; Supplementary Figure 1). Figure 5 demonstrates a comparison of fold changes between the two cultivars inoculated and un-inoculated with Sss in gene expression levels, as measured by RNA-Seq and RT-qPCR. This includes 10 selected genes identified by RNA-Seq that are associated with functions related to disease defense. There was a significant Spearman correlation between fold change expression by the two techniques ($R^2 = 0.79$, $P < 0.05$).

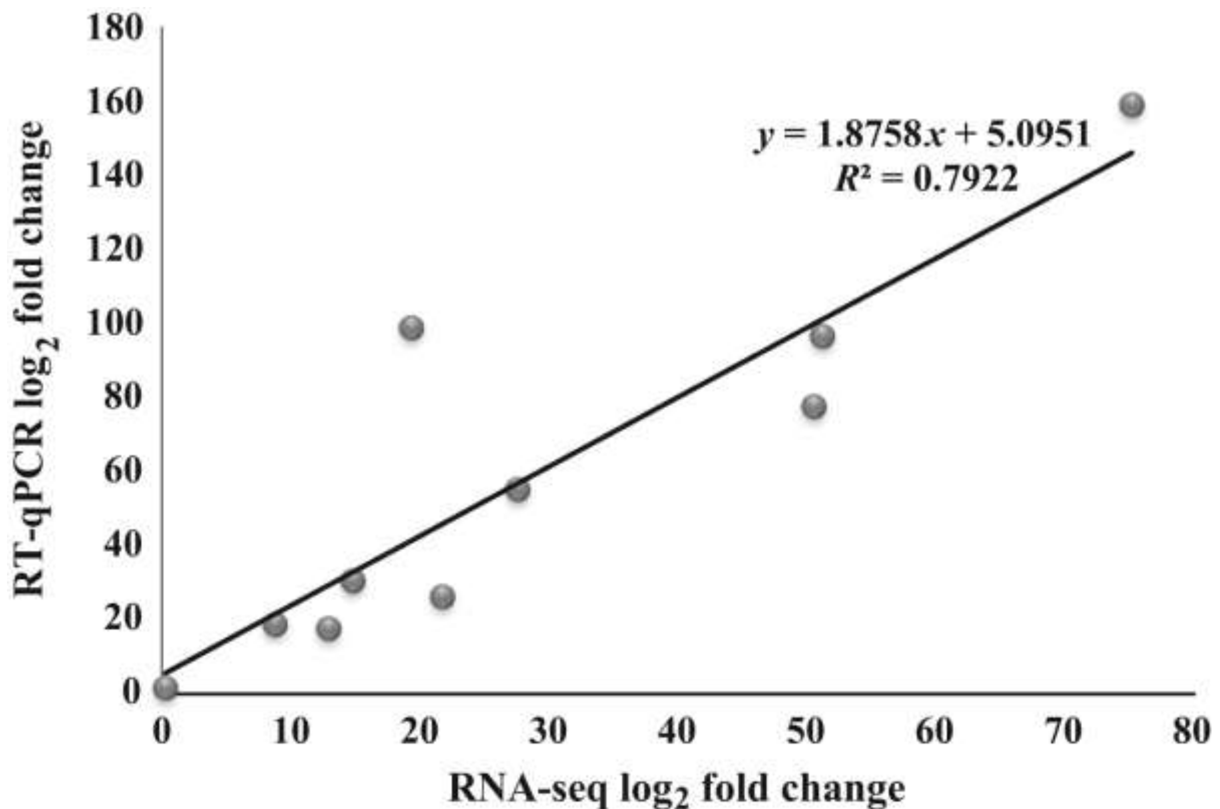

Figure 5. Comparisons of relative gene expression as determined by RNA-sequencing and reverse transcriptase quantitative PCR (RT-qPCR) for the 10 selected defence-related genes.

The RT-qPCR analysis performed indicated that nine of the selected defense-related genes were significantly up-regulated in the inoculated plants compared to the un-inoculated control plants (Figure 6). Even though the expression of the genes increased at 7 WAE in both cultivars, all the genes assessed remained constant (1-fold) for all the control plants at both time points. Differences in the gene expression levels were observed between the inoculated tolerant and susceptible cultivars at 7 and 15 WAE. The highest gene up-regulation with a change of 158.6-fold was observed with the *StWRKY6* gene in the tolerant cultivar compared to the susceptible cultivar, which had a lower up-regulation of 26.8-fold at 7 WAE (). The expression level of the *StNBS* gene was up-regulated by 98.5-fold and 21.9-fold in the tolerant and susceptible cultivars, respectively at 7 WAE. This was followed by up-regulation of *StMRNA* (96.1-fold), *StTOSB* (77.3-fold), *StSN1* (54.9-fold), *StPRF* (30.4-fold), *StSN2* (26-fold), *StLOX* (18.7-fold) and *StUDP* (17.6-fold) in the tolerant cultivar, which was significantly higher ($P < 0.05$) than the up-regulation levels of the same genes in the susceptible cultivar at 7 WAE . Conversely, no increase in expression was observed for the *StDEF* gene in the tolerant cultivar at either time-point. However, *StDEF* was up-regulated by 9-fold at 7 WAE, with an increase of 26.8-fold at 15 WAE in the susceptible cultivar . Expression of *StNBS*, *StMRNA*,

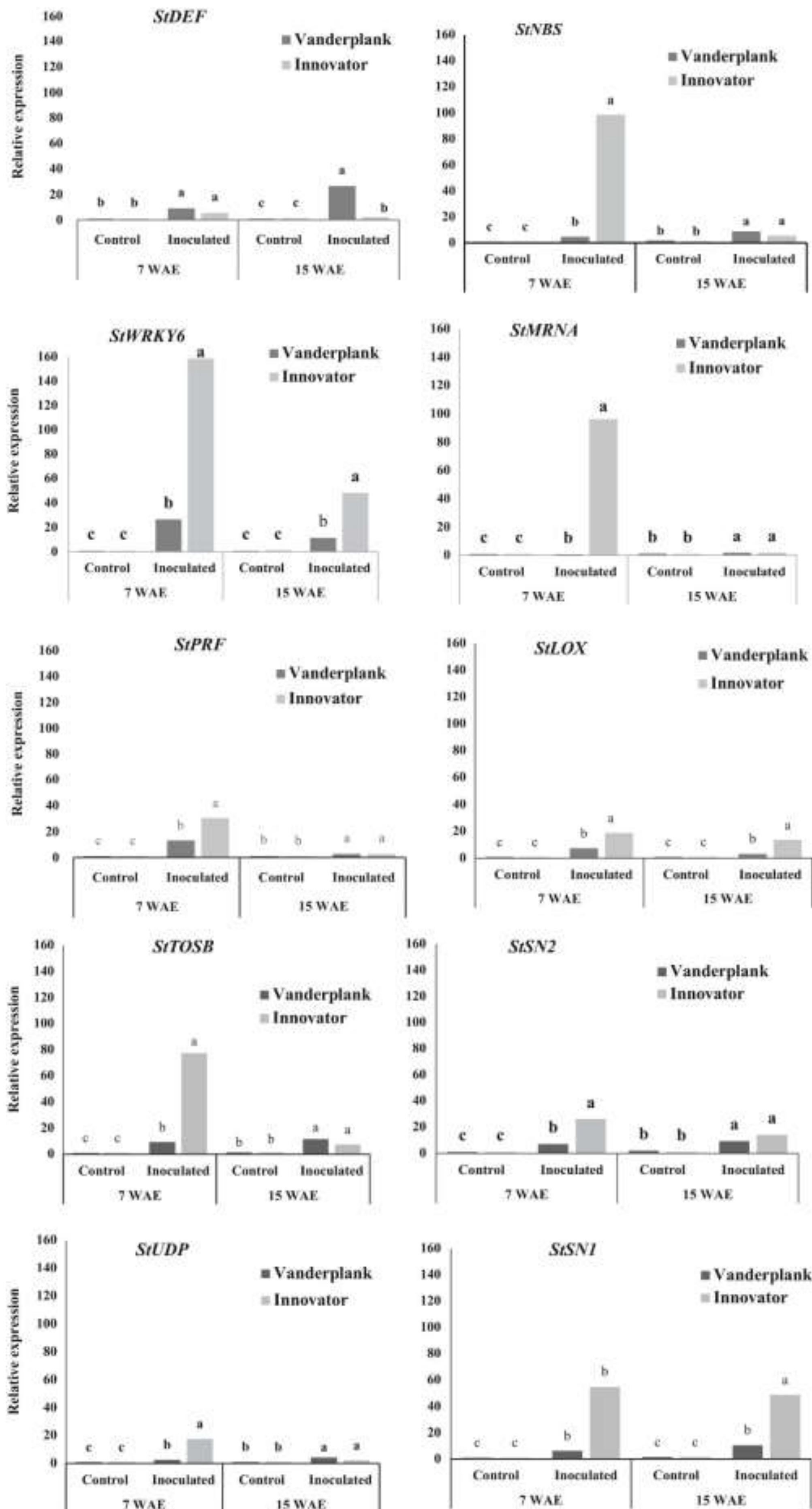
StDEF
StNBS
StWRKY6
StMRNA
StPRF
StLOX
StTOSB
StSN2
StUDP
StSN1
Vanderplank
Innovator
Relative expression
Control
Inoculated
7 WAE
15 WAE

Figure 6. Relative expression levels of selected potato defence genes in tolerant and susceptible potato cultivars. Expression levels of *StTOSB*, *StSN2*, *StUDP*, *StSN1*, *StPRF*, *StLOX*, *StWRKY6*, *StMRNA*, *StDEF* and *StNBS* were measured in Innovator (tolerant) and Vanderplank (susceptible) cultivars inoculated and uninoculated with *Spongospora subterranea* f. sp. *subterranea*. Amplification of *StEF*α-1 and β-*tubulin* gene expression was used to normalize the expression value in each sample. The relative expression values were determined against the average values of the uninoculated control samples. Data represent fold change of gene expression at 7 weeks after emergence (WAE) and at 15 weeks after emergence. The average of three replicates is shown and different letters show significant differences (Student's *t*-test: $P < 0.05$) between the two cultivars.

StUDP, StTOSB and *StPRF* genes was highly up-regulated at 7 WAE in the tolerant cultivar, but showed a sudden decrease of 3.7-fold, 2-fold, 2.2-fold, 7.5-fold and 2.8-fold, respectively at 15 WAE . On the other hand, expression of the four genes, *StWRKY6*, *StSN2*, *StLOX*, and *StSN1*, was highly up-regulated at 7 WAE, persisted and remained up-regulated with fold increases of 48.3-fold, 14-fold, 13.3-fold and 48.7-fold respectively, at 15 WAE in the tolerant cultivar.

Discussion

Next-generation transcriptomic sequencing was used in this study to identify genes that are differentially expressed in tolerant and susceptible potato cultivars in response to Sss infection at two different time points (7 WAE and 15 WAE). The differentially expressed genes involved in potato tuber Sss defense were validated using RT-qPCR. Several putative defense related genes were identified and were differentially expressed in the inoculated plants compared with the un-inoculated plants.

Differentially expressed genes related to plant defense responses, including the Defensin J1-2 gene, were identified in both cultivars. Furthermore, genes involved in pathogen perception were differentially expressed in both cultivars. These included signalling genes such as WRKY transcription factors (e.g. *StWRKY6*) and pathogen detection genes (e.g. NBS-LRR protein). However, the highest number of up-regulated defense response genes was observed in the inoculated tolerant cultivar. This observation is similar to the findings by Dees *et al.* (2016) who reported a pronounced up-regulation of defense related genes including the disease resistance protein At4g27190 in a *Streptomyces turgidiscabies* infected potato cultivar, compared to the susceptible cultivar. Evers *et al.* (2003) also found that most of the differentially expressed genes were up-regulated in the resistant compared to the susceptible potato cultivar upon infection by *Phytophthora infestans*.

The WRKY domain-containing protein is a well-established marker for defense responses of plants against pathogens (Rushton *et al.,* 2010). Hence, *StWRKY6* was significantly up-regulated in the tolerant potato cultivar compared to the susceptible cultivar.

The WRKYs are one of the largest families of transcriptional factors in plants and are associated with regulation of transcriptional reprogramming related to plant immune responses (Yogendra *et al.,* 2015). However, several studies have focused on model plants like Arabidopsis (Pandey & Somssich, 2009) and tobacco (Menke *et al.,* 2005). Few members of the WRKY superfamily in potatoes have been isolated or functionally characterized. For instance, a significantly higher up-regulation of the *StWRKY1* gene was reported in the resistant potato cultivar compared to the susceptible cultivar upon infection of potatoes by *Phytophthora infestans* (Yogendra *et al.,* 2015). Similarly, *AtWRKY33* was highly up-regulated in the tolerant cultivar compared to susceptible cultivar in response to *Pectobacterium carotovorum* subsp. *brasiliense* potato stem infection (Kwenda *et al.,* 2016).

Lipoxygenase (*StLOX*) gene is one of the potato tubers' main storage proteins and is known to play a key role in resistance of tubers to powdery scab, through deposition of suberin to the skin (Perla *et al.,* 2014). Comparably in this study, *StLOX* was highly up-regulated at the early stages of disease development and remained high until harvest of the tubers. More so, the *StLOX* gene is one of the key markers associated with late blight resistance in potatoes (Trognitz *et al.,* 2001); regulating a number of suberin- and/or non suberin- mediated pathways in potatoes (Perla *et al*., 2014). Other than the *StLOX* gene, three more genes (*StWRKY6*, *StSN2* and *StSN1*) showed a persistent expression behavior, from the early stage of disease development to maturity of the tubers, in the tolerant cultivar as opposed to the susceptible cultivar. Similarly, Dees *et al.* (2016) reported an early and sustained response of potatoes to *S. turgidiscabies* infection in a resistant cultivar but not in a susceptible cultivar. In-depth research is suggested for further exploration into the expression and role of these genes during potato tuber storage.

The up-regulation of *StSN1* and *StSN2* in the tolerant cultivar compared to the susceptible cultivar underlines the importance of these genes during plant disease defense. Snakin-1 (SN1) and snakin-2 (SN2) are two cell wall antimicrobial peptides that were isolated from potato tubers and were found to be active against important fungal and bacterial plant pathogens (Berrocal-Lobo *et al.,* 2002). They are components of both the constitutive and inducible defense barriers, specifically in storage and reproductive plant organs (Berrocal-Lobo *et al.,* 2002). Therefore, the *StSN1* and *StSN2* expression levels were highly up-regulated in the tolerant cultivar, while only a slight up-regulation was observed in the susceptible cultivar during infection of the tubers. This was in agreement with Berrocal-Lobo *et al.* (2002) who reported *StSN2* up-regulation after infection of potato tubers with *Botrytis cinerea*. Moreover, overexpression of the *StSN1* gene was reported to result in transgenic potato

resistance to *Rhizoctonia solani* and *Pectobacterium carotovorum* subsp. *carotovorum* (Almasia *et al.,* 2008).

The NBS-LRR protein (*StNBS*) up-regulation was induced at early stages of powdery scab disease development in the tolerant cultivar, as opposed to the susceptible cultivar. Defense response by NBS-LRR proteins induces effector-triggered immunity (ETI) and several of these NBS-LRR proteins recognize effectors secreted by pathogens that in turn activate downstream signaling pathways, leading to activation of plant defense response against various classes of pathogens (Dubey & Singh, 2018).

Phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play an important role in defense responses against pathogens in different plants (Shah, 2003). Therefore, the expression levels of potato SA marker genes were monitored in the current study. The findings of this study suggested that the tolerant potato cultivar Innovator employs the salicylic acid (SA) pathway hormonal responses against infection by Sss. This was evident in the up-regulation of three SA marker genes; *StWRKY6* (PGSC0003DMG400029207), *StUDP* (PGSC0003DMG400015601) and *StMRNA,* (GSC0003DMG402007388), while *StUDP* and *StMRNA* were down-regulated in the susceptible cultivar. The involvement of these genes in SA signaling in potatoes was previously reported by Wiesel *et al.* (2015). These genes were specifically up-regulated when potato plants were treated with SA hormone compared to ET and JA treatments (Wiesel *et al*., 2015). Generally, the SA hormonal pathway is related to plants' responses to biotrophic pathogens such as Sss; whereas JA and ET are related to defenses against necrotrophic pathogens (Derksen *et al.,* 2013).

It was noted that the *StDEF* gene was significantly up-regulated in the susceptible cultivar, while down-regulated in the tolerant cultivar, suggesting its involvement as a susceptibility-related gene to Sss in potatoes, although further investigations are required to validate this finding. Late blight susceptibility genes were identified in potatoes, where WRKY transcription factor 6 was found to be one of the susceptibility proteins (Sahu *et al.,* 2014), while it was highly up-regulated in the current study. Similarly, susceptibility-related genes against necrotrophic pathogens such as MYC2 basic helix-loop-helix-leucine zipper (bHLH) transcriptional factors (e.g. PGSC0003DMG400007010 and PGSC0003DMG400012237) were differentially expressed in a susceptible cultivar upon potato stem infection by *Pectobacterium carotovorum* subsp. *brasiliense* (Kwenda *et al.,* 2016).

The results of this study showed that increased mRNA accumulation for many plant defense genes is more rapid and pronounced during the early stages of Sss disease development. The products of major resistance (R) genes recognize and interact with elicitors

produced by pathogens, and the proteins encoded by defense response genes initiate signal transduction, leading to defense responses of host plants. The defense-response genes are usually race non-specific (Wen *et al.,* 2003), and thus, may have potential for improving the disease resistance of crop plants.

Quantitative analysis of defense response genes identified by RNA-seq contributes to the understanding about the roles of genes involved in powdery scab resistance. The RT-qPCR analysis revealed up-regulation of nine defense response genes in the tolerant cultivar, compared to the susceptible cultivar, indicating their potential involvement in the defense responses to Sss infection. While the limited number of cultivars and timed harvest points used in this study somewhat limit the interpretation of results in this study, there is an indication that Innovator mounts an early and durable response to infection by Sss, while Vanderplank is not able to sustain the defense response until harvest of plants. Future studies screening a larger number of cultivars at numerous timed harvest points will be beneficial in supporting the outcomes of this study. Selection of disease response genes from potatoes, their expression and functional analysis may facilitate the development of biomarkers, which then could be utilised in the development of Sss resistant cultivars by marker-assisted breeding.

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Conflict of interest

The authors declare no conflict of interest.

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