



Transcriptome signatures of tomato leaf induced by *Phytophthora infestans* and functional identification of transcription factor *SpWRKY3*

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Abstract

Key message *SpWRKY3* was identified as a resistance gene to *Phytophthora infestans* from *Solanum pimpinellifolium* L3708 and its transgenic tomato showed a significant resistance to *P. infestans*. This finding reveals the potential application of *SpWRKY3* in future molecular breeding.

Abstract Transcription factors (TFs) play crucial roles in the plant response to various pathogens. In this present study, we used comparative transcriptome analysis of tomatoes inoculated with and without *Phytophthora infestans* to identify 1103 differentially expressed genes. Seven enrichment GO terms (level 4) associated with the plant resistance to pathogens were identified. It was found that thirty-five selected TF genes from GO enriched term, sequence-specific DNA binding transcription factor activity (GO: 0003700), were induced by *P. infestans*. Of these TFs, the accumulation of a homologous gene of WRKY (*SpWRKY3*) was significantly changed after *P. infestans* induction, and it was also isolated from *P. infestans*-resistant tomato, *Solanum pimpinellifolium* L3708. Overexpression of *SpWRKY3* in tomato positively modulated *P. infestans* defense response as shown by decreased number of necrotic cells, lesion sizes and disease index, while the resistance was impaired after *SpWRKY3* silencing. After *P. infestans* infection, the expression levels of *PR* genes in transgenic tomato plants overexpressed *SpWRKY3* were significantly higher than those in WT, while the number of necrotic cells and the reactive oxygen species (ROS) accumulation were fewer and lower. These results suggest that *SpWRKY3* induces *PR* gene expression and reduces the ROS accumulation to protect against cell membrane injury, leading to enhanced resistance to *P. infestans*. Our results provide insight into *SpWRKY3* as a positive regulator involved in tomato–*P. infestans* interaction, and its function may enhance tomato resistance to *P. infestans*.

Introduction

Plants are exposed to a diversity of microorganisms during their entire life cycle (Derksen et al. 2013). To survive, plants develop efficient defenses against pathogen attack. During the pathogen attack, the pathogens produce effectors

to increase their pathogenicity. However, plants can sense the pathogens using an innate immune system that consists of two-way communication, PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (El Hadrami et al. 2011; Guttman et al. 2014). These two efficient strategies are complex physiological, biochemical and molecular processes, which play important roles through signaling transduction pathways to modulate regulatory proteins (e.g., transcription factors and protein kinases) and pathogenesis related proteins (Buscaill and Rivas 2014; Amorim et al. 2017). Previous study showed that transcription factors (TFs) as key components of plant defense mechanisms activated the expression of stress-related genes. They acted through sequence-specific interactions with *cis*-regulatory DNA elements in the promoters of stress-related genes (Garner et al. 2016). For example, an AP2/ERF TF acted in the recognition of GCC-box element with the sequence AGC CGCC, binding to the promoter regions of *PR* genes (such as *PR1–PR5*), and modulating their expressions against the

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pathogens (Ohme-Takagi and Shinshi 1995; Amorim et al. 2017). In addition, a TF MYB30 positively regulated *Arabidopsis thaliana* defense and associated cell death responses to bacteria through transcriptional activation of some genes related to very-long-chain fatty acid (VLCFA) metabolism (Canonne et al. 2011).

WRKY TFs comprise a large family of plant TFs, which play important roles in many biological processes, including plant hormone signal transduction, fruit development, leaf senescence and abiotic and biotic stresses (Li et al. 2015; Cheng et al. 2017; Jiao et al. 2017; Ye et al. 2017). The WRKY family is classed into three major groups based on the number of WRKY domains and the type of zinc finger motif (Huang et al. 2012; Guo et al. 2014). In plant–pathogen interaction, WRKY TFs implicated in the regulation of plant defense mechanisms (PTI and ETI) against pathogens by recognizing the W-box (TTGACC/T) in promoter regions of some genes (Rushton et al. 2010). The mitogen-activated protein kinase (MAPK) signaling pathway is involved in PTI. In *Arabidopsis*, pathogen attack activated an MAPK module, MEKK1–MKK1/2–MPK4, which led to the release of *AtWRKY33* and *MKS1* from MPK4–*MKS1*–*AtWRKY33* complex. The *MKS1*–*AtWRKY33* recognized the W-box in the promoter region of *phytoalexin deficient 3 (PAD3)* that was involved in synthesis of antimicrobial camalexin and modulated its expression (Qiu et al. 2008; Ishihama and Yoshioka 2012). Meanwhile, the MAPKs phosphorylated *NbWRKY8* in *Nicotiana benthamiana*, resulting in the enhancement of DNA-binding and transactivation activities in disease resistance signaling pathway (Ishihama et al. 2011). WRKYs also were involved in ETI. *Arabidopsis AtWRKY52/RRS1* was a complex of a WRKY TF and a TIR-NB-LRR protein conferring ETI immunity against the bacterial pathogen *Ralstonia solanacearum*. Mutation of the WRKY domain of *AtWRKY52* led to an autoimmune response, suggesting that the WRKY domain normally has a repressor function in plant immune responses (Deslandes et al. 2002, 2003; Noutoshi et al. 2005; Tasset et al. 2010). *Hordeum vulgare HvWRKY2* was a regulator of the defense response. A NBS-LRR protein, MAL recognized the powdery mildew AVR effector, resulting in the inhibition of the *HvWRKY2* function (Shen et al. 2007; Ishihama and Yoshioka 2012).

Tomato as an important model plant for fleshy fruit development and especially for plant–pathogen interactions has been studied extensively (Cui et al. 2017). Its genome sequencing has been finished in 2012 (Tomato Genome Consortium 2012). In addition, tomato also is a major crop plant constituting a major worldwide agricultural industry. Tomato late blight (LB) caused by the oomycetes pathogen *Phytophthora infestans* is one of the most devastating diseases of field-grown tomatoes (Zhang et al. 2014). This oomycetes, which has a worldwide distribution,

causes serious economic loss for field-grown tomatoes, and therefore, is regarded as a major threat to tomato production (Luan et al. 2016). For example, in Inner Mongolia of China and the USA, LB caused the loss of tomato production in early 2000s (Nowicki et al. 2012; Zhang et al. 2013). In plant–*P. infestans* interaction, *P. infestans* can produce effectors to increase their pathogenicity, such as AVR1, AVR2, AVR3a, PexRD2, IPI-O1/4 and others (King et al. 2014; Du et al. 2015; Chen and Halterman 2017; Turnbull et al. 2017; Wawra et al. 2017), while plants develop efficient defenses against *P. infestans* attack, such as the production of resistance (R) proteins. Currently, more than 60 potato *R* genes against *P. infestans*, have been identified, located, cloned, and studied (Rodewald and Trognitz 2013; Zhang et al. 2014). Compared with intensive studies in potato, studies on LB resistance in tomato were very limited. Only several tomato *R* genes have reported, such as *Ph-1*, *Ph-2*, *Ph-3*, *Ph-4* and *Ph-5* (Luan et al. 2015). In our previous studies, we identified and analyzed the function of *P. infestans*-induced genes, miRNAs and lncRNAs in tomato. For example, a *P. infestans*-induced TF, WRKY1 was involved in resistance to *P. infestans* (Li et al. 2015). Overexpression of miR172 in tomato enhanced the tomato resistance to *P. infestans* (Luan et al. 2018). In addition, we also found that a tomato lncRNA, lncRNA16397 conferred tomato resistance to *P. infestans* by co-expressing *glutaredoxin* (Cui et al. 2017). Although the study on the tomato–*P. infestans* interaction have got progressions, the molecular mechanisms of their interaction are also beyond understanding. It is necessary to study tomato resistance mechanisms against *P. infestans* and identify the key resistance genes which are used in disease-resistance breeding of tomato transgene. Therefore, in this study, we performed the RNA-Seqs in leaves of tomato inoculated with and without *P. infestans*. A resistance gene, *SpWRKY3* induced by *P. infestans* was identified and its function was analysed by transgenic approach. These results will benefit not only understanding the molecular mechanisms of tomato–*P. infestans* interaction but also future molecular breeding.

Materials and methods

Plant growth conditions and treatments

The resistant tomato to *P. infestans*, *Solanum pimpinellifolium* L3708, was chosen as the host plant and grown in a greenhouse under 16 h light within a temperature range of 22–28 °C. For the tissue-specific expression analysis, the root, stem and leaf were harvested, respectively. *P. infestans* was cultured in oat medium in the dark at 20 °C. The treated tomato plants (5–6-leaf stage) were inoculated with a suspension of *P. infestans* spores (10⁶ zoospores/ml) before being placed at

100% relative humidity in the dark to ensure spore germination at 20 ± 1 °C. The controls were treated with the same volume of sterile water and grown under the same conditions. Leaf tissues were collected at 0, 1, 2, 3, 4 and 5 day post inoculation (dpi). SA (2 mM), MeJA (0.1 mM) and ABA (0.1 mM) were applied to spray the seedlings. The leaves were collected after 0, 2, 4, 8, 12 and 24 h, respectively. All samples were quickly frozen in liquid nitrogen according to Li et al. (2015). The leaf tissues were stored at -80 °C for storage until RNA isolation.

RNA isolation and Illumina sequencing

Total RNA was extracted using Trizol kit (TaKaRa, Dalian, China). Its quantity and quality were measured by Bioanalyzer. Total RNA from the leaf induction by *P. infestans* (SpPi) at 3 dpi was sent for RNA-Seq library construction and high-throughput sequencing was provided by LC Biotech in Hangzhou, China. The libraries were sequenced on an Illumina HiSeq 2000 sequencer with 100 bp paired-end reads.

Reads mapping

Raw reads obtained from the HiSeq 2000 were preprocessed to filter out 5' adapters, 3' adapters, shorter reads and low quality reads. The clean reads were mapped to the tomato genome iTAGv2.3 (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Slycopersicum) using the spliced read aligner TopHat (Trapnell et al. 2009).

Differential expression analysis

The transcriptome of tomato *S. pimpinellifolium* without *P. infestans* induction (Sp) (Cui et al. 2017) was used to identify the differentially expressed genes (DEGs) though comparing with the transcriptome of SpPi sample. In these two transcriptome, the expression of one gene was measured with normalized counts of reads by its respective length using Cufflinks (Trapnell et al. 2012). Fragments Per Kilobase of exon model per Million mapped reads (FPKM) was applied to represent the normalized expression value. The DEGs between these two samples were identified using DEGseq (Anders and Huber 2010) with *P* value less than 0.05 and fold change more than 1. The fold change was calculated according to the equation:

$$\text{Fold change} = \log_2 \frac{\text{FPKM}_{\text{SpPi}}}{\text{FPKM}_{\text{Sp}}}$$

Gene ontology enrichment analysis

The significantly expressed GO terms were selected by GO enrichment analysis. The differences of the frequency

of assignment of GO terms in the DEG set were compared with the expressed genes in SpPi and Sp samples (*P* value < 0.05). Functional groups encompassing DEGs were identified based on GO analysis, and pathway analysis according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, with manual reannotation based on the several databases and literature search.

Sequence analysis of WRKYs and cloning of *SpWRKY3* gene

The domains of all WRKY TFs were examined for the receiver domain using InterPro (<http://www.ebi.ac.uk/interpro/scan.html>). To clone the full-length CDS of a differentially expressed WRKY gene (Solyc02g088340.2, named *SpWRKY3*), synthesis of cDNA used the total RNA from *S. pimpinellifolium* L3708 as template using Reverse Transcriptase M-MLV (TaKaRa, Dalian, China), and a pair of primers (*c-WRKY3F* and *c-WRKY3R*) for *SpWRKY3* were designed by Primer Premier 5 software based on tomato genome sequence (Solyc02g088340.2) and the information of RNA-Seq data (Supplementary Table S1). The forward primer was with *Bam*HI site and the reverse primer was with *Sac*I site. Products after gel extraction purification were ligated with T-Vector pMD 19 (Simple) (TaKaRa, Dalian, China) and then sequenced.

Sequence similarity analyses were performed using the BLAST program against rice and *Arabidopsis* genome. The promoter analysis was performed using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The CDS of *SpWRKY3* was determined using BioEdit, and then translated into the corresponding amino acid sequence.

The composition and physicochemical character, signal peptide, trans-membrane topological structure and hydrophobicity or hydrophilicity were analyzed by ProtParam at <http://web.expasy.org/protparam>, SignalP at <http://www.cbs.dtu.dk/services/SignalP>, TMHMM at <http://www.cbs.dtu.dk/services/TMHMM> and ProtScale at <http://web.expasy.org/protscale>. In addition, subcellular location and nuclear localization signals were also predicted using Euk-mPLoc Program (<http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/>) and NLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), respectively. The multiple sequence alignment was performed using ClustalX1.83 with the default settings.

Virus-induced gene silencing constructs and *Agrobacterium*-mediated virus infection

Virus-induced gene silencing (VIGS) was used to suppress the expression of *SpWRKY3* using TRV-based vectors (pTRV1 and pTRV2). The *SpWRKY3* was amplified using gene-specific primers (*v-WRKY3F* and *v-WRKY3R*,

Supplementary Table S1) and cloned into the pTRV2 vector. All TRV-VIGS constructs were transformed into *Agrobacterium tumefaciens* strain GV3101. Equal volumes (OD₆₀₀ = 2) of *A. tumefaciens* carrying pTRV1 and suspensions containing pTRV2-derived constructs or pTRV2 empty vector were mixed prior to infiltration into leaves of 1–2-week-old tomato plants. pTRV2 empty vector was used as the negative control in this study. Plants were maintained for 4 weeks, and leaflets were harvested from several plants for isolation of RNA and quantitative RT-PCR (qRT-PCR) analysis to assess the degree of silencing (Li et al. 2013).

Construction of overexpression plasmid and generation of *SpWRKY3* transgenic tomato

The overexpression plasmid was constructed based on the pBI121 vector. The CDS of *SpWRKY3* was digested with *Bam*HI and *Sac*I and then cloned into *Bam*HI and *Sac*I restriction sites of the pBI121 vector, replacing the *GUS* gene under the control of cauliflower mosaic virus (CaMV) 35S promoter. The recombinant plasmid was transformed into *A. tumefaciens* strain GV3101 by freeze–thaw method.

The cotyledons of *S. lycopersicum* Zaofen No.2 were infected with *A. tumefaciens* strain GV3101 containing the modified plasmid pBI121-*SpWRKY3* via *Agrobacterium*-mediated leaf disk method. The resistant buds were selected on 1/2MS agar medium containing 25 mg L⁻¹ kanamycin and 200 mg L⁻¹ carbenicillin disodium. After obtaining T₀ kanamycin-resistant plants, the presence of the transgene in the regenerating plantlets was further confirmed using PCR with a specific 35S forward primer and *SpWRKY3* reverse primer (Supplementary Table S1) (Li et al. 2015). The expression level of *SpWRKY3* in these selected positive transgenic lines was further examined by qRT-PCR.

Based on the methods of Al-Abdallat et al. (2015) and Huang et al. (2013), we used the DNA of the transgenic tomato as template to identify the transgene copy number by qRT-PCR. Because both the marker gene, *neomycin phosphotransferase II* (*nptII*) and the target gene are located on the same T-DNA and the copy number of both genes should be consistent in the same transgenic tomato, the *nptII* gene was chosen to identify the transgene copy number in the transgenic tomato.

Disease resistance analysis of silencing and overexpressed tomato

Detached-leaves from silencing and overexpressed tomato were inoculated with 20 µl *P. infestans* zoospore suspension (1 × 10⁶ zoospores/mL) and then placed on filter paper in a petri dish according to the method of Li et al. (2015). The whole plants were sprayed to run off with the same zoospore suspension. The inoculated leaves and

whole plants were maintained in the dark at high humidity for 1 d at 20 ± 1 °C, and then moved to the greenhouse at 20 ± 1 °C with a 16 h light and 8 h dark photoperiod cycle. After 7 days, the leaf symptoms were photographed and the diameter of lesion was measured.

The leaf area occupied by blight lesions and stem area showing blight symptoms was estimated by the scale described by Li et al. (2015). The resistance of a plant was indicated by the disease index (DI). The DI was calculated as described by Li et al. (2015).

Histochemical assays and measurements of physiological parameters

According to the method previously described by Li et al. (2015), and NBT and DAB staining were performed to measure H₂O₂ and O²⁻, respectively.

Peroxidase (POD) and superoxide dismutase (SOD) activities were measured following the protocol according to a previously described (Xia et al. 2009; Su et al. 2015). Chlorophyll was extracted using 80% (v/v) acetone and analyzed using UV spectrophotometry. The Portable Photosynthesis System CIRAS-2 was used to measure photosynthesis parameters, such as photosynthetic rate and stomatal conductance (Li et al. 2014). In addition, the malonaldehyde (MDA) content and relative electrolyte leakage (REL) were measured according to a previously described method (Cao et al. 2007; Li et al. 2015).

Expression analysis by qRT-PCR

The relative quantity of all genes in the present study was performed using qRT-PCR. Supplementary Table S1 showed the information of all primers. In addition, the tomato *actin* was used as an internal housekeeping gene. All reactions were carried out thrice with three independent biological replicates. qRT-PCRs were performed with the SYBR[®] Premix Ex Taq[™] II kit (TaKaRa) according to the manufacturer's protocol and using the Rotor Gene 3000 Real-time PCR machine (Corbett Research, Mortlake, Vic., Australia). Transcript levels of each mRNA were determined and normalized with the level of tomato *actin* using the 2^{-ΔΔCT} method.

Statistical analysis

All data are expressed as the mean ± SDs from three independent experiments. We used Duncan's multiple range test for the significance (*P* value < 0.05).

Results

High-throughput sequencing and mapping

Illumina-based next-generation sequencing was performed on tomato induced by *P. infestans* at 3 dpi. As shown in Table 1, a total of approximately 45 million raw reads were generated. After the short reads, low-quality sequence and ambiguous nucleotides were removed from raw reads, approximately 44 million (98%) clean reads were used for further analysis.

All clean reads were aligned to the tomato genome using TopHat. Of these clean reads, 32,709,617 (more than 74%) were mapped reads, and 32,201,083 (approximately 72%) were unique mapped reads (Table 1). Compared with the reference annotation, a number of unique mapped reads were mapped to exons (95.03%), introns (2.01%) and intergenic regions (2.96%).

Identification of DEGs

After FPKM for each gene was calculated, the DEGs were identified in two libraries, SpPi and Sp. Based on the criteria of fold change > 1 and *P* value < 0.05, a total of 1103 genes showed significantly differential expression including 855 up-regulated genes in SpPi sample and 248 down-regulated genes (Fig. 1a, b and Supplementary Table S2). A Venn diagram of the DEGs illustrated that 237 genes was expressed only in SpPi and 27 was no expression signal (Fig. 1c).

Table 1 Summary of sequencing results of tomato sample infected with *P. infestans*

Raw data		Clean data		Clean ratio	Mapped reads	Unique mapped reads
Reads	Base	Reads	Base			
45,0969,92	6.76G	44,198,526	6.63G	98.01%	32,709,617	32,201,083

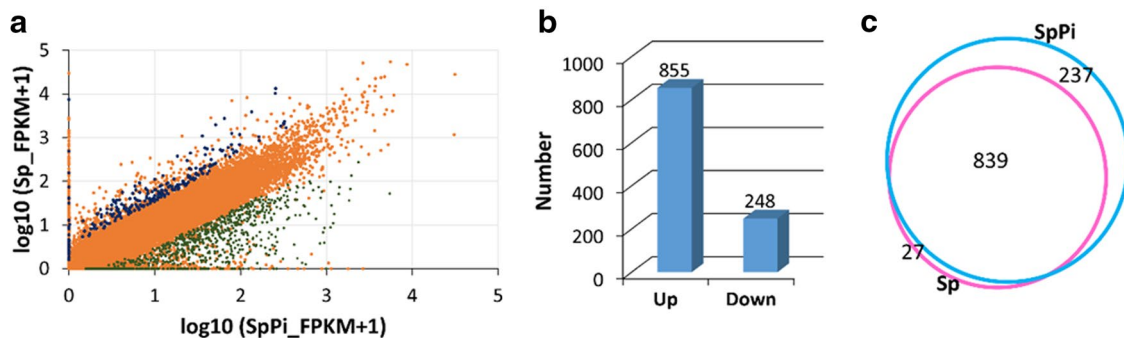


Fig. 1 Identification and characterization of DEGs between tomatoes inoculated with and without *P. infestans*. **a** Scatter diagram of gene expression between SpPi and Sp samples. The y-axis represents the logarithm of FPKM_{Sp} plus 1 and the x-axis represents the logarithm

of FPKM_{SpPi} plus 1. **b** The number of DEGs. **c** A Venn diagram showing DEGs that are commonly expressed in SpPi and Sp samples as well as those specifically expressed under one but not the other

GO enrichment and KEGG pathway analyses

Based on the likely functions, we attempted to categorize the 1103 DEGs by GO enrichment and KEGG pathway analysis. All DEGs were classified into the terms of biological process, cellular component and molecular function using Gene Ontology Consortium (<http://www.geneontology.org/>). GO enrichment analysis revealed that the 75 significant GO terms were over-represented with *P* value < 0.05 (Supplementary Table S3). Seven GO-Class-4 terms were retained as informative for further pathway analysis (Table 2), including oxidation–reduction process (GO: 0055114), response to stress (GO: 0006950), response to biotic stimulus (GO: 0009607), biosynthetic process (GO: 0009058), apoplast (GO: 0048046), oxidoreductase activity (GO: 0016491), sequence-specific DNA binding transcription factor activity (GO: 0003700).

Based on GO enrichment analysis, 225 genes from seven GO-Class-4 terms were performed the KEGG analysis. 80 KEGG pathways were annotated, including Plant–pathogen interaction (ko04626), MAPK signaling pathway–plant

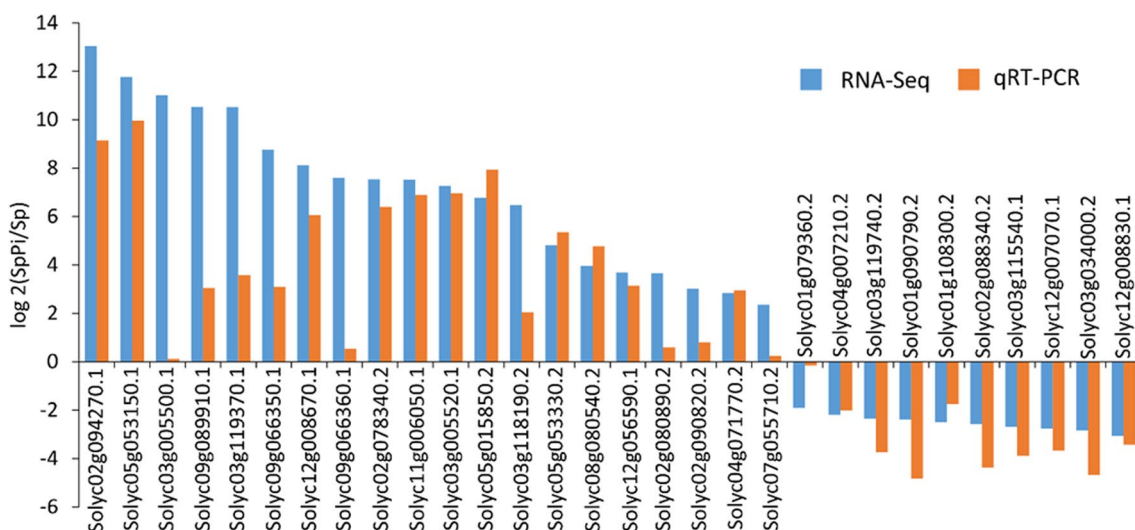


Fig. 2 Comparison of gene expression patterns obtained using RNA-Seq and qRT-PCR validation. The x-axis shows genes in three tissues validated in this study; y-axis shows the \log_2 ratio of expression in SpPi versus Sp. Tomato *actin* expression was used as a control

Table 2 GO term (Level 4) enrichment result of differentially expressed genes in tomato following *P. infestans* challenge

GO ID	GO term	GO function	Gene no.
GO:0055114	Oxidation–reduction process	BP	136
GO:0006950	Response to stress	BP	9
GO:0009607	Response to biotic stimulus	BP	7
GO:0009058	Biosynthetic process	BP	19
GO:0048046	Apoplast	CC	6
GO:0016491	Oxidoreductase activity	MF	66
GO:0003700	Sequence-specific DNA binding transcription factor activity	MF	41

BF biological process, CC cellular component, MF molecular function

(ko04016), Plant hormone signal transduction (ko04075), Phenylalanine metabolism (ko00360), Cysteine and methionine metabolism (ko00270), and alpha-Linolenic acid metabolism (ko00592) (Supplementary Table S4). These pathways are associated with interaction between plant and pathogen, biosynthesis and signal transduction of hormone related to plant resistance to pathogen.

Expression profiling of transcription factor genes in tomato induced by *P. infestans*

To understand the expression profiles of TF genes in tomato induced by *P. infestans*, the expression patterns of thirty-five selected TF genes from GO enriched term, sequence-specific DNA binding transcription factor activity were performed in response to *P. infestans* stress using qRT-PCR (Supplementary Table S5). Heat map representation for transcript

expression fold change in response to *P. infestans* is shown in Fig. 3. All the expression of TF genes was significantly induced by *P. infestans*. Under *P. infestans* induction, the trends of the expression of AP2/ERFs, WRKYs, BHLHs and HSFs were first increased and then decreased, and reached the peak at 1 dpi or 2 dpi. The expression levels of GATAs increased gradually from 0 to 1 dpi and 3–4 dpi, and decreased during 1–3 and 4–5 dpi. The maximum of expression level was at 1 and 4 dpi.

WRKY domain identification and SpWRKY3 cloning

WRKY TF has been most widely studied in plant–pathogen interaction. Eight WRKY TFs were identified in this study, of which, only Solyc02g088340.2 (*SIWRKY3*) contained two WRKY domains, and others contained only one. Based on these structure characteristics and their sequence similarity and topology, we subdivided the eight members of the WRKY gene family into three subgroups (Supplementary Table S6). *SIWRKY3* was clustered with Subgroup I.

One 1383 bp CDS fragment, named *SpWRKY3* (a homologous gene of *SIWRKY3*), was cloned from *S. pimpinellifolium* L3708, encoding polypeptides of 460 amino acids. After BLAST analysis, there was only one base difference in the CDS of between *SpWRKY3* and *SIWRKY3*. Protein domain analysis by InterPro predicted two WRKY domains followed by C-X₄₋₅-C-X₂₂₋₂₃-HX-H-type zinc-finger motifs in this sequence (Supplementary Fig. S1). BLASTp search against the tomato, rice and *Arabidopsis* genome showed that the identity of *SpWRKY3* amino acid sequence was 100% to *SIWRKY3* and closer genetic relationship with *AtWRKY3*, *AtWRKY4*, *OsWRKY4* and *OsWRKY85* in rice and *Arabidopsis*.

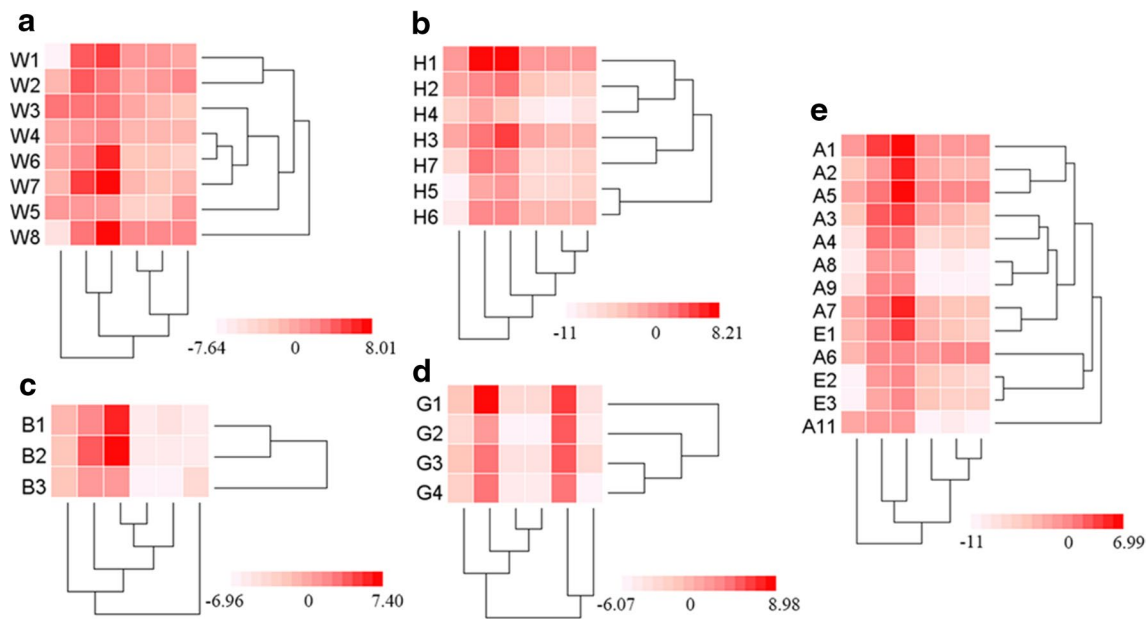


Fig. 3 The qRT-PCR expression analysis of the TF genes response to *P. infestans*. Tomato leaves were treated with *P. infestans* at 0, 1, 2, 3, 4 and 5 dpi. Relative expression levels of these genes are analyzed by qRT-PCR, and \log_2 -transformed fold-change values are used for cre-

ating the heatmap. **a** WRKY family. **b** HSF family. **c** BHLH family. **d** GATA family. **e** AP2/ERF family. Tomato *actin* expression was used as a control

The calculated molecular weight and theoretical pI of *SpWRKY3* were 50.9 kDa and 6.2, respectively. The prediction of the SignalP program showed that this protein did not contain a signal peptide (Supplementary Fig. S2). This protein was hydrophilic and outside the membrane, which indicated that it was a no trans-membrane topological protein. After analysis of subcellular location and nuclear localization signals, it was shown that *SpWRKY3* was located in nuclear and contained nuclear localization sequences (273 aa- 283 aa) (Supplementary Fig. S1). Multiple sequence alignment was performed on the WRKY proteins using ClustalX1.83. The result confirmed the highly conserved nature of WRKY domain (Supplementary Fig. S3).

The promoter analysis of *SpWRKY3* and *SIWRKY3* were also performed. The BLAST result of the *SpWRKY3* against the *S. pimpinellifolium* WGS Contigs (cshl_1.00) (<https://solgenomics.net/tools/blast/>) showed that *SpWRKY3* located in the antisense strand between 3047 bp and 5450 bp of contig: 6744965. Because the length of contig: 6744965 is 6161 bp, we only got a 711 bp-sequence of the promoter. Meanwhile, the promoter sequence of *SIWRKY3*, a 711 bp-sequence also downloaded from Sol Genomics Network (<https://solgenomics.net/>). The identity of both these promoter sequences was 97%. The promoter analysis showed that the promoter sequences of *SpWRKY3* and *SIWRKY3* contained 20 and 19 *cis*-elements, respectively (Table S7). The *cis*-element, 5' UTR Py-rich stretch and TCA element only existed in promoter sequence of *SpWRKY3*, and ACE

only existed in promoter sequence of *SIWRKY3*. 5' UTR Py-rich stretch conferred high transcription levels and TCA element was involved in salicylic acid responsiveness. These suggest that the function of WRKY3 between *S. pimpinellifolium* and *S. lycopersicum* may be different.

Tissue-specific expression and expression patterns of *SpWRKY3* after various stresses

qRT-PCR was used to determine relative tissue distribution of *SpWRKY3* gene expression in three tomato tissues including root, stem, leaf. The *SpWRKY3* gene was mainly expressed in leaf, with limited expression observed in root and stem (Fig. 4a).

To explore whether *SpWRKY3* is involved in tomato response to plant hormones, the expression patterns of *SpWRKY3* in tomato leaves were measured using qRT-PCR after treatment with JA, SA and ABA. All the trends of the expression of *SpWRKY3* were first increased and then decreased. The transcript of *SpWRKY3* reached the highest peaks at 2, 4 and 8 h after SA, JA and ABA treatment, respectively (Fig. 4d–f).

Silencing of *SpWRKY3* renders the tomato susceptible to *P. infestans*

We investigated a possible role for *SpWRKY3* in the tomato resistance to *P. infestans* using TRV-based VIGS system to

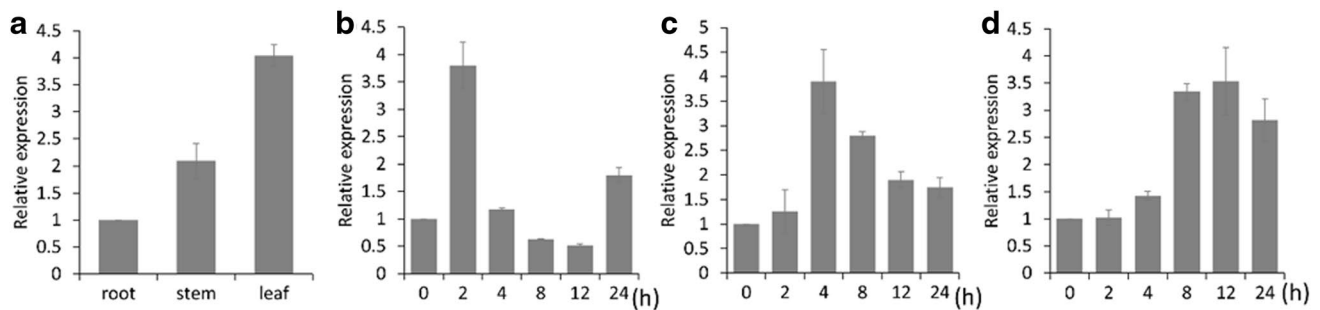


Fig. 4 The expression patterns of *SpWRKY3* in different tissues and in response to various treatments by qRT-PCR. **a** Tissue-specific expression of *SpWRKY3* in tomato root, stem and leaf. **(b–d)** Expression patterns of *SpWRKY3* in tomato leaves at the indicated time and

treated with SA (**b**), MeJA (**c**) and ABA (**d**). Data are the mean \pm SD of three independent experiments. Samples marked with various letters show a significant difference at $P < 0.05$. Tomato *actin* expression was used as a control

down-regulate the expression of *SpWRKY3* in the resistant tomato *S. pimpinellifolium* L3708. After 4-week *Agrobacterium* infiltration, qRT-PCR was performed to check the transcript levels of *SpWRKY3*. The results demonstrated that the expression level of *SpWRKY3* decreased by approximately 70% in pTRV2-*SpWRKY3* tomato compared with the control, pTRV2 tomato (Fig. 5a).

Having demonstrated that the VIGS constructs reduced the expression of *SpWRKY3* in tomato, we assessed the disease phenotypes for pTRV2 and pTRV2-*SpWRKY3* tomato plants. Detached-leaves inoculated with *P. infestans*, and seven days after the infection, the leaves from

pTRV2-*SpWRKY3* tomato exhibited more LB symptoms (Fig. 5b). In the whole-plant inoculation assay, the disease index (DI) was compared between pTRV2 and pTRV2-*SpWRKY3* tomato plants infection with *P. infestans*. 7 days after inoculation, pTRV2 tomato plants exhibited lower DI compared with the pTRV2-*SpWRKY3* tomato plants (Fig. 5c), and late blight lesions were clearly more pronounced in pTRV2-*SpWRKY3* tomato plants. These results indicate that silencing of *SpWRKY3* in tomato results in enhancement of the susceptibility to *P. infestans*.

Four key resistance genes from PR gene family were analyzed by qRT-PCR to determine whether they were

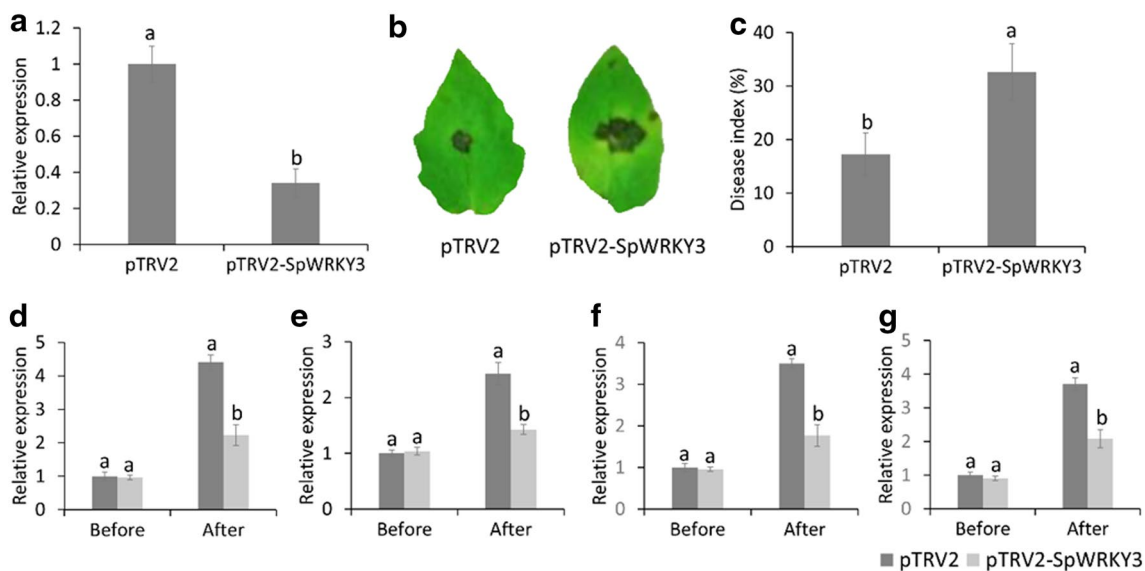


Fig. 5 Silencing of *SpWRKY3* seriously compromises *P. infestans* resistance in tomato. **a** Relative transcripts levels of *SpWRKY3* in pTRV2 and pTRV2-*SpWRKY3* tomato plants. **b** Disease signs on the detached leaves from pTRV2 and pTRV2-*SpWRKY3* tomato plants at 7 dpi. **c** Assessment of DI for pTRV2 and pTRV2-*SpWRKY3* tomato plants at 7 dpi. **d–g** Relative expression levels of *PR1* (**d**),

PR2 (**e**), *PR3* (**f**) and *PR5* (**g**) genes in the leaves from pTRV2 and pTRV2-*SpWRKY3* tomato plants before and after inoculation with *P. infestans*. Data are the mean \pm SD of three independent experiments. Samples marked with various letters show a significant difference at $P < 0.05$. Tomato *actin* expression was used as a control

affected in pTRV2-*SpWRKY3* tomato against *P. infestans*. Before *P. infestans* infection, the mRNA levels of four *PR* genes were not significantly different between pTRV2 and pTRV2-*SpWRKY3* tomatoes. After *P. infestans* infection, their expression levels were significantly increased in these tomatoes. However, the expression levels in pTRV2-*SpWRKY3* tomatoes were lower than those in pTRV2 tomatoes (Fig. 5d–g).

Effective enhancement of tomato resistance to *P. infestans* by overexpression of *SpWRKY3*

To further explicit the function of *SpWRKY3* in tomato–*P. infestans* interaction, a plasmid for the overexpression of *SpWRKY3* was constructed on the basis of pBI121 vector and the *pBI121-SpWRKY3* was introduced into tomato via *Agrobacterium*-mediated leaf disk method. After callus induction and shoot regeneration, eight representative positive transgenic lines were confirmed by selection on medium containing kanamycin. After genomic DNA-PCR detection based on specific primers (Supplementary Table S1), transgenic lines exhibited the expected transgene-specific band, but the non-transgenic lines (L4 and L7) and WT were not found (Fig. 6a). In addition, transgenic lines, L1, L5 and

L6 had single insertion event by qRT-PCR analysis (Supplementary Table S8). Compared to the WT, the transgenic lines expressed a higher level of *SpWRKY3*, with the levels of *SpWRKY3* in L1, L5 and L6 being approximately 10.84, 12.41 and 11.83 fold, respectively. So, these three transgenic lines were selected for further analysis (Fig. 6b).

To determine the function of *SpWRKY3* in tomato–*P. infestans* interaction, we examined the disease phenotype using a detached transgenic leaf inoculation assay. Compared with the WT leaves inoculation with *P. infestans*, the leaves of the transgenic plants exhibited fewer disease symptoms (Fig. 6c). The diameter of lesion also revealed increased resistance to the pathogen in the case of the transgenic plants, as shown by the presence of smaller lesion compared to WT (Fig. 6d).

The whole-plant inoculation assay also was performed and the DI was calculated at seven days after infection with *P. infestans*. As shown in Fig. 6e, the transgenic lines had lower DI than the WT plants. The disease symptom included the formation and development of brown-black. To further confirm the symptom, the content of chlorophyll was detected. Figure 6f showed that it was no significant difference between the WT and transgenic lines before inoculation. After inoculation, the accumulation of chlorophyll

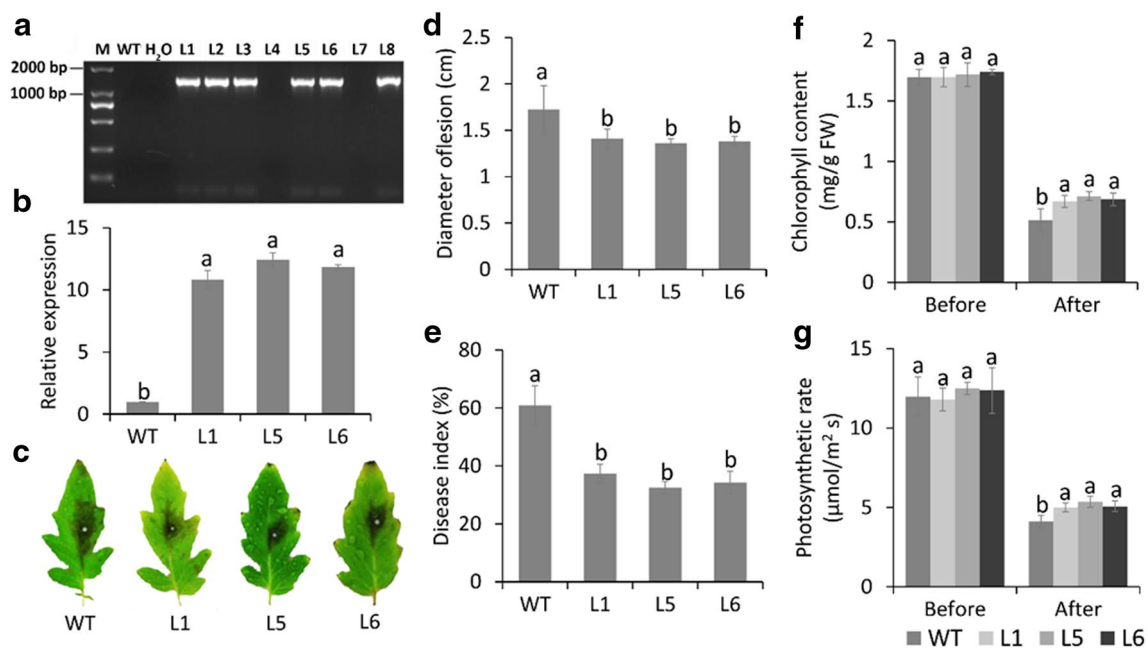


Fig. 6 Increased resistance to *P. infestans* inoculation in transgenic tomato. **a** Identification of transgenic lines by genomic DNA-PCR based on a pair of specific primers. M, DL2000 DNA Marker; WT, untransformed wild-type tomato; L1–8, independent transgenic tomato lines overexpressing *SpWRKY3*. **b** The expression levels of *SpWRKY3* in selected transgenic lines (L1, L5 and L6). **c** Phenotypes of the detached leaves from WT and transgenic lines at 7 dpi. **d** Lesion sizes on detached leaves produced by *P. infestans*. **e** Dis-

ease index of WT and transgenic lines at 7 dpi. **f** Chlorophyll content of WT and transgenic lines before and after inoculation with *P. infestans*. **g** Photosynthetic rate of WT and transgenic lines before and after inoculation with *P. infestans*. The data are presented as the mean \pm SD of three independent experiments. At least six individual plants per line are used for each experiment. The values indicated by the different letters are significantly different at $P < 0.05$, as determined using Duncan's multiple range tests

content was significantly higher in the transgenic lines than in WT plants. Meanwhile, the photosynthetic rate also was detected and it was higher in transgenic lines than in WT plants (Fig. 6g).

The expression levels of *PR* genes were investigated in WT and transgenic lines before and after *P. infestans* inoculation. In transgenic lines, the expression levels of *PR* genes are higher than WT plants. After inoculation with *P. infestans*, the expression levels of these genes also were significantly increased in *SpWRKY3*-overexpressing tomato compared to WT plants (Fig. 7).

Overexpression of *SpWRKY3* decreased membrane damage by ROS scavenging pathway in tomato inoculated with *P. infestans*

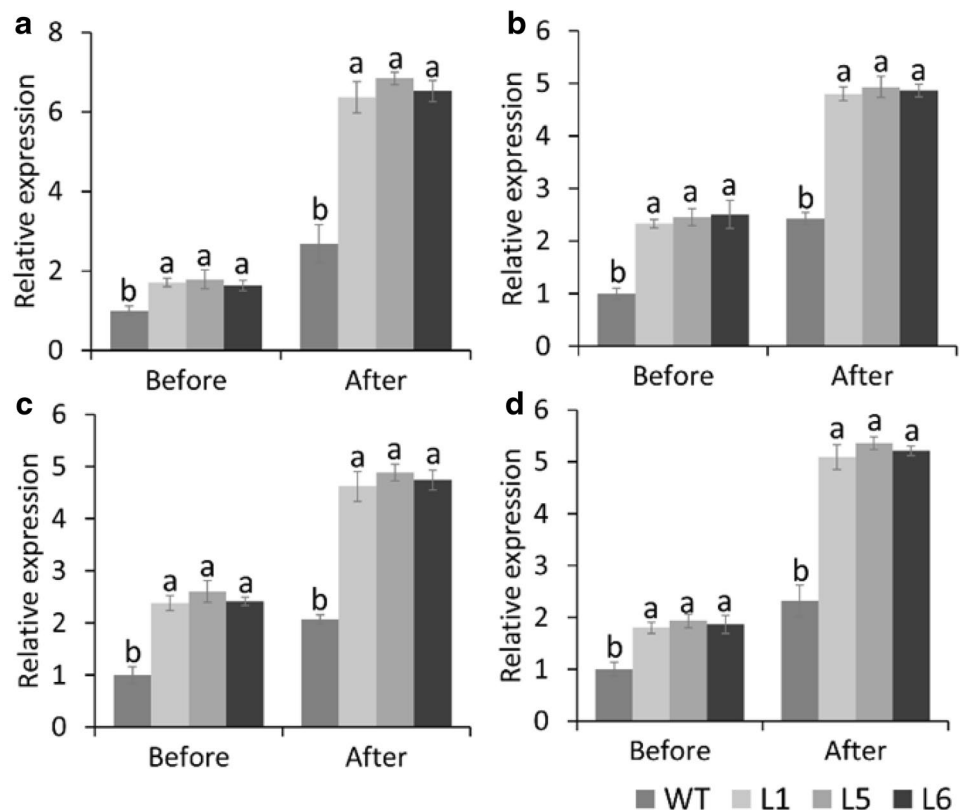
Reactive oxygen species (ROS) plays an important role in plant–pathogen interaction. Low levels of ROS can act as signaling molecules in response to pathogen infection, but late massive ROS generations that are toxic to the cell may lead to peroxidation of lipids, damage to cellular membranes, disease susceptibility and ultimately cell death. The main species of ROS, H_2O_2 and O_2^- were detected using DAB and NBT staining of WT and transgenic plants at 7 dpi, respectively. The dark-brown colored polymeric oxidation products from H_2O_2 accumulation increased in

both WT and transgenic lines leaves, whereas the leaves of WT exhibited more substantial staining (Fig. 8a). Similarly, NBT staining for O_2^- was performed in all leaves. After inoculation, the staining of WT leaves was deeper than transgenic lines (Fig. 8b). The SOD and POD are key antioxidant enzymes of ROS scavenging system. After inoculation, the activities of POD and SOD were dramatically higher in transgenic plants than those in WT plants (Fig. 8c, d). Similarly, the expression levels of *SOD* and *POD* genes were no significant change between WT and transgenic lines at first, but after inoculation, their expression levels were significantly up-regulated in *SpWRKY3*-transgenic tomato plants (Fig. 8e, f).

MDA is widely recognized as an indicator of lipid peroxidation. Membrane damage in plants can be estimated by measuring leakage of electrolytes, which are evaluated by determining REL. As shown in Fig. 8g, h, there was no significant difference between WT and transgenic lines before inoculation with *P. infestans*. However, after inoculation, the accumulations of MDA content and REL were remarkably lower in the transgenic lines than in the WT plants.

These results suggest that overexpression of *SpWRKY3* may regulate antioxidant to reduce the accumulation of ROS and alleviate cell membrane injury after late infection with *P. infestans*.

Fig. 7 The expression levels of *PR* genes in the WT and transgenic lines before and after inoculation with *P. infestans*. **a** *PR1*. **b** *PR2*. **c** *PR3*. **d** *PR5*. Data are the means \pm SD of three independent experiments. Samples marked with various letters show a significant difference at $P < 0.05$. Tomato *actin* expression was used as a control



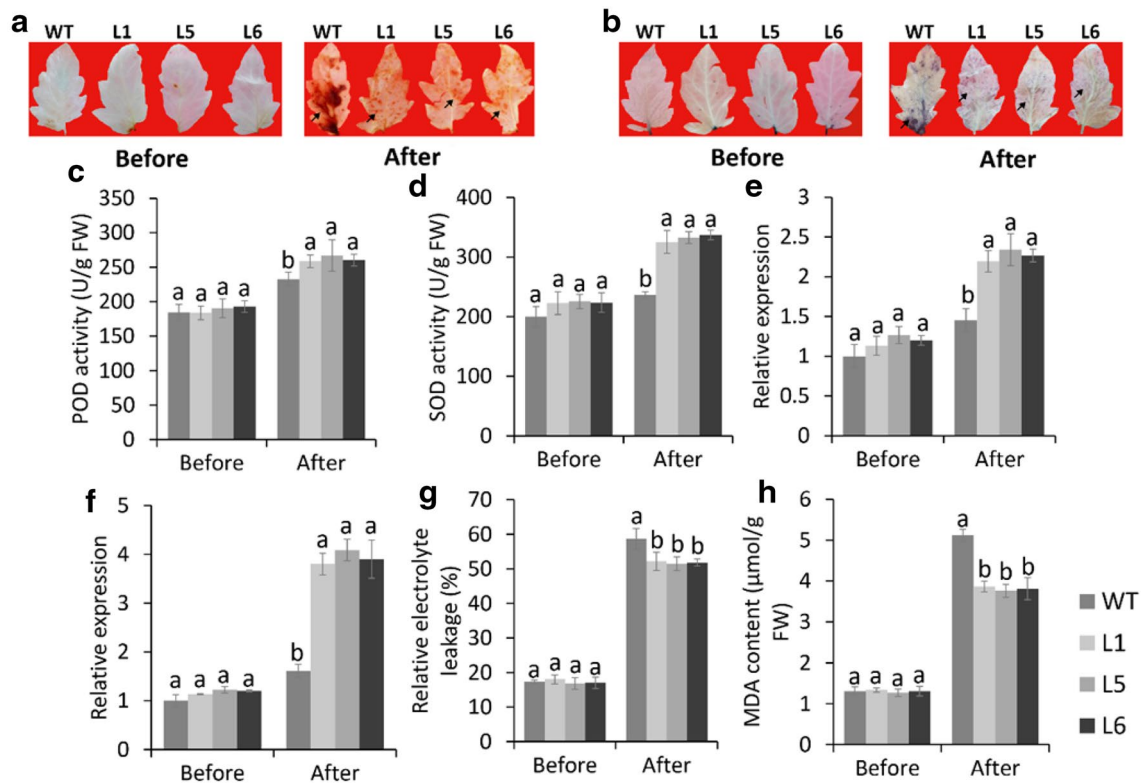


Fig. 8 Effective decrease of the level of ROS to prevent membrane damage by overexpression of *SpWRKY3*. **a** NBT staining for O_2^- . **b** DBA staining for H_2O_2 . **c** POD activity. **d** SOD activity. **e** The expression level of *POD* gene. **f** The expression level of *SOD* gene. **g** MDA content. **h** Relative electrolyte leakage. The physiological traits are analyzed before and after inoculation *P. infestans*. The data

are presented as the mean \pm SD of three independent experiments. At least six individual plants per line are used for each experiment. The values indicated by the different letters are significantly different at $P < 0.05$, as determined using Duncan's multiple range tests. The arrow shows the positions of infection with *P. infestans*

Discussion

Tomato LB caused by *P. infestans*, is very difficult to control and the cause of severe yield losses in tomato. Although many studies have investigated the interaction of *P. infestans* with tomato, the genetic basis and molecular mechanism of tomato LB resistance is still poorly understood and there were lack of resistant varieties. To achieve the purposes, the comparative transcriptome analysis via high throughput sequencing technologies was used to identify 1103 DEGs in tomato induced by *P. infestans*. GO enrichment analysis revealed these DEGs were assigned at 75 significant GO terms. Seven GO terms (level 4) were associated with plant resistance to pathogen (Table 2). Of these seven GO terms, most of differentially expressed TFs were assigned with GO term, sequence-specific DNA binding transcription factor activity. Many TFs have been suggested to play an important role for transcriptional reprogramming associated to plant stress response.

In this study, eight WRKY TF was involved in the enriched GO term, sequence-specific DNA binding

transcription factor activity. Of these eight WRKY TFs, it is interesting that only *SIWRKY3* contained two WRKY domains was clustered with Subgroup I (Huang et al. 2012) and RNA-Seq results showed the expression level of *SpWRKY3* (a homologue of *SIWRKY3*) was decreased in SpPi. *SpWRKY3* was cloned and isolated from *S. pimpinellifolium* L3708, which was also induced by *P. infestans*. The BLASTp results showed that *SpWRKY3* was closer genetic relationship with *AtWRKY3*, *AtWRKY4*, *OsWRKY4* and *OsWRKY85* in rice and *Arabidopsis*, which suggests *SpWRKY3* may have similar functions with them in plant–pathogen interaction. In rice, *OsWRKY85* was significantly increased by pathogen treatment (Ryu et al. 2006). *OsWRKY4* acted as a transcriptional activator mediating defense responses toward *Rhizoctonia solani* (Wang et al. 2015; Peng et al. 2016). Similar to the function of rice WRKYs, the functional analysis based on silencing and overexpression lines indicated that *AtWRKY3* and *AtWRKY4* had a positive role in plant resistance to necrotrophic pathogens and *AtWRKY4* had a negative effect on plant resistance to biotrophic pathogens (Lai et al. 2008).

Meanwhile, *SpWRKY3* was also a positive regulator in tomato resistance to *P. infestans*. The transgenic tomato plants that overexpressed *SpWRKY3* displayed less serious disease symptoms than WT plants after infection with *P. infestans*, as shown by decreased number of necrotic cells, lesion sizes and DI, while the resistance was impaired after *SpWRKY3* silencing (Figs. 5 and 6).

WRKY TFs regulate the expression of a set of defense-related genes in different defense signaling pathways, thus enhancing the resistance of plant to pathogen (Pandey and Somssich 2009; Amorim et al. 2017). After induction by pathogens, most of WRKYs can activate defense genes pathogenesis related (PR) genes. In this study, overexpression of *SpWRKY3* in transgenic tomato plants induced the expression of PR genes (such as *PR1*, *PR2*, *PR3* and *PR5*) (Fig. 7), and *SpWRKY3* silencing led to down-regulating the transcripts of these PR genes (Fig. 5d–g). The members of PR gene family included β -1,3-glucanase, thaumatin-like, chitinase, protease inhibitor, defensin, thionin and others. Tomato PR1, PR2 (β -1,3-glucanase) and PR5 (thaumatin-like) are SA-dependent genes, whereas PR3 (chitinase) are marker genes for JA signaling (Seo et al. 2008; Segarra et al. 2013). By promoting the activation of JA- and SA-dependent genes, WRKYs are involved in SA and JA signaling pathways and play a variety of roles. Rice *OsWRKY6* positively regulated the expression of the *OsPR10a*, a SA-dependent gene by binding directly the *OsPR10a* promoter (Choi et al. 2015). In *Arabidopsis*, *AtWRKY70* positively regulated the expression of the *PR2* and *PR5* and negatively regulated the expression of the JA-dependent genes (Li et al. 2004). Contrastingly, *AtWRKY75* effectively acted as a positive regulation of JA-dependent gene (*Defensin 1.2*) and suppressed the expression of SA-dependent gene (*PR1*) (Chen et al. 2013). The enhanced resistance in the transgenic lines overexpressing *OsWRKY30* was associated with activated expression of JA synthesis-related genes *PR3* (Peng et al. 2012). Interestingly, *Arabidopsis AtWRKY28* was involved in positive regulation of both JA and SA-responsive pathways (Chen et al. 2013), while overexpression of poplar *PtrWRKY89* in *Arabidopsis* showed negative regulations of both SA- and JA-related signaling pathways by reducing the expression levels of marker genes of the pathways (Jiang et al. 2016). In this study, both the SA-dependent genes and JA-dependent gene were induced and suppressed in the transgenic lines overexpressing *SpWRKY3* (Fig. 7) and silencing *SpWRKY3* tomato (Fig. 5d–g), respectively. Meanwhile, *SpWRKY3* transcripts were also up-regulated by SA and JA (Fig. 4d, e). Thus, *SpWRKY3* may act as an integrator between JA- and SA-dependent pathways by inducing the expression of PR genes.

ROS network plays essential roles in signal transduction of resistance to pathogen (Mittler et al. 2004; Wi et al. 2012). Following pathogen infection, ROS are rapidly

produced to inhibit pathogen growth by preventing pathogen from entering the cell or inducing resistant genes (Bradley et al. 1992; Kotchoni and Gachomo 2006; Wi et al. 2012; Vie et al. 2017). However, later generation of massive amounts of ROS is toxic to the cell and may lead to cell membrane damage, enhance susceptibility and cause apoptosis (Cui et al. 2017). The ROS scavenging system of plant regulates the steady-state level of ROS to prevent oxidative damage. In this system, POD and SOD play important roles to scavenge ROS (Kotchoni and Gachomo 2006).

WRKYs played important roles in ROS bursts as well as in ROS scavenging in plant–pathogen interaction. Previous work showed that WRKYs phosphorylated by MAPK was required for plant immune ROS bursts by activation of NADPH oxidase in *N. benthamiana* (Adachi et al. 2015). In ROS scavenging system, overexpression-*GhWRKY15* tobacco leaves infected with TMV (10 days post-inoculation), CMV (14 days post-inoculation) and *Colletotrichum gossypii* (7 days post-inoculation), accumulated lower levels of H_2O_2 relative to the WT plant, and the activities of POD was significantly increased in the transgenic tobacco (Yu et al. 2012). Transgenic tomato plants that overexpressed *SpWRKY3* displayed lower ROS than WT plants after infection with *P. infestans* at 7 dpi (Fig. 8a, b). Meanwhile, the activities of SOD and POD were significantly increased in the transgenic tomato plants (Fig. 8c–f). In addition, the number of necrotic cells of transgenic tomato plants was less, as shown by decreased MDA content and REL (Fig. 8g–h). Thus, *SpWRKY3* may activate ROS scavenging system to reduce the level of ROS to prevent the damage of tomato cell membrane in tomato–*P. infestans* interaction.

In conclusion, we used RNA-Seq to investigate genome-wide gene expression difference in the leaves of tomatoes inoculated with and without *P. infestans*. A number of differentially expressed TFs were identified and qRT-PCR showed that all these TFs were induced by *P. infestans*. Of these TFs, *SpWRKY3* was a positive regulatory factor in tomato resistance to *P. infestans*. Overexpression of *SpWRKY3* in tomato modulated *P. infestans* defense response, while the resistance was impaired after *SpWRKY3* silencing. We also proposed that *SpWRKY3* was involved in positive regulation of both JA and SA-responsive pathways by inducing the expression of PR genes. *SpWRKY3* played important roles in the ROS scavenging pathway, where it reduced ROS accumulation and alleviated cell membrane injury after *P. infestans* infection. Thus, this mechanism would allow a response to *P. infestans* stress through upregulation of *SpWRKY3*, which upregulated PRs and reduced ROS accumulation to protect against cell membrane injury, thus promoting tomato resistance to *P. infestans*. And this also provides the genetic basis and molecular mechanism of tomato LB resistance for molecular breeding.

Author contribution statement YL and JM conceived and designed the experiments. JC and YL wrote the paper. JC analyzed the RNA-Seq data. JL, PX and NJ performed the experiments.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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