A Tomato Nucleotide Binding Sites–Leucine-Rich Repeat Gene Is Positively Involved in Plant Resistance to *Phytophthora infestans*

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ABSTRACT

The nucleotide binding sites–leucine-rich repeat (NBS-LRR) genes are key regulatory components of plant to pathogens. *Phytophthora infestans*-inducible coding sequence encoding an NBS-LRR (*SpNBS-LRR*) protein in tomato (*Solanum pimpinellifolium* L3708) was cloned and characterized based on our RNA-Seq data and tomato genome. After sequence analysis, *SpNBS-LRR* was identified as a hydrophilic protein with no transmembrane topological structure and no signal peptide. *SpNBS-LRR* had a close genetic relationship to RPS2 of *Arabidopsis thaliana* by phylogenetic analysis. In addition, *SpNBS-LRR* gene was mainly expressed in root, with low expression observed in leaf and stem. To further investigate

the role of *SpNBS-LRR* in tomato–*P. infestans* interaction, *SpNBS-LRR* was introduced in susceptible tomatoes and three transgenic lines with higher expression level of *SpNBS-LRR* were selected. These transgenic tomato plants that overexpressed *SpNBS-LRR* displayed greater resistance than wild-type tomato plants after infection with *P. infestans*, as shown by decreased disease index, lesion diameters, number of necrotic cells, *P. infestans* abundance, and higher expression levels of the defense-related genes. This information provides insight into *SpNBS-LRR* involved in the resistance of tomato to *P. infestans* infection and candidate for breeding to enhance biotic stress-resistance in tomato.

Late blight, caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases of potato and tomato (Whisson et al. 2016). In the right environmental conditions, this disease can cause great losses in potato and tomato production (Axel et al. 2012; Fry et al. 2015; Sliwka 2004). Enormous economic damage amounting to an estimated \$5 billion worldwide annually is caused by *P. infestans* (Axel et al. 2012; Haverkort et al. 2009; Judelson and Blanco 2005). In tomato production, epidemics of late blight in the United States caused \$46 million and \$66 million economic losses of fresh market and processing tomatoes, respectively, in 2009 (Nowicki et al. 2012). Meanwhile, the total losses in processing tomato reached approximately 638,900 tons in China's main growing area, Bayannur city, the central and western part of Inner Mongolia in 2007 (Zhang et al. 2013). Because of the constant change of the global population of *P. infestans* and emergence of aggressive new strains, it is difficult to prevent and control late blight (Fry et al. 2015; Kamoun et al. 2015). There are still no effective methods thus far to control its damage due to the lack of understanding about the interaction mechanism between tomato and *P. infestans*. It is very urgent and significant that we study tomato resistance mechanisms against *P. infestans* and consider breeding *P. infestans*-resistant tomato.

Plants evolve various mechanisms to protect themselves against the challenge of pathogens (Bhattarai et al. 2016; Chen et al. 2016; Dangl and Jones 2001). One of the efficient defense mechanisms is described by the gene-for-gene interaction between a host disease resistance (R) gene and a pathogen avirulence (avr) gene (Chen et al. 2016; Flor 1956; Song et al. 2015). The *NBS-LRR* resistance genes

form the largest R-gene family among plant genomes, which encode proteins containing nucleotide binding sites (NBS) and leucine-rich repeat (LRR) domains (Ellis and Jones 1998; Zhao et al. 2016). It is believed that the NBS domain is involved in regulation of the signal conduction through conformational change (Inoue et al. 2013; Takken et al. 2006), whereas the function of the LRR domain is recognizing pathogens based on protein–protein interactions (Guo et al. 2016; Kobe and Kajava 2001). In *Arabidopsis*, the effectors avrB, avrRpt2, and avrPphB into plant leaves induces a defense response, which exclusively possess the corresponding NBS-LRR proteins RPM1, RPS2, and RPS5 (Gopalan et al. 1996; Leister et al. 1996; Warren et al. 1998).

Previous studies showed that some R genes encoding NBS-LRR proteins had characterized *NBS-LRR* genes governing rice blast resistance (*Pi64*) (Ma et al. 2015), cotton Verticillium wilt resistance (*GbRvD*) (Yang et al. 2016), tobacco resistance to *P. parasitica* (*VaRGAI*) (Li et al. 2017), wheat powdery mildew resistance (*Pm3b* and *Pm8*) (Hurni et al. 2013; Yahiaoui et al. 2004), and others. In the family *Solanaceae*, a large number of NBS-LRR-encoding genes were identified from their genomes (Qian et al. 2017; Wei et al. 2016). After the infection of pathogens such as tomato leaf curl New Delhi virus, *Xanthomonas perforans*, and *Verticillium dahliae*, *NBS-LRR* genes in tomato and potato expressed increased expression (Du et al. 2015; Kushwaha et al. 2015; Yang et al. 2015). Likewise, *NBS-LRR* genes could play crucial roles in the resistance of tomato and potato to *P. infestans*, potato virus X, and other pathogens (Brommonschenkel et al. 2000; Cai et al. 2013; Sacco et al. 2007; van der Vossen et al. 2003).

In our previous work, we identified a lot of *NBS-LRR* genes in tomato *Solanum pimpinellifolium* L3708 and *S. lycopersicum* Zaofen No.2 inoculated with *P. infestans* by analysis of the RNA-Seq data. The expression of these genes was increased after *P. infestans* infection. Here, we report that a *P. infestans*-inducible coding sequence (CDS) encoding an NBS-LRR (*SpNBS-LRR*) protein in tomato was cloned and characterized based on our RNA-Seq data and tomato genome. It has been submitted to the National Center for Biotechnology Information (NCBI) as GenBank number LC057399. To more thoroughly understand the function of *SpNBS-LRR*, transgenic

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tomato plants that overexpressed *SpNBS-LRR* were generated and assayed the resistance to *P. infestans*. The wild-type (WT) tomato plants displayed more serious disease symptoms than transgenic tomato plants overexpressed *SpNBS-LRR* after infection with *P. infestans*, as shown by higher disease index, increased number of necrotic cells, and lesion diameters. This study provides key clues in understanding the roles of *SpNBS-LRR* in tomato defense response to *P. infestans*.

MATERIALS AND METHODS

Plant and pathogen. *S. pimpinellifolium* L3708, a wild tomato species reported as possessing high resistance to a wide range of *P. infestans* isolates, was used as the material for clone and expression analysis of *SpNBS-LRR*. *S. lycopersicum* Zaofen No.2, which is susceptible to pathogens, was used as the material for transient expression and tomato transformation. The seeds were germinated at 28 to 30°C. The plants were grown in the greenhouse at 25 ± 3°C under 16 h light. Leaf tissue samples for gene clone were collected from 6-week-old L3708 tomato plants. *P. infestans* strain P12103 used in the experiments was provided by Professor Shan from Northwest A&F University of China. It was maintained on oat medium at 20°C in dark (Luan et al. 2016).

Cloning of *SpNBS-LRR*. Total RNA was extracted from the leaf tissue samples with RNAiso Plus (TaKaRa, Dalian, China) following the manufacturer's instructions. The cDNA for molecular cloning was synthesized with reverse transcription M-MLV (TaKaRa).

A pair of primers for *SpNBS-LRR* was designed by Primer Premier 5 Software based on tomato genome sequence (*Solyc02g036270*) and the information of RNA-Seq in our previous studies. The forward primer was with *Bam*HI site and the reverse primer was with *Sac*I site. The PCR system was 20 µl volumes, which contained 1 µl of cDNA, 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 2 µl of dNTP mixture (2.5 mM each), 2 µl of 10× *LA* PCR buffer II (Mg²⁺ Plus), 0.2 µl of *TaKaRa LA* *Taq*, and 12.8 µl of ddH₂O. The PCR amplification program used consisted of an initial denatured step at 94°C for 5 min, 32 cycles at 95°C for 30 s, 66°C for 30 s, and 72°C for 3 min, and a final elongation step at 72°C for 10 min performed with *TaKaRa* PCR Thermal Cycler Dice Gradient (TP600). Products after gel extraction purification were ligated with T-Vector pMD 19 (Simple) (TaKaRa) and then sequenced.

Sequence analysis. Sequence similarity analyses were performed using the BLAST program at NCBI (<https://www.ncbi.nlm.nih.gov/blast>). The CDS of *SpNBS-LRR* was determined using BioEdit, and then translated into the corresponding amino acid sequence. The composition and physicochemical character, signal peptide, transmembrane topological structure, and hydrophobicity or hydrophilicity were analyzed by ProtParam at <https://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 <https://web.expasy.org/protscale>. The multiple sequence alignment was performed using ClustalX 2 with the default settings. The domains were predicted by InterPro (<http://www.ebi.ac.uk/interpro/scan.html>). The logos of conserved domains were made by online software WebLogo (<http://weblogo.berkeley.edu/logo.cgi>). Phylogenetic trees were constructed using the neighbor-joining (NJ) and maximum likelihood (ML) method with Poisson correction by MEGA6.06.

Construction of overexpression plasmid and transient expression. The overexpression plasmid was constructed based on the pBI121 vector. The CDS of *SpNBS-LRR* was digested with *Bam*HI and *Sac*I and then cloned into *Bam*HI-*Sac*I cut pBI121 vector, replacing the GUS gene under the control of cauliflower mosaic virus 35S promoter. The recombinant plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw method (Höfgen and Willmitzer 1988).

A sample *A. tumefaciens* from culture was centrifuged at 4,000 rpm/min and the sediment was resuspended by MMA (MES at

10 mM, AS at 20 µM, and MgCl₂ at 10 mM). Then, *S. lycopersicum* Zaofen No.2 epidermal cells were infected by *A. tumefaciens*. *A. tumefaciens* with empty vector was used as a control. After 0, 1, 2, 3, 4, and 5 days, the infection areas of leaves were collected for RNA extraction. Then the infiltrated leaf regions were inoculated with *P. infestans* (~10⁶ zoospores/ml) and the number of disease spots was counted at 1, 2, 3, 4, and 5 days postinoculation (dpi).

Generation of *SpNBS-LRR* transgenic tomato lines and *P. infestans* resistance analysis. The cotyledon of tomato *S. lycopersicum* Zaofen No.2 were infected with *A. tumefaciens* strain GV3101 containing the modified plasmid pBI121-*SpNBS-LRR* via *Agrobacterium*-mediated leaf disk method. The resistant buds were selected on 1/2MS agar medium containing kanamycin at 25 mg liter⁻¹ and carbenicillin disodium at 200 mg liter⁻¹. After obtaining kanamycin-resistant plants, the presence of the transgene in the regenerating plantlets was further confirmed using PCR with neomycin phosphoryltransferase II (*nptII*) gene specific primers (Li et al. 2015). The expression level of *SpNBS-LRR* in these selected positive transgenic lines was further examined by semiquantitative RT-PCR and quantitative RT-PCR (qRT-PCR), respectively.

Detached-leaf inoculation was performed according to the method of Li et al. (2015) with some modifications. The detached leaves from WT and transgenic tomato plants were inoculated with 20 µl of *P. infestans* zoospore suspension (~10⁶ zoospores/ml) and then placed on filter paper in a petri dish. 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 day 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 5 days, the leaf symptoms were photographed and the diameter of lesion was measured. The diameter was measured using crossing method. The maximum and minimum length of the lesion were examined, respectively, and then, their average number was calculated as lesion diameter. Each experiment was carried out three times.

The leaf area occupied by blight lesions and stem area showing blight symptoms was estimated by the scale described by Li et al. (2015). Disease grades were categorized from 0 to 6 based on the lesion area: 0, no symptoms; 1, lesion area smaller than 5% of the total leaf area, no symptoms on the stem; 2, 5 to 15% of leaves area infected, no symptoms on the stem; 3, 15 to 30% of leaves area infected, no symptoms on the stem; 4, 30 to 60% of leaves area infected and 10 to 30% of the stem are blighted; 5, 60 to 90% of leaves area infected and 30 to 50% of the stem are blighted; and 6, larger than 95% leaves area infected and stem fully blighted or plant death. The resistance of a plant was indicated by the disease index (DI):

$$DI = \frac{\sum (DG_i \times n_i) \times 100}{n \times DG_{max}}$$

where DG_i is the value of disease grades, n_i is the number of plants with each disease grade, and n is the total number of plants. Upon image acquisition, first the leaves were cut from tomato plants, and then the leaves were spread out on the white board smoothly and the images were captured using a camera. Last, the images were imported into ImageJ software to calculate the lesions, according to the introduction from the website (<https://imagej.nih.gov/ij/>). Each experiment was carried out three times.

In addition, the leaves were collected for the malonaldehyde (MDA) content detection and trypan blue staining assays, which were also performed as described by Li et al. (2015).

qRT-PCR analysis. The sequences of tomato *PR1*, *PR2*, and *PR5* were obtained from GenBank (Molinari et al. 2014). The GenBank accession numbers are Y08804, NM001247229, and NM001247422, respectively. The homolog sequences of *Arabidopsis* *PDF1.2*, *Solyc04g072470* (*PDF-like 1*), and *Solyc07g009260* (*PDF-like 2*) were downloaded from a tomato genome database

A BLAST search against the tomato genome showed that the putative *Sp*NBS-LRR sequence was most similar to the tomato NBS-LRR family. The identity of the amino acid sequence was 99% similar to *Solyc02g036270.2*, a member of the NBS-LRR family. The domain analysis result showed this protein contains an NB-ARC domain, a coiled coil region, and a LRR region (Fig. 1A). The NB-ARC domain, located at 154 to 419 aa contained P-loop NTPases and AAA+ ATPase subdomain, and a coiled coil region and an LLR region were located at 427 to 447 aa and 460 to 757 aa, respectively (Fig. 1A).

The cloning, characterization, and expression analysis of *SpNBS-LRR*. Through PCR and sequencing, one 2,316-bp CDS fragment (*SpNBS-LRR*) was cloned from *S. pimpinellifolium* L3708 and submitted to NCBI as GenBank accession number LC057399. It could encode a protein composed of 771 amino acids. The calculated molecular weight and theoretical pI of *SpNBS-LRR* are 87.4 and 5.74 kDa, respectively. The signal peptide prediction result shows that *SpNBS-LRR* does not contain a signal peptide (Supplementary Fig. S1). This protein is hydrophilic because the grand average of hydropathicity (GRAVY) is -0.178 (Supplementary Fig. S2). The transmembrane topological structures of *SpNBS-LRR* were predicted by TMHMM, and the result shows the peptide chains of *SpNBS-LRR* outside the membrane, which indicates that it is not a transmembrane topological protein (Supplementary Fig. S3).

The *Sp*NBS-LRR sequence was aligned with ath_RPS2 and ath_RPP13 from *Arabidopsis thaliana*, ptc_RGH and ptc_RGH2 from *Populus trichocarpa*, sly_Tm-2 from *S. lycopersicum*, osa_NBS-LRR2 and osa_NBS-LRR1 from *Oryza sativa*, and cch_BS2 from *Capsicum chacoense* (Supplementary Fig. S4). The alignment revealed the motifs of NBS-LRRs were conserved, such as P-loop, kinase-2, and GLPL motifs (Supplementary Fig. S5). In addition, *Sp*NBS-LRR belonged to CC-NBS-LRR subclass because of the existence of coiled coil region. In order to further determine the identity of the *Sp*NBS-LRR, phylogenetic analysis was performed based on amino acid sequences using the NJ and ML methods, respectively. As shown in Figure 1B and Supplementary Figure S6, the *Sp*NBS-LRR had a close genetic relationship to *A. thaliana* RPS2, supported by high bootstrap values. Thus, we speculated the *Sp*NBS-LRR may have similar functions with RPS2 in plant-pathogen interaction pathway.

Fig. 1. Sequence, phylogenetic, and expression analysis of *SpNBS-LRR*. **A**, Nucleotide sequence and deduced amino acid sequence of *SpNBS-LRR* CDS. The deduced amino acid sequence is shown under the nucleotide sequence. The NB-ARC domain is indicated in top box I, the CC domain is indicated in middle box II, and the LRR domain is indicated in bottom box III. P-loop NTPases subdomain is shaded underlined and AAA+ ATPase subdomain is shaded by medium gray. **B**, Phylogenetic analysis of NBS-LRRs, including *SpNBS-LRR*, and another 13 different sequences by the maximum likelihood methods (bootstrapping = 1,000) using MEGA 6.06 software. The other NBS-LRR sequences aligned were *ath_RPS2* (GenBank AAK38117.1), *ath_RPP13* (GenBank AEE78169.1), and *ath_ADR1* (GenBank AEE31605.1) from *Arabidopsis thaliana*, *osa_NBS-LRR1* (GenBank CCD28565.1), *osa_NBS-LRR3* (GenBank CCD28563.1), *osa_NBS-LRR6* (GenBank CCF78562.1) and *osa_NBS-LRR7* (GenBank CCD33203.1) from *Oryza sativa*, *sly_Tm-2* (GenBank AAV87531.1) and *sly_hero* resistance protein (GenBank CAD29729.1) from *Solanum lycopersicum*, *ptc_RGH* (GenBank EEE93227.2) and *ptc_RGH2* (GenBank EEE93225.2) from *Populus trichocarpa*, *can_nematode* resistance protein (GenBank ACI43068.1) from *Capsicum annuum*, and *cch_BS2* (GenBank AAF09256.1) from *Capsicum chacoense*. **C**, qRT-PCR analysis of expression patterns of *SpNBS-LRR* in different tissues, including root, stem, and leaf. Data are the means $\pm$ SEs of three independent experiments. Letters indicate significant differences among samples ($P < 0.05$). Tomato actin expression was used as a control.

The qRT-PCR was used to determine relative expression level of *SpNBS-LRR* gene in three tomato tissues including root, leaf, and stem. The *SpNBS-LRR* gene was mainly expressed in root, with low expression observed in leaf and stem (Fig. 1C).

Construction of pBI-*SpNBS-LRR* plasmid and its over-expression by agrobacterium infiltration. To further investigate the role of *SpNBS-LRR*, a plasmid for the overexpression of *SpNBS-LRR* was constructed on the basis of the pBI121 vector. The CDS of the *SpNBS-LRR* was cloned into *Bam*HI-*Sac*I cut pBI121, placing it under the control of the cauliflower mosaic virus 35S promoter (Fig. 2A). Verified by restriction enzyme identification, PCR, and sequence analysis, the plasmid of pBI-*SpNBS-LRR* was successfully constructed. The pBI121-*SpNBS-LRR* plasmid was mobilized into *A. tumefaciens* strain GV3101 by the freeze-thaw method (Höfgen and Willmitzer 1988). As shown in Supplementary Figure S7B, the result of PCR proved that pBI121-*SpNBS-LRR* was successfully mobilized into GV3101. *A. tumefaciens* with and without *SpNBS-LRR* were introduced into leaves of *S. lycopersicum* Zaofen No.2 by agrobacterium infiltration. The qRT-PCR analysis showed that the expression level of *SpNBS-LRR* was significantly up-regulated, reaching the highest point at 3 days after agrobacterium infiltration (Fig. 2B).

Three days after the transient overexpression of *SpNBS-LRR*, the leaves were infected with *P. infestans*. Resistance analysis showed that the extent of the disease was more severe and the lesions were significantly larger in control group than in the transient-overexpression-*SpNBS-LRR* group. As showed in Figure 2C and D, differences in the disease spots between control group and transient-overexpression-*SpNBS-LRR* group were not significant ($P < 0.05$) at 0 and 1 dpi. From 2 dpi, the average number of disease spots on the leaves overexpressing *SpNBS-LRR* was significantly less than that on the control group. These results suggested that transient overexpression of *SpNBS-LRR* may have enhanced the resistance of tomato against *P. infestans*.

***SpNBS-LRR* enhances the resistance of tomato to *P. infestans*.** To investigate whether *SpNBS-LRR* is involved in tomato-*P. infestans* interaction, pBI121-*SpNBS-LRR* was introduced into tomato. After callus induction and shoot regeneration, 10 lines of transgenic tomato plants were confirmed by their abilities to form roots in medium containing kanamycin and PCR identification with neomycin phosphoryltransferase II (*npII*) gene specific primers (Fig. 3A). The expression level of *SpNBS-LRR* in these selected positive transgenic

lines revealed that the transcript was the most in OE3, OE6, and OE8 (Fig. 3B). Meanwhile, the results of qRT-PCR analysis showed the expression level of *SpNBS-LRR* was approximately 10-fold, 12-fold, and 9-fold increased in OE3, OE6, OE8 compared with that in WT, respectively (Fig. 3C).

We first examined the disease phenotype using detached leaf inoculation assay. After inoculation with *P. infestans*, the disease symptoms were more severe on the leaves of WT compared with the disease symptoms on the leaves of *SpNBS-LRR* overexpressing lines. Trypan blue staining showed that the number of dead cells on the leaves of OE3, OE6, and OE8 was significantly less than the number on the WT, and the lesion diameter was also significantly smaller in transgenic lines (Fig. 3D and E).

In the whole-plant inoculation assay, the leaf area occupied by blight lesions was clearly more pronounced in WT at 5 dpi (Fig. 4A). The disease index (DI) also was scored at 5 dpi. A lower DI in transgenic lines was observed compared with that in WT plants (Fig. 4B). The transcript levels of *P. infestans Actin* gene were used to indicate *P. infestans* growth in plant by qRT-PCR (Jiang et al. 2016) and the abundance of *P. infestans* was significantly increased in WT compared with the transgenic lines (Fig. 4C). After trypan blue staining, darker blue marks in inoculated leaves indicated the cell death was more prominent in WT than in transgenic lines (Fig. 4D). In addition, MDA is widely recognized as an indicator of lipid peroxidation about membrane damage (Li et al. 2015). After inoculation, the accumulations of MDA content were significantly lower in the transgenic lines than in WT, suggesting transgenic plants did not suffer from severe membrane damage (Fig. 4E). These results suggested to some extent that overexpression of *SpNBS-LRR* in tomato resulted in an enhancement of its resistance to *P. infestans*.

The transcript levels of pathogenesis-related genes were also investigated by qRT-PCR in WT and transgenic lines. The genes *PR1*, *PR2*, and *PR5* were considered as markers for salicylic acid (SA)-dependent systemic acquired resistance (SAR) and plant defensin (*PDF*) and are involved in response to jasmonic acid (JA). Compared with WT, the expression levels of all these genes were significantly increased in transgenic lines (Fig. 5).

DISCUSSION

Many plant-pathogen interactions are determined by the presence of resistance genes that enable plants to recognize pathogens

Fig. 2. Construction of 35S:*SpNBS-LRR* plasmid and transient overexpression of *SpNBS-LRR* in tomato leaves. **A**, Schematic representation of pBI121-*SpNBS-LRR* plasmid showing the sites for restriction enzymes. **B**, qRT-PCR validation of relative expression levels of *SpNBS-LRR* in control and transient-overexpression-*SpNBS-LRR* groups at 0, 1, 2, 3, 4, and 5 days after agrobacterium infiltration. Error bars indicate standard error (SE; $n = 3$) and letters indicate significant difference among samples ($P < 0.05$). Tomato actin expression was used as a control. **C**, Leaf phenotypes of tomato leaves in control and transient-overexpression-*SpNBS-LRR* groups at 0 to 5 days after *Phytophthora infestans* inoculation. Scale bars = 0.5 cm. **D**, Average number of disease spots on tomato leaves in control and transient-overexpression-*SpNBS-LRR* groups at 2, 3, 4, and 5 days postinoculation (dpi), respectively. Error bars indicate SE ($n = 6$) and letters indicate significant difference among samples ($P < 0.05$).

and activate inducible defenses (Sekhwal et al. 2015). Previous studies on late blight showed that more than 60 potato resistance genes against *P. infestans* have been located, identified, and cloned (Rodewald and Trognitz 2013; Zhang et al. 2014). Compared with intensive studies on potato, those on late blight resistance in tomato are very limited (Zhang et al. 2014). Several resistance genes against *P. infestans* in tomato, such as *Ph-1*, *Ph-2*, *Ph-3*, *Ph-4*, and *Ph-5*, have been reported and mapped on chromosome 7, 10, 9, 1, and 10, respectively (Black et al. 1996; Bonde and Murphy 1952; Foolad et al. 2008; Kim and Mutschler 2005; Merk et al. 2012). Our previous works focused on identification and functional analysis of tomato resistance genes (Li et al. 2015), miRNAs (Luan et al. 2015, 2016), and lncRNAs (Cui et al. 2017) associated with tomato resistance to *P. infestans*.

NBS-LRR proteins, the largest class of resistance proteins, have been established to play important roles in plant effector-triggered immunity. In this study, we isolated an *NBS-LRR* gene (*SpNBS-LRR*) associated with resistance to *P. infestans* from *S. pimpinellifolium* L3708. After inoculation of *P. infestans*, the *SpNBS-LRR* gene showed low-level expression at first and then rapidly increased in our previous works (Jiang et al. 2018). Similar to our previous results, de Vries et al. (2018) showed that the tomato NBS-LRRs (including *Solyc02g036270*) were responsive to *P. infestans* stress. A *CC-NBS-*

LRR gene in the resistant *Vitis riparia* was expressed at a very low level under noninfected conditions; however, its transcript reached the highest level at 12 h postinoculation in response to *Plasmopara viticola* infection (Kortekamp et al. 2008).

Based on the results of phylogenetic analysis, this *SpNBS-LRR* had a close genetic relationship to *A. thaliana* RPS2, supported by high bootstrap values (Fig. 1B). This showed that the *SpNBS-LRR* maybe had similar functions with RPS2 in plant–pathogen interaction. In previous studies, RPS2 was involved in *A. thaliana*–*Pseudomonas syringae* DC3000 interaction (Cherkis et al. 2012; MacQueen et al. 2016). During interaction, RPS2 can bind the RIN4 and triggers ETI when RIN4 is cleaved by the bacterial effector AvrRpt2 (Axtell and Staskawicz 2003; Mackey et al. 2003). Two members of hypersensitive induced reaction protein (HIR) family, AtHIR1 and AtHIR2, form complexes with RPS2 in *Arabidopsis*, contributing to RPS2-mediated ETI (Qi et al. 2011). In addition, another effector, AvrRpm1, also induces RPS2-dependent responses (Cherkis et al. 2012; Kim et al. 2009).

In tomato, 252 NBS-LRR genes were identified from the whole genome of *S. lycopersicum*, which were divided into six subgroups (Liu et al. 2014). In recent years, some tomato *NBS-LRR* genes were cloned and their functions were explored. An *NBS-LRR* resistance gene, *Tm-2²*, was cloned from tomato and verified its resistance to

Fig. 3. Identification of transgenic tomato lines and disease symptoms on the detached leaves from wild type (WT) and transgenic lines at 5 days after inoculation of *Phytophthora infestans*. **A**, PCR-based genotyping of *SpNBS-LRR* overexpression transgenic and WT tomato. +, positive control using pBI121-*SpNBS-LRR* plasmid DNA as PCR template; lanes 1 to 10, transformed tomato plant line 1 to 10 (OE1 to OE10); –, negative control using untransformed WT tomato. **B**, Semiquantitative RT-PCR analysis of *SpNBS-LRR* expression levels in transgenic tomatoes. Tomato actin expression was used as a control. **C**, The expression level of *SpNBS-LRR* in selected transgenic lines OE3, OE6, and OE8. Tomato actin expression was used as a control. **D**, Trypan blue staining of the detached leaves from WT and transgenic lines at 5 days after point infection of *P. infestans*. Scale bars = 0.5 cm. **E**, Measurement of the diameter of lesion in *P. infestans* inoculated leaves after point infection. Data are the means $\pm$ standard errors of three independent experiments. Letters indicate significant differences among samples ($P < 0.05$).

Fig. 4. Resistance of transgenic and wild type (WT) tomatoes after whole-plant inoculation with *Phytophthora infestans*. **A**, Disease symptoms on the leaves of WT and transgenic lines at 5 days postinoculation. Scale bars = 0.5 cm. **B**, Disease index. **C**, Transcript accumulation of *P. infestans Actin* gene in these inoculated plants. Tomato actin expression was used as control. **D**, Trypan blue staining. Scale bars = 0.5 cm. **E**, Malonaldehyde (MDA) content. Data are the means $\pm$ standard errors of three independent experiments. Letters indicate significant differences among samples ($P < 0.05$).

Fig. 5. Relative expression levels of some defense-related genes in the leaves of wild type (WT) and transgenic tomato plants. PR1, GenBank accession Y08804; PR2, GenBank accession NM001247229; PR5, GenBank accession NM001247422; PDF-like-1, Solyc04g072470; and PDF-like-2, Solyc07g009260. Data are the means $\pm$ standard errors of three independent experiments. Letters indicate significant differences among samples ($P < 0.05$). Tomato actin expression was used as a control.

tomato mosaic virus by transforming it to susceptible tomato plants (Lanfermeijer et al. 2003). Another member of tomato *NBS-LRR* gene family, *Prf*, could enhance the resistance of tomatoes to *P. syringae* by associating with Pto (Gutierrez et al. 2010). The *Ph-3*, encoding a CC-NBS-LRR protein, was isolated from *S. pimpinellifolium* by map-based cloning and demonstrated its resistance to *P. infestans* (Zhang et al. 2014). In addition, silencing of *NBS-LRRs* in tomato rendered it susceptible to *Fusarium oxysporum* (Ji et al. 2018; Ouyang et al. 2014). In this study, *SpNBS-LRR* was introduced into tomato *S. lycopersicum* Zaofen No.2, which is susceptible to *P. infestans*. Compared with untransformed WT plants, overexpression of *SpNBS-LRR* increased *P. infestans* resistance in transgenic tomato plants, which was mainly demonstrated by decreased disease index, lesion diameters, number of necrotic cells, and *P. infestans* abundance (Figs. 3 and 4). This provides insight into *SpNBS-LRR* and its involvement in the resistance of tomato to *P. infestans*.

Plants can be induced to switch on defense reactions to a broad range of pathogens as a result of prior exposure to pathogens. Induced resistance is expressed locally, at the site of the infection or systemically, at sites remotely located from the initial infection (Métraux et al. 2002). SAR and induced systemic resistance (ISR) are two forms of induced resistance (Choudhary et al. 2007). It has been shown that SAR and ISR are dependent on SA and JA signaling, respectively (Choudhary et al. 2007). Notably, *PR1*, *PR2*, and *PR5* are marker genes for SA signaling (Molinari et al. 2014), and *Arabidopsis PDF1.2* are marker genes for JA signaling (Penninckx et al. 1996, 1998). Overexpression of a pepper *PR1* gene in tobacco plants enhanced resistance to *P. nicotianae*, *Ralstonia solanacearum*, and *P. syringae* pv. *tabaci* (Sarowar et al. 2005). Likewise, transgenic rice plants that overexpressed a *PDF* gene, *Rs-AFP2*, showed enhanced resistance to two of the most important pathogens of rice, *Magnaporthe oryzae* and *Rhizoctonia solani* (Jha and Chattoo 2010). In this study, the expression levels of *PR1*, *PR2*, *PR5*, and *PDF-like* (the homolog of *Arabidopsis PDF1.2*) genes were significantly increased in transgenic lines that overexpressed *NBS-LRR* (Fig. 5). Similarly, Pik-H4, an NBS-LRR protein interacting with OsBIH-D1 enhanced the accumulation of PR (Liu et al. 2017). These results suggest that NBS-LRR might induce the expression of *PR* and *PDF* genes and is involved in SAR and ISR in tomato-*P. infestans* interactions.

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