Tomato NAC Transcription Factor SISR1 Positively Regulates Defense Response against Biotic Stress but Negatively Regulates Abiotic Stress Response

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Abstract

Biotic and abiotic stresses are major unfavorable factors that affect crop productivity worldwide. NAC proteins comprise a large family of transcription factors that play important roles in plant growth and development as well as in responses to biotic and abiotic stresses. In a virus-induced gene silencing-based screening to identify genes that are involved in defense response against Botrytis cinerea, we identified a tomato NAC gene SISR1 (Solanum lycopersicum Stress-related NAC1). SISR1 is a plasma membrane-localized protein with transactivation activity in yeast. Expression of SISR1 was significantly induced by infection with B. cinerea or Pseudomonas syringae pv. tomato (Pst) DC3000, leading to 6–8 folds higher than that in the mock-inoculated plants. Expression of SISR1 was also induced by salicylic acid, jasmonic acid and 1-amino cyclopropane-1-carboxylic acid and by drought stress. Silencing of SISR1 resulted in increased severity of diseases caused by B. cinerea and Pst DC3000. However, silencing of SISR1 resulted in increased tolerance against oxidative and drought stresses. Furthermore, silencing of SISR1 accelerated accumulation of reactive oxygen species but attenuated expression of defense genes after infection by B. cinerea. Our results demonstrate that SISR1 is a positive regulator of defense response against B. cinerea and Pst DC3000 but is a negative regulator for oxidative and drought stress response in tomato.

Citation: Liu B, Ouyang Z, Zhang Y, Li X, Hong Y, et al. (2014) Tomato NAC Transcription Factor SISR1 Positively Regulates Defense Response against Biotic Stress but Negatively Regulates Abiotic Stress Response. PLoS ONE 9(7): e102067. doi:10.1371/journal.pone.0102067

Editor: Hua Lu, UMBC, United States of America

Received March 7, 2014; Accepted June 13, 2014; Published July 10, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included in the manuscript.

Funding: This work was supported by the National Basic Research Program of China (2009CB119005), the National Key Technology R & D Program of China (2011BAD12B04), the National High-Tech R & D Program (No. 2012AA101504) and the Research Fund for the Doctoral Program of Higher Education of China (20120101110070). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Plants constantly encounter various biotic (i.e. pathogen infection) and abiotic (i.e. drought, high salinity and extreme temperature conditions) stresses that significantly affect both biomass growth and yield production. However, plants have developed to equip with a sophisticated signaling networks to precisely regulate defense responses against pathogen attack and abiotic stress. Upon perception of the environmental cues, initiation of the signaling network ultimately leads to activation of a large set of genes, which are regulated by different types of transcription factors (TFs). Thus, TFs are critical regulatory factors in modulating the temporal and spatial expression of the genes involved in defense response. During the last decade, numerous TFs belonging to the different families such as NAC, ERF, MYB, WRKY, and bZIP families have been identified to play important roles in regulating plant responses to biotic and abiotic stresses [1–5]. The NAC (NAM/ATAF/CUC) TFs are unique plant TFs [6] and comprise a large family with more than 100 members in rice, Arabidopsis, tobacco, potato, soybean, grapevine and poplar [7–13]. Structurally, the NAC proteins contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal domain [6]. Recent extensive studies have implicated NAC proteins as important components in different aspects of plant development including formation of boundary cells of the meristem, cell division and expansion, lateral root development, leaf senescence, secondary wall biosynthesis, and flowering time [for reviews, see 6,14–18]. In addition, the involvement and function of the NAC proteins in plant responses to biotic and abiotic stresses have been well documented not only in model plants but also in various crop plants [for reviews, see 4,15,19,20]. Thus, it is believed that the NAC proteins can be used as useful functional gene resources for improvement of biotic and abiotic stress tolerance in crops [15,19,21].

Accumulating evidence demonstrates that the NAC proteins play critical roles in regulation of plant defense responses against different types of pathogens. The first line of evidence came from the identification of the potato gene StNAC, which was induced by pathogen attack [22]. Recent functional studies by altering expression of individual NAC gene through knockout/knockdown and overexpression approaches have identified a number of NAC genes that are involved in defense responses of different plants to pathogen infection. In Arabidopsis, at least eight NAC proteins such as ATAF1, ATAF2, ANAC019, AAC042, ANAC055, ANAC072, CBNAc1 and NT6 were identified as negative
regulators of defense responses against different types of pathogens including Botrytis cinerea, Alternaria brassicicola, Fusarium oxysporum and Pseudomonas syringae pv. tomato [23–31]. In rice, OsNAC6, RIM1, OsNAC4, ONAC122 and ONAC131, were shown to function in regulation of disease resistance against M. oryzae and Rice dwarf virus, and hypersensitive cell death, respectively [32–37]. Similarly, the barley HvNAC6 was demonstrated to increase penetration resistance and promote basal resistance against virulent Blumeria graminis f. sp. hordei, respectively [38,39]. Grapevine VvNAC1 and pepper CaNAC1 were also involved in regulation of disease resistance response [40,41]. Interestingly, the potato and Nicotiana benthamiana NTP1 and NTP2 were found to be targeted by an RxLR effector Pi03192 from Phytophthora infestans [42]. Thus, it is clear that the NAC proteins participate in many aspects of plants-pathogen interactions, acting as regulators of immune responses or as targets of pathogen effectors.

Figure 1. Sequence alignment and phylogenetic tree analysis of SISRN1 with other plant NAC proteins. A. Sequence alignment of SISRN1 with NbNTP2 and AtNAC017. The numbers on the left indicate amino acid positions of the proteins used. Shared amino acid residues are shown in black background. Conserved NAC domain and putative transmembrane motif are indicated. B. Phylogenetic tree analysis of SISRN1 with other plant NAC proteins. Phylogenetic tree was constructed by neighbour-joining method using MEGA program version 6.05. SISRN1 in the tree is indicated by an arrow. Plant NAC proteins used and their GenBank accessions are as follows: Arabidopsis AtNAC017 (AAK32826), AtNAC016 (AAD39614) and AtNAC013 (AEE31534), Nicotiana benthamiana NbNTP2 (AGY49287), potato StNTP2 (AGY49285) a tomato predicted NAC (Solyco04g072220) and rice Os09g32040 (BAF25461).

doi:10.1371/journal.pone.0102067.g001
Recent functional analyses have also provided direct evidence supporting that the NAC proteins function as important components in complex signaling processes during plant abiotic stress responses (for reviews, see [4,15,19,20]). A number of NAC proteins have been shown to play important roles in plant tolerance to drought and salinity stress. Such NAC proteins include the Arabidopsis ANAC019, ANAC035, ANAC072, ANAC096, ANAC2, ATAF1, ATAF2 and RD26 [25,43–47], rice SNAC1/OsNAC1, OsNAC5, SNAC2/OsNAC6, OsNAC09, OsNAC045, OsNAC052 and OsSNAP [32,48–55], and wheat TaNAC69 [56]. Some of NAC proteins have also been implicated in oxidative, temperature and nutrient stresses, for examples, ANAC013 and NTL4 in oxidative stress [57,58], ANAC042 and NTL4 in oxidative stress [57,58], ANAC013 and NTL4 in oxidative stress [57,58], ANAC019 and TaNAC69 [59,60], and wheat TaNAC69 [56]. Some of NAC proteins have also been implicated in oxidative, temperature and nutrient stresses, for examples, ANAC013 and NTL4 in oxidative stress [57,58], ANAC042 and NTL4 in oxidative stress [57,58], ANAC019 and TaNAC69 [59,60], and wheat TaNAC69 [56].

Materials and Methods

Plant growth and treatments

Tomato (Solanum lycopersicum) cv. Suhong 2003 was used in this study. Tomato plants were grown in a growth room at 22°C under a 16 hr light and 8 hr dark regime. For analysis of gene expression in response to defense signaling hormones, 4-week-old tomato plants were treated by foliar spraying with 100 μM MeJA (Sigma-Aldrich, MO, USA), 100 μM 1-amino cyclopropane-1-carboxylic acid (ACC) (Sigma-Aldrich, MO, USA), 1 mM salicylic acid (SA) (Sigma-Aldrich, MO, USA) or water as a control. Alternatively, fully expanded leaves were detached from four-week-old plants and subjected to drought stress by stopping watering for a period until wilting symptom appeared or watered every two days as controls. Alternatively, fully expanded leaves were detached from four-week-old plants and subjected to drought stress treatment by stopping watering for a period until wilting symptom appeared or watered every two days as controls. Experiments were performed with at least three biological replicates. For each biological replicate, at least five plants were used. The data are shown as means ± SD. The significance of differences between the treatments was determined using one-way ANOVA and Tukey's HSD test (P < 0.05).

Cloning of \textbf{SISR1} and bioinformatics analysis

Extraction of total RNA using Trizol reagent (Takara, Dalian, China) was performed according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using the AMV-reverse transcriptase (Takara, Dalian, China) according to the manufacturer’s instructions. The full-length cDNA of \textbf{SISR1} was PCR amplified using gene-specific primers \textbf{SISR1-orf-1F} (5' - ATG AAG AAT TTG TCT GAT TCT TCT GAT-3') and \textbf{SISR1-orf-1R} (5' - TGG CAA GAT GCC AAA TGA TAG AAC A-3'). The PCR product was cloned into pMD19-T vector (Takara, Dalian, China) and confirmed by sequencing. Similarity searches of nucleotide and amino acid sequences were carried out using BLAST program at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignment and phylogenetic tree construction were performed by ClustalW method using MegaAlign program in LaserGene software.

Subcellular localization

The coding sequence of \textbf{SISR1} was PCR amplified using primers \textbf{SISR1-gfp-1F} (5' - AGT GGA TCC ATG AAG ATG TTT GAG TTA TCT GCT GAT TC-3'), a BamHI site underlined) and \textbf{SISR1-gfp-1R} (5' - GGG TCT AGA TTA AGA GGA GAT GGG TCT CCT-3', an XbaI site underlined). The PCR product was cloned into pFGC-eGFP vector to yield pFGC-SISR1 construct. The recombinant plasmid pFGC-SISR1 and pFGC-eGFP (as a control) were introduced into onion epidermal cells by the particle bombardment method. Particle bombardment was performed with a PDS-1000 (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions, using gold particles coated with plasmid DNA under a slight vacuum at a helium pressure of 1100 psi. After bombardment, the onion peels were incubated with liquid Murashige-Skoog (MS) medium for 24 hr and GFP was detected with a confocal laser scanning microscope (Zeiss LSM 510 META; argon laser excitation wavelength, 488 nm).

Transcription activation assay in yeast

The coding sequence of \textbf{SISR1} was PCR amplified using primers \textbf{SISR1-vigs-1F} (5' - AGA GTA TGG GCC TCC CTT CCT AAG-3'), a SalI site underlined) and \textbf{SISR1-vigs-1R} (5' - TCT GAT-3') (as a control) were introduced into yeast strain AH109. The transformed yeast was cultivated on the SD/Trp - and SD/Trp + His + medium for 3 days at 28°C, followed by addition of x-gal. The transcriptional activity of the fusion protein was evaluated according to the corresponded growth situation and production of blue pigments after the addition of x-gal of the transformed yeast cells on SD/Trp + His + medium.

Construction of \textbf{VIGS} vectors

For construction of \textbf{VIGS} vectors, a 372 bp fragment of \textbf{SISR1} was amplified with a pair of gene-specific primers \textbf{SISR1-vigs-1F} (5' - GAT GTA TGC AGC GAT AAG ATG TTT GAG TTA TCT GCT GAT TC-3'), a SalI site underlined) and \textbf{SISR1-vigs-1R} (5' - ATG TAA CTC GAG TGC CTC ATG CAA CTC TCG CT-3', an XbaI site underlined) and cloned into pYL156, yielding TRV-SISR1 construct. For construction of TRV-GUS, a 396 bp fragment of the GUS gene was amplified with primers GUS-vigs-1F (5' - GCT CGG AGG GAT GAC GCT TCT CCT AAG-3', an EcoRI site underlined) and SISR1-vigs-1R (5' - ATG TAA CTC GAG TGC CTC ATG CAA CTC TCG CT-3', an XbaI site underlined) and cloned into pYL156, yielding TRV-SISR1 construct. The recombinant plasmids TRV-SISR1 and TRV-GUS were transformed into \textit{A. tumefaciens} strain GV3101 by electroporation.

Agroinfiltration for \textbf{VIGS} and transient expression

Agrobacteria carrying TRV-SISR1, TRV-GUS, pFGC-SISR1 or pFGC-eGFP were grown in YEP liquid medium with...
50 μg/mL kanamycin, 50 μg/mL rifampicin and 25 μg/mL gentamicin to OD<sub>600</sub> = 0.8–1.0. Cells were centrifuged and resuspended in infiltration MES buffer (pH5.7, 10 mM MES, 10 mM MgCl<sub>2</sub> and 150 μM acetosyringone). For VIGS agroinfiltration, agrobacteria carrying TRV-GUS or TRV-SlSRN1 were mixed with agrobacteria carrying TRV1 in a ratio of 1:1 and maintained at OD<sub>600</sub> = 1.5 for 3 hr at room temperature. The mixed agrobacteria suspension was infiltrated into the abaxial surface of the 2-week-old seedlings using a 1 mL needleless syringe. Efficiency of the VIGS protocol was evaluated using phytoene desaturase (PDS) gene as a marker of silencing in tomato plants according to Liu et al. [71]. The VIGS-infiltrated plants were allowed to grow for three weeks under same condition as mentioned above and then used for all experiments. For transient expression agroinfiltration, agrobacteria carrying pFGC-SlSRN1 or pFGC-eGFP empty vector were infiltrated into leaves of 4-week-old plants using a 1 mL needleless syringe. Leaf samples were collected 2 days after agroinfiltration for analyzing the expression level of SISRNI and were used for disease assays.

**Disease assays**

Inoculation of tomato plants with *B. cinerea* was carried out as described previously [72]. Briefly, spores were collected from 10-day-old *B. cinerea* cultures and resuspended in 4% maltose and 1% peptone buffer to a concentration of 1×10<sup>6</sup> spores/mL. Detached fully expanded leaves were inoculated by drop inoculation method according to a previously reported procedure [72]. For whole plant inoculation, 4-week-old plants were sprayed with spore suspension or buffer as mock inoculation control. The inoculated leaves or plants were covered with a transparent plastic film and kept in a growth chamber with similar conditions as for plant growth. Diameters of each lesion were recorded 4 days post inoculation (dpi). Leaves from at least ten individual plants were used in each independent experiment.
Abiotic stress assays

For oxidative stress assays, fully expanded leaves from TRV-SlSRN1- or TRV-GUS-infiltrated plants were collected 2 weeks after VIGS infiltration and rinsed with sterile distilled water. Leaf discs (13 mm in diameter) were made by a hole puncher from at least 6 individual plants for each experiment and were incubated in 1/2 MS buffer supplemented with 20 mM H2O2 or without H2O2 (as a control) for 3 days under illumination condition at moderate light intensity (200 μmol m⁻² s⁻¹). Measurement of chlorophyll content was performed as described before [73] and the content of chlorophyll was calculated according to the formula Chl (A+B) = 5.24A664 + 22.24A648. In drought stress assays, the TRV-SlSRN1- or TRV-GUS-infiltrated plants were allowed for further growth with normal watering regime for 2 weeks after VIGS infiltration and then were subjected to drought stress by stopping watering for a certain period of time until the wilting symptoms were obvious. Measurement of the relative water content (RWC) in leaves was performed as described before [74]. Fully expanded leaves from 6 individual plants were detached to measure the leaf fresh weight (W_f), turgid leaf weight (W_t), and dry weights (W_d) and relative water contents (RWC) were calculated from the equation RWC (%) = (W_f - W_d)/W_f × 100% [74]. All experiments were repeated independently for three times.

Quantitative RT-PCR analysis of gene expression

Silencing efficiency of SlSRN1 in TRV-SlSRN1-infiltrated plants, expression of SlSRN1 and defense genes were analyzed by qRT-PCR. Tomato SlActin was used as a reference gene with primers of SlActin-1F (5’-GCA TGA ACG CTA GAT GGA AG-3’) and SlActin-1R (5’-ATA CCC ACC ATC ACA CCA GTA T-3’). Primers for SlSRN1 and other defense genes are as follows: SlSRN1-q-1F, 5’-GGA TGG TCG ACT AGA AGT CAC ATC TC-3’; SlSRN1-q-1R, 5’-CCA AGG TGA TCC ATC TCC AGA A-3’; SPr1a-q-1F, 5’-TCT TGT GAG GCC CAA ATT TC-3’; SPr1a-q-1R, 5’-ATA GTG TGG CCT CTC GGA CA-3’; SPr1b-q-1F, 5’-CCA GGA CTA TCT TGC GGT TC-3’; SPr1b-q-1R, 5’-GAA CCT AAG CGA CGA TAG AC-3’; SPr5-q-1F, 5’-AAT TGC TGG CATT ATTA ATG GTG C-3’; SPr5-q-1R, 5’-TAG CAG ACC GCT TAA GAT GC-3’. Relative expression was calculated using 2⁻ΔΔCT method. The experiments were repeated independently with three biological replicates using SYBR Green PCR master mix kit (Takara, Dalian, China) in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions.

Detection of reactive oxygen species

Detection of H2O2 and superoxide anion (O2⁻) in leaf tissues was performed by 3,3-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively, according to the methods described previously [75,76]. Leaf samples were collected from inoculated plants at 0 and 24 hr after inoculation with B. cinerea and dipped into DAB solution (1 mg/mL, pH 3.8) for staining of H2O2, or in NBT solution (1 mg/mL NBT in 10 mM Na2SO3 and 10 mM phosphate buffer, pH 7.8) for staining superoxide anion. Accumulation of H2O2 and superoxide anion in leaves was visualized using a digital camera.

Figure 3. Expression of SlSRN1 in responses to pathogen infection and treatments with defense signaling hormones. Four-week-old tomato seedlings were inoculated by spore suspension of B. cinerea (A), bacterial suspension of Pst DC3000 (B) or similar volume of buffer solution as mock-inoculation control or treated by foliar spraying with 1 mM SA, 100 mM MeJA, 100 mM ACC solutions or sterilized distill water as a control (C). Leaf samples were collected at different time points after inoculation or treatment as indicated. Total RNA was extracted and used for qRT-PCR analysis. Data presented are the means ± SD from three independent experiments and different letters above the columns indicate significant differences at p<0.05 level.

doi:10.1371/journal.pone.0102067.g003

Pst DC3000 grown overnight in King’s B liquid medium containing 25 μg/mL rifampicin were diluted and re-grown in 10 mM MgCl2 to OD600 = ~1.0. Bacteria were collected and resuspended in 10 mM MgCl2 to OD600 = 0.0002. Four-week-old plants were vacuum infiltrated with bacteria suspensions and then kept in a growth chamber with high humidity. For measurement of bacterial growth curve, leaf punches from six individual plants were surface sterilized in 70% ethanol for 10 sec, homogenized in 200 μL of 10 mM MgCl2, diluted in 10 mM MgCl2, and plated on KB agar plates containing 100 μg/mL rifampicin.

PLOS ONE | www.plosone.org 5 July 2014 | Volume 9 | Issue 7 | e102067
Results

Characterization of SISR1

To explore the molecular basis of defense response in tomato against necrotrophic fungal pathogens, we performed VIGS-based screening to identify genes that are involved in defense response to B. cinerea. In these VIGS fast screenings, dozens of genes were chosen and examined for altered phenotype of disease caused by B. cinerea. It was observed that knockdown of a gene encoding for a NAC transcription factor led to increased severity of disease caused by B. cinerea. This putative NAC gene, designated as SISR1 for Solanum lycopersicum stress-related NAC1, was chosen for further study. A full-length cDNA, SGN-U320122, and two partial cDNAs, SGN-U585287 and SGN-U587626, for SISR1 were identified by blast searches against the tomato genomic database (http://solgenomics.net/). The SISR1 gene corresponds to the predicted locus Solyc12g056790 on chromosome 12 and comprises 2 introns and 3 exons. After cloning and confirmation of the sequence, the full-length cDNA of SISR1 is 1987 bp with an open reading frame of 1773 bp, which encodes a protein of 590 residues with a calculated molecular mass of 66.5 kDa and a theoretical pI of 4.8. The SISR1 protein contains a conserved NAM domain.

Figure 4. SISR1 positively regulates resistance response against B. cinerea. A–C. Silencing of SISR1 led to enhanced susceptibility to B. cinerea. A. Silencing efficiency in TRV-SISR1-infiltrated tomato plants. Two-week-old seedlings were infiltrated with agrobacteria carrying TRV-SISR1 or TRV-GUS and leaf samples were collected 4 weeks after VIGS treatment. The transcript of SISR1 was analysed by qRT-PCR. B and C. Disease phenotype and lesion size on detached leaves of TRV-SISR1 or TRV-GUS-infiltrated plants after inoculation with B. cinerea, respectively. D–E. Transient overexpression of SISR1 resulted in increased resistance to B. cinerea. D. Expression of SISR1 in pFGC-SISR1- or pFGC-eGFP-infiltrated plants. Leaves of 3-week-old seedlings were infiltrated with agrobacteria carrying pFGC-SISR1- or pFGC-eGFP vector and leaf samples were collected 3 days after infiltration. E and F. Disease phenotype and lesion size on detached leaves of pFGC-SISR1- or pFGC-eGFP-infiltrated plants after inoculation with B. cinerea, respectively. Data presented are the means ± SD from three independent experiments and different letters above the columns indicate significant differences at $p<0.05$ level.

doi:10.1371/journal.pone.0102067.g004
SISR1 is localized on the plasma membrane and has transactivation activity

To determine the subcellular localization of SISR1, a SISR1-GFP fusion construct was generated and introduced into onion epidermal cells by particle bombardment. When transiently expressed, the SISR1-GFP fusion protein was localized exclusively on the plasma membrane of onion epidermal cells but not in the nucleus, while the GFP fluorescence was observed throughout the entire cytoplasm and the nucleus without specific compartment localization (Fig. 2A). This result indicates that the SISR1 is localized to the plasma membrane of cells.

Transactivation activity of SISR1 was examined using a yeast assay system. As shown in Fig. 2B, both yeast transformants carrying pBD-SISR1 and pBD vector grew well on SD/Tryptophan medium. However, only yeast transformants containing pBD-SISR1 were able to grow on the SD/Trp His− medium and produced a blue pigment after the addition of x-α-gal, showing a β-galactosidase activity, whereas transformants containing the pBD empty vector did not. These results indicate that SISR1 has transactivation activity in yeast cells.

Induced expression of SISR1 by pathogens and defense signaling hormones

To explore the possible involvement of SISR1 in tomato disease resistance response, we analyzed the expression dynamics of SISR1 in response to infection by different types of pathogens and treatment with defense signaling hormones. The expression of SISR1 in mock-inoculated plants maintained unchanged in B. cinerea-inoculated plants during the experimental period (Fig. 3A). However, the expression level of SISR1 started to increase at 12 hr post inoculation (dpi), peaked at 24 hpi and maintained at a very high level until 72 hpi, showing increases of 3–8 folds over that in mock-inoculated plants during the experimental period (Fig. 3A). Similar expression dynamic of SISR1 was observed in plants after infection with Pst DC3000 (Fig. 3B). In Pst DC3000-inoculated plants, the expression level of SISR1 increased at 12 hpi, peaked at 24 hpi and maintained at a relatively high level until 72 hpi, giving increases of 6–8 folds over that in mock-inoculated plants (Fig. 3B). In defense signaling hormone-treated plants, expression of SISR1 was induced by SA, JA and ACC, showing increases of approximately 4–7 folds over that in control plants at 12 and 24 hr after treatment (Fig. 3C). These results indicate that expression of SISR1 can be induced by infection of B. cinerea and Pst DC3000 and by treatment with defense signaling hormones.

SISR1 is required for resistance against B. cinerea

To explore the possible function of SISR1 in disease resistance, we used the TRV-based gene silencing system [71] to knockdown the expression level of SISR1 in tomato plants and compared the phenotype between the silenced and the control plants after infection with B. cinerea or Pst DC3000. For this purpose, we made TRV-mediated VIGS constructs for SISR1 genes and performed standard VIGS procedure on two-week-old tomato seedlings. Only when the efficiency of the VIGS protocols was >90%, judged based on the appearance of bleaching phenotype in the pTRV-PDS-infiltrated plants, the TRV-SISR1-infiltrated plants in the same batch were used for various experiments three weeks after VIGS infiltration. The silencing efficiency for SISR1 under our experimental condition was ~70% (Fig. 4A), as examined by qRT-PCR analysis of the transcript level of SISR1 in the TRV-SISR1-infiltrated plants and compared with that in the TRV-GUS-infiltrated negative control plants.

We first examined the disease phenotype of the TRV-SISR1-infiltrated plants after inoculation with B. cinerea using a detached leaf inoculation assay. Under our disease assay conditions, typical disease symptom, e.g. necrotic lesions, was observed in the leaves from the TRV-SISR1- and TRV-GUS-infiltrated plants 2 dpi but the lesions in the leaves from the TRV-SISR1-infiltrated plants expanded much rapidly and were larger than those in the TRV-GUS-infiltrated plants (Fig. 4B). At 4 dpi, the lesion size in the leaves from the TRV-SISR1-infiltrated plants showed an average of 6.5 mm, giving an increase of 30% over that in the TRV-GUS-infiltrated plants (average of 4.7 mm for lesion size) (Fig. 4C). Meanwhile, we also explored whether transient expression of SISR1 in tomato leaves can confer an increased resistance against B. cinerea. As shown in Fig. 4D, the expression level of SISR1 in leaves of plants infiltrated with agrobacteria carrying pFGC-SISR1 construct increase significantly at 2 days.
after infiltration, leading to approximately 4 times higher over that in the control plants infiltrated with agrobacteria carrying pFGC-eGFP vector only. Disease assays revealed that the lesions on leaves of the pFGC-SlSRN1-infiltrated plants were smaller than those on leaves of the pFGC-eGFP-infiltrated plants (Fig. 4E), resulting in a reduction of 40% in size (Fig. 4F). These data indicate that silencing of SlSRN1 resulted in increased disease while transient overexpression led to reduced disease caused by B. cinerea, demonstrating that SlSRN1 plays an important role in resistance against B. cinerea.

**Silencing of SlSRN1 resulted in increased disease caused by Pst DC3000**

We further examined whether SlSRN1 is involved in resistance against Pst DC3000. In out experiments, necrotic lesions were observed in the inoculated leaves of the TRV-SlSRN1- and TRV-GUS-infiltrated plants; however, the lesions on leaves of the TRV-SlSRN1-infiltrated plants were larger than those in the TRV-GUS-infiltrated plants (Fig. 5A). At 2 dpi, the bacterial population in the inoculated leaves of the TRV-SlSRN1-infiltrated plants showed a 10-fold higher over that in the TRV-GUS-infiltrated plants (Fig. 5B). At 4 dpi, the bacterial population in inoculated leaves of the TRV-SlSRN1-infiltrated plants was measured to be $1.26 \times 10^9$ cfu/cm$^2$, showing a 16-fold increase in bacterial growth.
relative to that in the TRV-GUS-infiltrated plants (7.9 × 10^7 cfu/cm^2) (Fig. 5B). These results indicate that silencing of SlSRN1 resulted in enhanced disease severity and increased bacterial growth, implying the requirement of SlSRN1 for resistance against Pst DC3000.

Silencing of SlSRN1 affects defense response against B. cinerea

We first examined whether silencing of SlSRN1 affects accumulation of reactive oxygen species (ROS) upon infection of B. cinerea. No significant accumulation of H_2O_2 and superoxide anion was detected in leaves of the TRV-SlSRN1- and TRV-GUS-infiltrated plants at 0 hr after inoculation (Fig. 6A and B), indicating that silencing of SlSRN1 did not affect accumulation of ROS in tomato plants. At 24 hr after inoculation with B. cinerea, accumulation of superoxide anion and H_2O_2 in leaves of the TRV-SlSRN1-infiltrated plants showed significant increases as compared with those in the TRV-GUS-infiltrated plants (Fig. 6A and B), especially the increase of H_2O_2 accumulation in leaves of the TRV-SlSRN1-infiltrated plants (Fig. 6B).

We further examined the effect of SlSRN1 silencing on the expression of defense- and signaling-related genes in tomato plants after infection by B. cinerea. For this purpose, we analyzed and compared the expression levels of SlPR1a, SlPR1b, SlPR3 and STPK1b in the TRV-SlSRN1- and TRV-GUS-infiltrated plants after infection by B. cinerea. As shown in Fig. 6C, expression levels of SlPR1a and SlPR1b in the TRV-SlSRN1-infiltrated plants were significantly increased, showing 9–10 folds higher than those in the TRV-GUS-infiltrated plants after infection with B. cinerea. However, the expression levels of SlPR3 and STPK1b in the TRV-SlSRN1-infiltrated plants were markedly decreased, leading to a reduction of 75–90% as compared with those in the TRV-

Figure 7. Silencing of SlSRN1 increased tolerance to oxidative stress. Two-week-old seedlings were infiltrated with agrobacteria carrying TRV-SlSRN1 or TRV-GUS and leaf discs were taken from leaves of TRV-SlSRN1- or TRV-GUS-infiltrated plants at 2 weeks after VIGS infiltration. Leaf discs were soaked in 1/2 MS buffer supplemented with 20 mM H_2O_2 or H_2O (as a control). Phenotype (A) and relative chlorophyll contents (B) in leaf discs from TRV-SlSRN1- or TRV-GUS-infiltrated plants under oxidative stress. Photos and samples for analysis of chlorophyll contents were taken at 5 days after treatment. Data presented in (B) are the means ± SD from three independent experiments and different letters above the columns indicate significant differences at p<0.05 level.

doi:10.1371/journal.pone.0102067.g007
GUS-infiltrated plants after infection with B. cinerea (Fig. 6C). These data indicate that silencing of SlSRN1 affects the expression of a set of defense- and signaling-related genes upon infection with B. cinerea.

**SlSRN1 is required for tolerance to oxidative and drought stress**

To explore whether SlSRN1 has a function in abiotic stress response, we analyzed and compared the oxidative stress tolerance and drought tolerance of the TRV-SlSRN1- and TRV-GUS-infiltrated plants. In oxidative stress assays, leaf discs from leaves of the TRV-SlSRN1- and TRV-GUS-infiltrated plants were treated in H2O2 solution as an artificial oxidative stress condition. During 5 days of the experimental period, no significant phenotype appeared on the leaf discs from the TRV-SlSRN1- and TRV-GUS-infiltrated plants without H2O2 treatment (Fig. 7A). When treated with H2O2, bleaching and chlorosis symptom were observed in leaf discs from the TRV-SlSRN1- and TRV-GUS-infiltrated plants; however, bleaching and chlorosis symptoms in leaf discs from the TRV-SlSRN1-infiltrated plants were less severe than those of the TRV-GUS-infiltrated plants (Fig. 7A). This observation was further confirmed by measuring chlorophyll contents in leaf discs from the TRV-SlSRN1- and TRV-GUS-infiltrated plants after H2O2 treatments (Fig. 7B). Without H2O2 treatment, no significant difference in relative chlorophyll contents in leaf discs from the TRV-SlSRN1- and TRV-GUS-infiltrated plants was observed. However, relative chlorophyll contents in leaf discs from the TRV-SlSRN1- and TRV-GUS-infiltrated plants were dramatically decreased after treatments with H2O2 (Fig. 7B). Notably, relative chlorophyll contents, measuring approximately 29.5% at 5 days after treatment, in leaf discs from the TRV-SlSRN1-infiltrated plants were significantly higher than that, measuring about 12.2%, from the TRV-GUS-infiltrated plants (Fig. 7B). These results indicate that silencing of SlSRN1 strengthens oxidative stress tolerance in tomato.

In drought stress assays, the appearance and wilting phenotype in the TRV-SlSRN1-infiltrated plants was less severe than that in the TRV-GUS-infiltrated plants during 7 days of experimental period (Fig. 8A). At the time starting to drought treatment, RWCs in leaves of the TRV-SlSRN1- and TRV-GUS-infiltrated plants were
were similar, measuring approximately 84–87% (Fig. 8B). At 5 days after drought treatment, RWC in leaves of the TRV-SlSRN1-infiltrated plants (~72%) was significantly higher than that in the TRV-GUS-infiltrated plants (~53%), giving an increase of 36% in RWC as compared that in the TRV-GUS-infiltrated plants (Fig. 8B). These data indicate that silencing of SlSRN1 improves drought stress tolerance in tomato. To further confirm this conclusion, we analyzed the expression of SlSRN1 during drought stress. In the whole plant assays, the expression of SlSRN1 was significantly induced by drought stress, leading to a 5-fold increase over that in the control plants (Fig. 8C). This coincided with the up-regulated expression of two previously reported tomato drought-responsive genes, SlAREB1 [79] and SGN-213276 [80] (Fig. 8C). Similar expression pattern of SlSRN1 was also observed in the detached leaf assay. The expression level of SlSRN1 in drought stress-treated leaves started to increase at 1 hr after treatment and increased gradually during an experimental period of 5 hr (Fig. 8D) whereas the expression of SlSRN1 in water-saturated leaves remained unchanged during the experimental period (Fig. 8D). These data indicate that SlSRN1 is a drought stress-responsive gene in tomato.

Discussion

Regulation of gene expression at the transcriptional level is critical to activate effective defense responses upon biotic and abiotic stresses. The NAC proteins comprise a large family of transcription factors with more than 100 members in plant species whose genomes have been completely sequenced so far. Recently, genome-wide bioinformatics analysis identified 110 SlNAC genes in potato encoding for 136 proteins, including 14 membrane-bound NAC proteins [12]. It is possible that there are similar number of NAC genes and proteins in tomato. In this regarding, only a few of the tomato NAC proteins have been studied in detail for their biological functions, e.g. SINAC4 in fruit ripening and carotenoid accumulation [68], SINAM2 in flower-budary morphogenesis [67], SINAC1 in chilling tolerance [66] and in enhancing viral replication via interaction with replication accessory protein [70], GOBLET in determining leaflet boundaries of compound leaves [63]. The present study identified a pathogen-responsive tomato NAC gene SlSRN1 and demonstrated that SlSRN1 positively regulates defense response against biotic stress but negatively regulates tolerance to oxidative and drought stresses. Our findings from functional characterization of SlSRN1 expand the list of NAC proteins with known biological functions in the tomato NAC family.

The SlSRN1 protein contains a typical NAC domain at N-terminal and a putative transmembrane motif at C-terminal (Fig. 1A) and shows the highest levels of identity to NbNPT2 and potato StNTP2 [12] and Arabidopsis ANAC017 [77], ANAC016 [78] and ANAC013 [58]. The presence of a transmembrane motif and high level of sequence identity to recently functionally characterized NAC proteins suggest that SlSRN1 belongs to the membrane-bound transcription factors [81]. The ANAC017, NbNTP2 and StNTP2 were shown to target endoplasmic reticulum membrane [42,77]. Similarly, we also observed that the SlSRN1 was targeted to the plasma membrane when transiently expressed in onion epidermal cells (Fig. 2A). In our study, the SlSRN1 has transactivation activity in yeast cells (Fig. 2B), indicating that SlSRN1 is a transcriptional activator. Similarly, the ANAC013, ANAC016 and ANAC017, three closest homologs of SlSRN1 in Arabidopsis, were demonstrated to bind to cis-elements in promoters of their downstream target genes and initiate the expression of these targeting genes [58,77,78]. Recently, it was shown that the StNTP2 protein was released from the ER membrane after treatment with P. infestans culture filtrate, which can induce rapidly the expression of StNPT2, and accumulated in the nucleus [42]. Thus, it is likely that SlSRN1 and its homologs like StNTP2 have a dynamic subcellular localization to exert their biochemical function upon different environmental stress signals. Further identification of the SlSRN1-regulated target genes will provide direct evidence suggesting the mechanism of SlSRN1 in regulation of downstream gene expression.

Our VIGS-based experimental results demonstrate that SlSRN1 is a positive regulator of defense response against biotic stress including B. cinerea and Pst DC3000. Expression of SlSRN1 was dramatically induced by B. cinerea and Pst DC3000 within the early stage of infection (12–72 hr after inoculation), indicating that SlSRN1 is an early pathogen-responsive gene (Fig. 3). This is similar to the expression pattern of StNTP2 in response to treatment with culture filtrate of P. infestans, in which rapid induction of StNTP2 expression was observed at 3 hr after treatment [42]. Another, expression of SlSRN1 was induced significantly by SA, JA and ACC, three well-known defense signaling hormones (Fig. 3C). Collectively, pathogen- and defense signaling hormone-inducible expression features imply that SlSRN1 is positively involved in defense response against pathogen infection. Direct evidence supporting a role for SlSRN1 in disease resistance response came from our VIGS-based experiments. In our study, we found that silencing of SlSRN1 resulted in increased severity of diseases caused by both B. cinerea and Pst DC3000 whereas transient expression of SlSRN1 led to decreased severity of disease by B. cinerea (Fig. 4 and 5). This is in line with the observations that silencing of NbNPT2, showing high level of identity to SlSRN1 (Fig. 1B), markedly increased severity of disease caused by P. infestans in N. benthamiana [42]. Therefore, we concluded that SlSRN1 is required for disease resistance against pathogens with different lifestyles such as B. cinerea and Pst DC3000, representing necrotrophic and hemibiotrophic pathogens, respectively. It is worthy to note that data from further study on the function of SlSRN1 in disease resistance against other pathogens will be helpful to understand whether SlSRN1 acts as a global regulator of disease resistance response against different types of pathogens.

ROS accumulated during B. cinerea infection has been implicated in susceptible response against necrotrophic fungi like B. cinerea [82]. Silencing of SlSRN1 increased accumulation of ROS in tomato leaves after infection with B. cinerea (Fig. 6A and B), which may be due to the changes in activity of superoxide dismutase and catalase induced by B. cinerea [83,84]. Thus, it is likely that silencing of SlSRN1 promotes the B. cinerea-induced accumulation of ROS and thus attenuates disease resistance to this pathogen. On the other hand, expression of SlPR1a and SlPR1b was induced significantly but expression of SlPR5 and StTPK1b was reduced markedly in SlSRN1-silenced plants after infection of B. cinerea (Fig. 6C). The PRI gene is mainly regulated through SA-mediated signaling pathway against biotrophic pathogens [83]. The unregulated expression of SlPR1a and SlPR1b after infection of B. cinerea, similar to our previous observation that silencing of SlMPK4 led to increased expression of SlPR1a [86], was observed in some mutants (e.g. Arabidopsis atw2/y33) with reduced disease resistance to B. cinerea [87]. The reduced expression of StTPK1b (Fig. 6C), a regulator of the ethylene (ET)-dependent signaling pathway [72], indicates that silencing of SlSRN1 attenuates the ET-dependent signaling pathway, which is important for resistance to necrotrophic pathogens [82]. However, increased disease caused by Pst DC3000 in SlSRN1-silenced plants and induction of SlSRN1 expression by SA and JA might indicate that SlSRN1 also
functions in SA- and/or JA signaling pathways. Thus, it is possible that SlSRN1 positively regulates defense response against different types of pathogens probably through the SA- and JA/ET-mediating signaling pathways. Further study is required to elucidate the molecular mechanism by which SlSRN1 regulates the signaling pathways involved in activation of defense responses against different pathogens.

The involvement of NAC proteins in abiotic stress response has been well documented (for reviews, see [4,15,19,20]). We found in this study that silencing of SlSRN1 resulted in increased tolerance to oxidative and drought stress (Fig. 7 and 8), indicating that SlSRN1 is a negative regulator of tolerance to oxidative and drought stress. The function of SlSRN1 in oxidative stress tolerance is similar to ANAC016 but contrary to ANAC013 [58,78]. It was shown that mutation in ANAC016 resulted in increased tolerance to oxidative stresses [78], whereas overexpression of ANAC013 increased tolerance to oxidative stress [58]. Previous studies have demonstrated that some membrane-bound NAC proteins including ANAC013 and ANAC017 functions in mitochondrial retrograde regulation (MRR) of the oxidative stress [58,77], ROS is thought to be one of the candidate signaling molecules for MRR [58]. The observation that the SlSRN1-silenced plants accumulated increased levels of ROS especially H₂O₂ after infection by B. cinerea (Fig. 6A and 6B) may indicate that silencing of SlSRN1 potentiate the ability of ROS production upon environmental stress signals. In this regard, the increased level of ROS in the SlSRN1-silenced plants during the oxidative stress may signal to initiate MRR and trigger oxidative stress tolerance. It was found that ANAC013 and ANAC017 mediate MRR-induced expression of a set of so-called mitochondrial dysfunction stimolus genes such as ALTERNATIVE OXIDASE1a and thus trigger increased oxidative stress tolerance [58,77]. Furthermore, we found that the leaf discs from the SlSRN1-silenced plants had relatively higher level of chlorophyll content than that in the control plants after treatment in H₂O₂ (Fig. 7B).

This is similar to the observation that mutations in ANAC016 resulted in delayed leaf senescence (i.e. stay green phenotype) under H₂O₂ stress [78]. On the other hand, the function of SlSRN1 in drought stress tolerance as a negative regulator is also opposite to ANAC017, which was shown to be a positive regulator of drought stress tolerance [77]. The difference between SlSRN1 and ANAC017 for their functions in drought stress tolerance might be partially due to different expression patterns and functional diversity of the membrane-bound NAC proteins in different plant species. Expression of ANAC017 was not induced by stress but mutation in ANAC017 [77], whereas the expression of SlSRN1 was induced by drought stress (Fig. 8C and 8D). Although the membrane-bound NAC proteins constitute a specific small group of the NAC family and show a high level of sequence similarity, they seem to have diverse functions in abiotic stress response. For example, ANAC013 and ANAC017 in Arabidopsis have opposite functions in oxidative stress tolerance [58,77]. Further detailed analysis of gene expression profiling between the SlSRN1-silenced and non-silenced plants will be helpful to identify the target genes that are regulated by SlSRN1 and will provide new insights into understanding the mechanism that SlSRN1 regulates abiotic stress tolerance.

Acknowledgments

We thank Professor Bizeng Mao (Institute of Biotechnology, Zhejiang University, Hangzhou, China) for her assistance in particle bombardment assays for analysis of SlSRN1 small RNA localization.

Author Contributions

Conceived and designed the experiments: FS BL. Performed the experiments: BL ZO YZ XL YH LH SL. Analyzed the data: FS BL DL. Contributed reagents/materials/analysis tools: DL HZ. Wrote the paper: FS.

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