Host Alternative NADH:Ubiquinone Oxidoreductase Serves as a Susceptibility Factor to Promote Pathogenesis of *Rhizoctonia solani* in Plants

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ABSTRACT

Phytopathogens have evolved mechanisms to utilize host genes (commonly known as susceptibility factors) to promote their pathogenesis. *Rhizoctonia solani* is a highly destructive fungal pathogen of various plants, including rice. We previously reported rice genes that were differentially regulated during *R. solani* pathogenesis. In this study, we analyzed the role of tomato homologs of two rice genes, isoflavone reductase (*IFR*) and alternative NADH:ubiquinone oxidoreductase (*NUOR*), as potential susceptibility factors for *R. solani*. Virus-induced gene silencing of *NUOR* in tomato resulted in compromised susceptibility against *R. solani*, whereas

IFR-silenced plants demonstrated susceptibility similar to that of control plants. *NUOR* silencing in tomato led to homogenous accumulation of reactive oxygen species (optimum range) upon *R. solani* infection. In addition, the expression and enzyme activity of some host defense and antioxidant genes was enhanced, whereas H₂O₂ content, lipid peroxidation, and electrolyte leakage were reduced in *NUOR*-silenced plants. Similarly, transient silencing of *OsNUOR* provided tolerance against *R. solani* infection in rice. Overall, the data presented in this study suggest that *NUOR* serves as a host susceptibility factor to promote *R. solani* pathogenesis.

Rhizoctonia solani is a soilborne necrotrophic fungal pathogen that causes catastrophic diseases in numerous plant species (Anderson et al. 2017; Ghosh et al. 2014; Pan et al. 1999; Zou et al. 2000). Extensive polymorphism in different strains enables *R. solani* to adopt diverse mechanisms to infect various hosts (Ghosh et al. 2019; Kuramae et al. 2003; Xia et al. 2017). *R. solani* accounts for great loss in crop yield and thereby poses a significant threat to socio-economic development. The practical solution to control this pathogen relies mainly on cultural practices and adoption of fungicides. Because of its complex lifestyle, coenocytic nature and despite significant efforts, understanding of *R. solani* remains elusive. Hence, developing resistance against this pathogen is a challenging task (Channamallikarjuna et al. 2009; Srinivasachary et al. 2011).

Plants have evolved strong defense responses in order to develop resistance against phytopathogens (Jones and Dangl 2006). However, the continuing arms race between plants and pathogens has led to the evolution of some pathogens that overcome plant defense responses (Engelhardt et al. 2018). Manipulation of host cell physiology has been used as a strategy by phytopathogens to promote susceptibility (Dobón et al. 2015; Schnepf et al. 2018). The host genes that promote disease susceptibility are referred to as susceptibility genes (S-genes) or factors. However, knowledge about how S-genes facilitate pathogen growth and infectivity is still

limited. One of the well-known examples of S-genes includes *Mildew resistance locus o* (*Mlo*) in barley. Loss-of-function mutation or RNAi-mediated silencing of *Mlo* genes provides broad-spectrum resistance against powdery mildew fungi in a wide range of plant species (Kusch and Panstruga 2017). Host gene expression profiling has been used as an effective method for identification of S-genes in plants (Hok et al. 2011; Santos et al. 2017). Downregulation of S-genes could play a pivotal role in compromising susceptibility against pathogens and thereby enhancing plant disease resistance.

We previously reported rice genes that were differentially regulated upon *R. solani* infection (Ghosh et al. 2017). Based on the contrasting expression dynamics of these genes in susceptible (PB1 and TP309) and partially resistant (TETEP) rice genotypes, we selected isoflavone reductase (*IFR*) and alternative NADH:ubiquinone oxidoreductase (an alternative oxidoreductase, abbreviated here as *NUOR*) as putative S-genes in this study. *IFR* is a crucial enzyme for isoflavonoid phytoalexin biosynthesis in plants and plays a major role in several biotic and abiotic stresses (Cheng et al. 2015; Kim et al. 2010). *NUORs* are mitochondrial components (single subunit enzymes) in plants involved in the electron transport chain (ETC) pathway. This gene also regulates metabolic homeostasis and gene expression for controlling various biotic and abiotic stresses (Kerscher 2000; Saha et al. 2016; Schertl and Braun 2014). Reports show the importance of mitochondrial components in controlling reactive oxygen species (ROS) accumulation during stress responses in *Arabidopsis* and tobacco (Cvetkovska et al. 2014; Moller 2001; Wu et al. 2015).

To validate the role of *IFR* and *NUOR* as putative S-genes, we adopted a gene silencing approach. Our data suggest that silencing of the *NUOR* gene compromises the pathogenesis of *R. solani* in tomato and rice. Furthermore, molecular and biochemical assays suggested that the silencing of *NUOR* could enhance host defense responses and promote homeostasis of ROS during *R. solani* infection.

MATERIALS AND METHODS

Identification and selection of putative S-genes. We previously reported rice genes that were commonly differentially regulated in two different susceptible rice genotypes (PB1 and TP309) upon *R. solani* infection (Ghosh et al. 2017). In addition, we

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analyzed the expression dynamics of these genes in *R. solani*-infected TETEP, a partially resistant rice cultivar (BioProject ID: PRJNA298635). We selected genes that were more than 2.5-fold upregulated in susceptible cultivars (PB1 and TP309) but down-regulated or not expressed in partially resistant rice (TETEP) as candidate S-genes (Supplementary Table S1). Based on expression dynamics, two genes—namely *OsIFR* (LOC_Os01g13610.1) and *OsNUOR* (LOC_Os07g37730.1)—were selected as putative S-genes in rice. Furthermore, the tomato orthologs of *IFR* and *NUOR* were identified by the BLASTx tool using the rice gene sequence as input. Induction of these genes in *R. solani*-infected plants (rice and tomato) was also validated by quantitative reverse transcription PCR (RT-qPCR).

Construction of recombinant vectors. The tobacco rattle virus plasmid (pTRV) vector was used to generate all recombinant clones for virus-induced gene silencing (VIGS) in tomato (Liu et al. 2002). Phytoene desaturase (*PDS*), which is a reporter gene responsible for carotenoid biosynthesis in plants and leads to the photobleaching phenotype in leaves upon gene silencing, was used as a positive control during the analysis (Benedito et al. 2004). For construction of recombinant pTRV2 vectors, the partial 374-bp fragment of *PDS* (accession NM_001247166.2), 569 bp of *IFR* (accession XM_004248665), and 513 bp of *NUOR* (accession XM_004232770) were PCR amplified from tomato cDNA using gene-specific primers with the *Xba*I/*Sac*I restriction enzymes (Supplementary Tables S2 and S3) and cloned in the pJET1.2 cloning vector (Thermo Scientific). Thereafter, the target gene fragments were subcloned into the pTRV2 vector at the *Xba*I/*Sac*I restriction sites in the antisense orientation. All of the vectors, pTRV1, pTRV2, and recombinant pTRV2 plasmids (pTRV2:PDS, pTRV2:IFR, and pTRV2:NUOR) were mobilized individually into *Agrobacterium* strain GV3101.

To construct the *OsNUOR* RNAi clone, the 345-bp fragment of *OsNUOR* was PCR amplified from rice cDNA and cloned in the sense and antisense orientation in a modified pGEMT vector containing an intron at the *Sph*I/*Nco*I and *Pst*I/*Nde*I restriction enzymes, respectively. In addition, the sense-intron-antisense cassette was subcloned into the pBI121 vector at the *Bam*HI/*Sac*I sites to generate a hairpin RNAi clone (pBI121:hp-*OsNUOR*). The constructs were mobilized into *Agrobacterium* strain LBA4404 for further study.

Gene silencing in tomato. Tomato seedlings at the four-leaf stage were used for the agroinfiltration assay. The primary culture of each *Agrobacterium* cell harboring a specific plasmid (pTRV1/pTRV2:0/pTRV2:PDS/pTRV2:IFR/pTRV2:NUOR) was grown in Luria-Bertani (LB) medium supplemented with rifampicin (50 mg/ml) and kanamycin (50 mg/ml) at 28°C in a 150-rpm incubator shaker overnight. In addition, a secondary culture was grown using 0.1% of the primary culture as inoculum and the bacterial cells were pelleted down by centrifugation at 5,000 rpm for 5 min. The bacterial pellet was resuspended in induction buffer (10 mM of $MgCl_2$, 10 mM of MES[2-(N-morpholino) ethane sulfonic acid], and 200 μ M of acetosyringone) keeping the $OD_{600} = 1.0$ and incubated at 25°C for 2 h with gentle shaking. *Agrobacterium* suspension with the specific pTRV2 plasmid (empty as well recombinants) was mixed with cells of the pTRV1 plasmid in a 1:1 ratio. Each mixture was infiltrated on the abaxial side of tomato seedlings (~10 days old) using a 1-ml needleless syringe and incubated in a growth chamber (Percival Scientific Inc.) at 25°C and 80% RH with 16-h/8-h day/night conditions (Liu et al. 2002). Each experiment was repeated three times with a minimum of 10 tomato seedlings per gene construct per repeat.

Gene silencing in rice. For transient agroinfiltration in rice, 30- to 45-day-old rice plants grown under control growth conditions (28°C; 80% RH and 16-h/8-h day/night conditions) were used. *Agrobacterium* strain LBA4404 harboring pBI121:0 (vector control) and pBI121:hp-*OsNUOR* was grown in LB medium supplemented with rifampicin (50 mg/ml) and kanamycin (50 mg/ml) at 28°C/150 rpm overnight. The *Agrobacterium* cells were pelleted and resuspended in induction buffer as described

above and inoculated in the midvein of rice leaves using a syringe, per the method described by Jha et al. (2007). Each experiment was repeated three times with four to five leaves infiltrated per construct and a minimum of four to five plants per construct per repeat.

Semiquantitative PCR and qPCR for gene expression studies. Total RNA was isolated from 200 mg of harvested leaf tissues using the RNeasy Plant Mini Kit (Qiagen) per the manufacturer's instructions and was quantified using a NanoDrop spectrophotometer (Thermo Scientific). Two micrograms of RNA was used for cDNA synthesis using a high-capacity cDNA synthesis kit (Applied Biosystems). Transcript accumulation of target genes was estimated by semiquantitative PCR (RT-PCR) using 2 μ l of 4-fold diluted cDNA as a template. DyNAzyme II Taq polymerase (Thermo Scientific) was used for RT-PCR with initial denaturation at 95°C for 3 min, 28 cycles at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The tomato actin gene was utilized as an endogenous control in each case.

The transcript level of target genes was determined through RT-qPCR using gene-specific primers and PowerUp SYBR Green Master Mix (Applied Biosystems) and 2 μ l of 4-fold diluted cDNA from corresponding plant samples as the template. Actin (for tomato) and 18S (for rice) genes were used to normalize the gene expression. The relative expression of target genes in test plants was determined using the comparative D cycle threshold (C_t) method $2^{-\Delta C_t}$ and fold change in expression was calculated using the $2^{-\Delta\Delta C_t}$ method over control plants (Livak and Schmittgen 2001; Schmittgen and Livak 2008). Each experiment was repeated thrice with three technical and three biological replicates.

***R. solani* infection assay.** The equal-sized sclerotia of the *R. solani* AG1-IA strain (BRS1) were collected from potato dextrose agar plates (HiMedia) at 28°C and were used to infect rice (*Oryza sativa* L. 'PB1') and tomato (*Solanum lycopersicum* 'Pusa Ruby').

For tomato infection, each sclerotium was attached on the abaxial side of young leaves by clipping tiny strips of aluminum foil. The infected plants were incubated in growth chambers preset at 28°C and 80% RH. A minimum of 10 plants per construct with at least two leaves per plant were infected with *R. solani* per experiment and at least three independent experiments were conducted during the study. Fungal pathogenicity in infected leaves was measured in terms of the extent of disease symptoms produced in each of the treated leaves. Thereafter, we developed a disease scale based on the severity of observed disease symptoms as follows: NS, no symptoms; mild, a few tiny necrotic spots; medium, fusion of tiny necrotic spots into large spots; and severe, fusion of many large necrotic spots and cell death. The estimated relative disease index in silenced and vector control plants at 3 and 7 days after *R. solani* infection was represented as a bar chart.

The sclerotium was attached inside tillers of the sheaths in order to infect rice, as described in Ghosh et al. (2017). A minimum of four to five different rice plants and four to five tillers per plant infected with *R. solani* were used during the analysis. The disease severity index was estimated by calculating the percentage of relative vertical sheath colonization (RVSC), as described by Ghosh et al. (2017). The experiment was independently repeated three times. The samples were harvested from the infected leaves/tillers and were used for detailed biochemical and molecular analysis.

Pathogen load quantification. *R. solani* biomass in infiltrated tomato plants was quantified by analyzing the abundance of its 18S rRNA using RT-qPCR. Expression was normalized using the tomato actin gene. Each experiment was repeated three times with three technical and biological replicates.

ROS accumulation (3,3'-diaminobenzidine staining). ROS accumulation was estimated by the 3,3'-diaminobenzidine (DAB) immunohistochemical staining method (Ghosh et al. 2017). *R. solani*-infected tomato leaves and rice sheaths were stained using 1 mg/ml of DAB solution (prepared in 50 mM of Tris acetate buffer, pH 5.0) and incubated in the dark at 25°C for 16 h with gentle

shaking. A mixture of ethanol/acetone (3:1 ratio) was used for destaining the leaves in a boiling water bath for 1 h, which resulted in the appearance of brown color patches in leaves/sheaths as a result of oxidation of DAB by H₂O₂. Images were captured using a digital camera (DS-Rs1; Nikon). The experiment was repeated three times with a minimum of 10 leaves/sheaths analyzed per treatment during each repeat.

H₂O₂ quantification. H₂O₂ quantification was performed in *R. solani*-infected tomato plants (agroinfiltrated with pTRV plasmids pTRV:0 and pTRV:NUOR) as described by Velikova et al. (2000). Infected leaf tissue (0.5 g) was homogenized and mixed in 2.0 ml of 0.1% (wt/vol) trichloroacetic acid (TCA) and centrifuged at 13,000 rpm for 10 min. The supernatant obtained was mixed with 1 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of potassium iodide solution. After 10 min of incubation, spectrophotometric measurement at 390 nm was carried out. The H₂O₂ concentration (in nanomoles per gram of fresh weight) was determined using a standard curve. The experiment was repeated three times with a minimum of 10 leaves analyzed per treatment during each repeat.

Lipid peroxidation (malondialdehyde) assay. A previously reported protocol for measuring malondialdehyde (MDA) content, a marker of lipid peroxidation, was followed (Hodges et al. 1999) with a few modifications. The emerging leaf samples from tomato plants (200 mg) were harvested, homogenized into a fine powder using a mortar and pestle, and mixed with 4 ml of 0.1% TCA. After vortexing and centrifugation at 8,000 × g for 15 min, 1 ml of the supernatant was mixed with 2 ml of the solution of 20% TCA and 0.5% 2-thiobarbituric acid. The mixture was heated at 95°C for 30 min, followed by cooling on ice, and absorbance was recorded at 450, 532, and 600 nm using a spectrophotometer (BMG Labtech). The MDA concentration was calculated using the following formula: MDA concentration (in micromoles/liter) = 6.45 × (OD₅₃₂ – OD₆₀₀) – 0.56 × OD₄₅₀. The experiment was repeated three times with a minimum of 10 leaves analyzed per treatment during each repeat.

Electrolyte leakage assay. To determine electrolyte leakage in tomato leaves, we followed a previously reported method (Kang et al. 2009) with slight modifications. Leaf discs (5 mm in diameter) from *R. solani*-infected pTRV:0 and pTRV:NUOR infiltrated plants were collected and incubated in 15 ml of deionized water for 4 h in a glass test tube at 25°C (for diffusion of solutes in water). The conductivity of each sample at 1, 2, and 3 days postinoculation (dpi) was recorded using a conductivity meter (LABMAN Scientific Instruments Pvt. Ltd.). To estimate total electrolyte leakage, samples were autoclaved at 121°C for 15 min and conductivity was again recorded after all tubes were cooled to 25°C. Electrolyte leakage was measured using the following formula: Percentage electrolyte leakage = (Conductivity of initial electrolyte leakage / conductivity of total electrolyte leakage) × 100. The experiment was repeated three times with a minimum of 10 leaves analyzed per treatment during each repeat.

Antioxidant activity assays. The following enzymatic assays were performed to determine the antioxidant activity in pTRV infiltrated tomato plants.

Catalase activity. To estimate catalase (CAT) activity, 0.5 g of leaf sample was homogenized with 2.0 ml of extraction buffer (100 mM of potassium phosphate buffer, pH 7.0, 1.0% polyvinyl pyrrolidone [PVP], and 0.5 mM EDTA). Upon centrifugation at 13,000 rpm for 10 min at 4°C, 0.2 ml of the supernatant (enzyme extract) was mixed with 100 mM of phosphate buffer (pH 7.0) and 12.5 mM of H₂O₂ in 2 ml of reaction solution. The change in absorbance (A₂₄₀) was monitored spectrophotometrically for 2 min compared with the control. Enzyme activity was calculated as micromoles of H₂O₂ per milligram of protein per minute by measuring the decrease in absorbance attributable to H₂O₂ dissociation using the coefficient of absorbance, ξ = 39.4/mM/cm (Azevedo et al. 1998).

Superoxide dismutase activity. Plant leaf samples (0.2 g) were homogenized in 1 ml of 100 mM phosphate buffer (pH 7.0) in order to estimate superoxide dismutase (SOD) activity. Upon centrifugation at 13,000 rpm for 10 min, the supernatant was collected and mixed with 2 ml of a reaction solution (100 mM of potassium phosphate, pH 7.8, 75 μ M of nitroblue tetrazolium, 20 mM of methionine, 50 μ M of riboflavin, and 0.1 mM of EDTA). The reaction mixture was incubated at 28°C for 10 min. Absorbance at 560 nm was measured using a spectrophotometer to determine SOD activity denoted as units per milligram of protein (Giannopolitis and Ries 1977).

Ascorbate peroxidase activity. To estimate ascorbate peroxidase (APX) activity, 0.2 g of leaf sample from tomato plants was homogenized in 1 ml of extraction buffer (100 mM of potassium phosphate buffer, pH 7.0, 1.0% PVP, and 0.5 mM of EDTA). Upon centrifugation at 13,000 rpm for 10 min at 4°C, 0.2 ml of the supernatant (enzyme extract) of each sample was added to the reaction solution (100 mM of potassium phosphate buffer, pH 7.0, 1.0 mM of ascorbic acid, and 0.25 mM of H₂O₂). The reaction mixture was incubated at 28°C for 10 min and absorbance was measured at 290 nm. APX activity was calculated using the molar extinction coefficient, ξ = 2.8/mM/cm, and unit activity was expressed as micromoles of ascorbic acid oxidized per milligram of protein per minute (Nakano and Asada 1981).

RESULTS

Selecting putative S-genes from interaction transcriptome data. RNA sequencing analysis of *R. solani*-infected rice leaves revealed that several genes were commonly upregulated in two different susceptible rice cultivars (PB1 and TP309) during *R. solani* pathogenesis (Ghosh et al. 2017). Among these genes, we observed some to be highly upregulated (>2.5-fold) in susceptible cultivars but downregulated and/or minimally expressed in the partially tolerant cultivar, TETEP. We selected two such genes (*OsIFR* and *OsNUOR*) as potential S-genes for further characterization. Using RT-qPCR, we observed that *OsIFR* and *OsNUOR* (Fig. 1A and B) were significantly upregulated during *R. solani* infection in PB1. However, expression of these genes was significantly downregulated in *R. solani*-infected TETEP at 3 dpi (Supplementary Fig. S1).

Generally, the transient gene silencing approach has its own limitations (e.g., gene silencing efficiency, duration of silencing) in monocots (Scofield and Nelson 2009). However, the VIGS approach has been widely used to silence genes in various plants, including tomato (Senthil-Kumar and Mysore 2011, 2014). Previous studies showed that *R. solani* strains belonging to different anastomosis groups can naturally infect and establish disease in tomato (Misawa and Kuninaga 2010; Taheri and Tarighi 2012). In this study, we observed that the *R. solani* AG1-IA strain (BRS1) infects and causes disease in tomato under laboratory as well as greenhouse conditions. Thus, we employed *Tobacco rattle virus* (TRV)-mediated VIGS for functional characterization of putative S-genes in tomato. The tomato orthologs of *OsIFR* (accession XP_004248713.1; identity: 40%) and *OsNUOR* (accession XP_004232818.1; identity: 76%) were identified using the BLASTx tool. Using RT-qPCR, we observed both *IFR* and *NUOR* (Fig. 1C and D) to be upregulated upon *R. solani* infection in tomato.

VIGS-mediated silencing of putative S-genes in tomato. TRV-VIGS-mediated silencing was carried out to characterize *IFR* and *NUOR* as potential S-genes against *R. solani*. The tomato seedlings were agroinfiltrated with pTRV:0 (vector control), pTRV:PDS (positive control), pTRV:IFR, and pTRV:NUOR. The photo-bleaching phenotype in emerging leaves of pTRV:PDS infiltrated plants indicated silencing of the *PDS* gene. On the other hand, no visual silencing phenotype was observed in the case of pTRV:0, pTRV:IFR, and pTRV:NUOR infiltrated plants (Fig. 2A). Furthermore, semiquantitative PCR and RT-qPCR analysis (Fig. 2B and C)

confirmed a significant decrease in the transcript level of targeted endogenous genes (*PDS*, *IFR*, and *NUOR*) in the respective VIGS plants compared with the empty vector (pTRV:0) infiltrated plants.

***R. solani* pathogenesis in silenced tomato.** We analyzed whether silencing of *IFR* and *NUOR* compromises the pathogenesis of *R. solani* in tomato. *R. solani*-infected leaves showed severe necrotic lesions in the pTRV:0 and pTRV:IFR infiltrated plants at 3 dpi, whereas minute necrotic lesions were observed in the infected leaves of pTRV:NUOR plants (Fig. 3A). By 7 dpi, the infection proceeded to other healthy leaves in the twigs of pTRV:0 and pTRV:IFR infiltrated plants. However, only the inoculated leaves showed necrotic symptoms and there was only limited spread of the disease to the other leaves in the twigs of pTRV:NUOR infiltrated plants (Fig. 3B). RT-qPCR analysis revealed down-regulated expression of *IFR* and *NUOR* in *R. solani*-infected leaves (3 dpi) of pTRV:IFR and pTRV:NUOR infiltrated plants, respectively (Fig. 3C). Because the primary infected leaves of pTRV infiltrated plants were completely necrotic and mostly degenerated at 7 dpi, we analyzed the expression of *IFR* and *NUOR* genes in the twigs. As shown in Figure 3D, we observed sustained silencing of these genes in *R. solani*-infected twigs at 7 dpi. We further monitored the abundance of *R. solani* 18S rRNA in the primary infected leaves at 3 dpi and in the twigs at 7 dpi to quantify the pathogen load. The pathogen load was significantly high in pTRV:0 and pTRV:IFR infiltrated plants, whereas pTRV:NUOR infiltrated plants exhibited a significantly reduced pathogen load at 3 and 7 dpi

(Fig. 3E and F). Overall, this suggested that *NUOR* silencing confers tolerance against *R. solani* infection in tomato. The disease index categorizing observed disease symptoms as mild, medium, and severe in pTRV:NUOR and pTRV:0 plants further supported enhanced tolerance in the *NUOR*-silenced plants (Supplementary Fig. S2).

***NUOR* silencing modulates ROS homeostasis and expression of mitochondrial genes in tomato.** *NUOR* is known to function as an additional electron entry and as an exit point in the mitochondrial ETC pathway in plants (Kerscher 2000). A recent study demonstrated that defects in the ETC pathway (mitochondrial complex I) cause induced accumulation of ROS in Arabidopsis (Soto et al. 2015). Interestingly, the emerging leaves of *NUOR*-silenced tomato plants showed enhanced ROS accumulation compared with that of pTRV:0, pTRV:PDS, and pTRV:IFR plants (Fig. 4A).

We further tested whether *NUOR* silencing modulates other components of the mitochondrial complex system, which can influence ROS homeostasis and defense. Based on previous studies (Meyer et al. 2009; Wu et al. 2015), we selected tomato orthologs of Arabidopsis genes (*NDUFA5*, *NDUFA9*, *NDUFS1*, *NDUFS4*, *NDUFS8*, *NDUFV1*, *CA2*, *SOM42*, *NAD1a*, and *NAD3*) encoding mitochondrial components to study alteration in their expression during *NUOR* silencing in tomato. Quantitative expression analysis revealed only *NDUFS1* and *NAD3* to be induced in pTRV:NUOR plants compared with vector control

Fig. 1. Expression analysis of putative susceptibility genes (S-genes) in rice and tomato. Relative expression of **A**, *OsIFR* and **B**, *OsNUOR* in a *Rhizoctonia solani*-infected rice cultivar (PB1). Relative expression of **C**, *IFR* and **D**, *NUOR* genes in tomato plants infected with *R. solani*. The expression of genes in infected samples (at different time points) was normalized with that of uninfected samples. Bars represent the mean value of three independent biological replicates. Error bars show the standard error of the mean. *Os* = *Oryza sativa*, *IFR* = isoflavone reductase and *NUOR* = alternative NADH:ubiquinone oxidoreductase, and dpi = days postinoculation.

plants, whereas expression of other mitochondrial genes was not affected (Supplementary Fig. S3). The upregulation of these genes in silenced plants may compensate for the decrease in *NUOR* activity.

***NUOR* silencing modulates ROS accumulation during pathogenesis of *R. solani* in tomato.** We also tested ROS accumulation in pTRV:*NUOR* and pTRV:0 tomato plants upon *R. solani* infection. Accumulation of localized ROS was observed around the infection site at 1 dpi in pTRV:0 infiltrated leaves. Homogenous ROS accumulation (uniform brown coloration in the whole leaf) was observed in pTRV:*NUOR* leaves (Fig. 4B). Furthermore, ROS production was enhanced at 2 to 3 dpi, leading to the appearance of localized larger brown spots in infected pTRV:0 leaves. Uniform brown coloration, along with a few small brown spots, was visible in *R. solani*-infected pTRV:*NUOR* leaves, suggesting homogenous ROS accumulation.

We also studied ROS accumulation at 7 dpi of *R. solani* infection. The primary infected leaves were mostly disintegrated in both pTRV:0 and pTRV:*NUOR* infiltrated plants, so we analyzed ROS accumulation in other leaves of the twig. Most of the leaves showed dark brown spots attributable to DAB staining in the pTRV:0 infiltrated plants, which correlated with necrotic disease lesions. However, uniform brown coloration was observed in the leaves of pTRV:*NUOR* plants, suggesting homogenous ROS accumulation (Fig. 4C). Taken together, this suggests that a preinduced ROS level in pTRV:*NUOR* plants may be associated with an induced defense response that controls *R. solani* infection.

Specific pathogenesis-related genes were induced in *NUOR*-silenced tomato upon *R. solani* infection. We dissected the expression of pathogenesis-related (PR) genes (*PR1*, *PR-P2*, *PR5*, *Nonexpressor of PR genes NP24* and *NPR1*) in pTRV:*NUOR* and pTRV:0 infiltrated tomato plants upon *R. solani* infection. Expression of *NPR1* and *PR5* was significantly upregulated in pTRV:*NUOR* plants compared with pTRV:0 plants (Fig. 5). However, there was no significant difference in the expression of *PR1*, *PR-P2*, and *NP24* in the infected pTRV:*NUOR* and pTRV:0 plants.

***NUOR* silencing alters host antioxidant expression and activity to suppress *R. solani* pathogenesis in tomato.** We further assessed the expression and enzyme activity of some of the antioxidant genes in pTRV infiltrated plants upon *R. solani* infection at 3 dpi. Expression of *CAT1*, *CAT2*, *Mn-SOD*, *APX1*, and *APX2* was upregulated in pTRV:*NUOR* infiltrated plants compared with pTRV:0 infiltrated plants (Fig. 6). Furthermore, enzyme activity of *CAT*, *SOD*, and *APX* was enhanced in pTRV:*NUOR* plants compared with pTRV:0 infiltrated plants (Fig. 7A to C). On the other hand, H_2O_2 content and MDA content (Fig. 7D and E) were relatively lower in pTRV:*NUOR* infiltrated plants compared with pTRV:0 infiltrated plants. However, only a marginal decrease in electrolyte leakage was observed in infected pTRV:*NUOR* plants compared with pTRV:0 plants (Fig. 7F).

Transient silencing of *OsNUOR* confers tolerance against *R. solani* infection in rice. To further substantiate *NUOR* as a potential S-gene, we assessed whether RNAi-mediated

Fig. 2. pTRV-based virus-induced gene silencing in tomato. **A**, Photobleaching phenotype in emerging leaves of pTRV:PDS infiltrated plants; leaves from pTRV:0, pTRV:IFR, and pTRV:*NUOR* infiltrated plants had no phenotypic alterations. **B**, Semiquantitative and **C**, quantitative real-time PCR reflecting downregulation of target genes (*PDS*, *IFR*, *NUOR*) in pTRV infiltrated plants at 20 days postinoculation. Bars represent the mean value of three independent biological replicates. Error bars show the standard error of the mean. Different letters above the bars indicate statistically significant differences at $P < 0.05$ (using one-way analysis of variance). TRV = Tobacco rattle virus, PDS = Phytoene desaturase, IFR = isoflavone reductase, and *NUOR* = alternative NADH:ubiquinone oxidoreductase.

transient silencing of *OsNUOR* provides tolerance against *R. solani* infections in rice. As shown in Supplementary Figure S4, the silenced rice plants (pBI121:hp-*OsNUOR*) showed relatively fewer disease symptoms compared with the empty vector (pBI121:0) infiltrated control plants. Furthermore, estimation of the disease index (in terms of RVSC) revealed a significant reduction in the pBI121:hp-*OsNUOR* plants compared with the pBI121:0 plants. DAB staining revealed large brown spots correlating with necrotic disease symptoms in pBI121:0 plants. However, relatively smaller brown necrotic spots were observed in pBI121:hp-*OsNUOR* plants. The homogenous induction of ROS in the uninfected pBI121:hp-*OsNUOR* plants suggested that *NUOR* silencing enhances ROS accumulation in rice. In addition, we observed enhanced expression of various antioxidant genes (*CATA*, *CATB*, *APX1*, *APX2*, and *Mn-SOD*) in pBI121-*OsNUOR* plants compared with pBI121:0 plants upon *R. solani* infection.

DISCUSSION

Plants induce strong defense responses to protect themselves from pathogens. However, pathogens have evolved strategies to overcome host defense responses. Understanding how pathogens

overcome host defense responses has long been of prime interest for plant pathologists. Studies suggest that some plant genes are hijacked by pathogens and are exploited as susceptibility factors to promote their pathogenesis (Lapin and Van den Ackerveken 2013; Mukhtar et al. 2011; Rodriguez-Moreno et al. 2018; Weßling et al. 2014). For example, silencing of S-genes enhanced resistance in transgenic potato plants against late blight disease caused by *Phytophthora infestans* (Sun et al. 2016). Similarly, silencing of grapevine susceptible genes provided resistance to powdery mildew disease (Pessina et al. 2016).

Our study provides evidence that *NUOR* (an alternative oxidoreductase) functions as a potential S-gene to promote *R. solani* infection in rice and tomato. *NUOR* functions as a mitochondrial component and is involved in the ETC pathway. It has been anticipated that downregulation of *NUOR* expression or mutation in *NUOR* may disturb the coordinated signaling cascade in the ETC pathway (Cvetkovska et al. 2014). Because mitochondrial components regulate constant ROS production, it was hypothesized that downregulation of *NUOR* expression may also modulate ROS production (Cvetkovska and Vanlerberghe 2012). A previous study revealed that silencing of alternative oxidases could enhance the production of ROS and reactive nitrogen species in tobacco plants

Fig. 3. *Rhizoctonia solani* pathogenesis in silenced tomato plants. Disease symptoms in *R. solani*-infected leaves of pTRV (pTRV:0, pTRV:IFR, and pTRV:NUOR) infiltrated plants at **A**, 3 days postinoculation (dpi) and **B**, 7 dpi. Red arrows indicate the site of *R. solani* infection. Relative transcript accumulation of *IFR* and *NUOR* using RT-qPCR in pTRV infiltrated plants at **C**, 3 dpi and **D**, 7 dpi of *R. solani* infection. RT-qPCR-based quantification of *R. solani* 18S rRNA abundance in pTRV:IFR and pTRV:NUOR plants compared with pTRV:0 infiltrated plants at **E**, 3 dpi and **F**, 7 dpi. Bars represent the mean value of three independent biological replicates. Error bars show the standard error of the mean. Different letters above the bars indicate statistically significant differences at $P < 0.05$ (using one-way analysis of variance). TRV = Tobacco rattle virus, IFR = isoflavone reductase, NUOR = alternative NADH:ubiquinone oxidoreductase, and RT-qPCR = quantitative reverse transcription PCR.

(Cvetkovska et al. 2014). In addition, benzothiadiazole-mediated inhibition of mitochondrial complex I enhanced ROS production (van der Merwe and Dubery 2006). In the present study, we observed homogeneous production of ROS being induced upon

NUOR silencing in tomato leaves. In addition, the silenced plants exhibited tolerance to *R. solani* infection, as we observed reduced disease symptoms as well as pathogen load in the infected tissues (Fig. 3).

Fig. 4. 3,3'-Diaminobenzidine staining reflecting ROS accumulation in silenced tomato plants. **A**, ROS accumulation in emerging leaves of pTRV infiltrated plants (pTRV:0, pTRV:PDS, pTRV:IFR, pTRV:NUOR) at 20 days postinoculation (dpi). **B**, ROS accumulation in leaves of pTRV:0 and pTRV:NUOR infiltrated plants infected with *Rhizoctonia solani* at 1, 2, and 3 dpi. **C**, ROS accumulation in leaves of pTRV:0 and pTRV:NUOR infiltrated tomato plants infected with *R. solani* at 7 dpi. ROS = reactive oxygen species, TRV = *Tobacco rattle virus*, PDS = phytoene desaturase, IFR = isoflavone reductase, and NUOR = alternative NADH:ubiquinone oxidoreductase.

Fig. 5. Expression analysis of different pathogenesis-related (*PR*) genes in pTRV infiltrated tomato plants upon *Rhizoctonia solani* infections at 3 days post-inoculation. Bars represent the mean value of three independent biological replicates. Error bars show the standard error of the mean. **P* < 0.05 and ***P* < 0.01 (statistically significant differences using the Student *t* test). TRV = *Tobacco rattle virus*, NUOR = alternative NADH:ubiquinone oxidoreductase, and NP = nonexpressor of PR genes.

R. solani infection leads to enhanced ROS production, which is helpful for the pathogen to cause necrosis in the infected plants (Ghosh et al. 2017; Torres et al. 2006). Similarly, we observed enhanced ROS accumulation localized surrounding the disease lesion in the infected

pTRV:0 plants (Fig. 5). However, ROS accumulation remained homogeneous upon *R. solani* infection in pTRV:NUOR plants. The antioxidant enzymes such as *CAT*, *SOD*, and *APX* were also induced in *R. solani*-infected pTRV:NUOR plants compared with pTRV:0 plants.

Fig. 6. Expression analysis of antioxidant genes in pTRV infiltrated tomato plants upon *Rhizoctonia solani* infection at 3 days postinoculation. Bars represent the mean value of three independent biological replicates. Error bars show the standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ (statistically significant differences using the Student *t* test). TRV = *Tobacco rattle virus*, NUOR = alternative NADH:ubiquinone oxidoreductase, CAT = catalase, SOD = superoxide dismutase, and APX = ascorbate peroxidase.

Fig. 7. NUOR-silenced tomato plants demonstrate higher proficiency in managing oxidative stress during *Rhizoctonia solani* infection. **A, B, and C**, Antioxidant enzyme (*CAT*, *SOD*, *APX*) activity at 3 days postinoculation (dpi), **D**, H_2O_2 content at 3 dpi, **E**, MDA content at 3 dpi, and **F**, electrolyte leakage at 1, 2, and 3 dpi in *R. solani*-infected tomato plants. Bars represent the mean value of three independent biological replicates. Error bars show the standard error of the mean. * $P < 0.05$ and ** $P < 0.01$ (statistically significant differences using the Student *t* test). CAT = catalase, TRV = *Tobacco rattle virus*, NUOR = alternative NADH:ubiquinone oxidoreductase, SOD = superoxide dismutase, APX = ascorbate peroxidase, and MDA = malondialdehyde.

Overall, this suggested that antioxidant machinery is induced upon *NUOR* silencing, which may prevent the pathogen-induced ROS burst. This tempts us to speculate that *NUOR* silencing induces an optimal level of ROS, which in turn may be helpful for inducing defense responses. Previous reports suggested that an increase in ROS level is associated with an enhanced defense response that serves as a barrier for fungal infection (Ali et al. 2018; Cvetkovska et al. 2014; Lamb and Dixon 1997; Tripathy and Oelmüller 2012). A decrease in H₂O₂ content, MDA content, and electrolyte leakage in *R. solani*-infected *NUOR*-silenced plants was quite noteworthy in this study. Previous studies suggested that decreases in H₂O₂ content, MDA content, and electrolyte leakage are associated with an induced defense response upon pathogen attack (Bundó and Coca 2017; Liu et al. 2013). Taken together, this suggested that the optimal level of ROS in *NUOR*-silenced plants potentially induces a defense response during *R. solani* infection. This was further supported by our observation that two host defense marker genes, *NPR1* and *PR5* (Ali et al. 2017; El-kereamy et al. 2011; Šestacova et al. 2016), were upregulated in *NUOR*-silenced plants upon *R. solani* infection.

Although *R. solani* is a necrotrophic pathogen, it is speculated that *R. solani* adopts a brief biotrophic phase before entering into the necrotrophic phase for nutrient acquisition and pathogenesis (Ghosh et al. 2017). Therefore, it appears that the optimal ROS-induced defense response in *NUOR*-silenced plants may suppress *R. solani* colonization in the biotrophic phase, and thereby delays its transition to the necrotrophic phase. Because *NUOR* is widely conserved in plants, the ability to manipulate its expression may serve as one of the reasons for the widespread success of *R. solani* as a phytopathogen. However, how *R. solani* manipulates host *NUOR* during pathogenesis remains to be elucidated in future research.

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