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Nitroso Glutathione Reductase GSNOR1 for Positive Regulation of Plant Resistance against *Phytophthora*

LIU Xingshao[1], SONG Siyu[2], GAO Xianxian[1], SHAN Weixing[1] and QIANG Xiaoyu[1]

(1. College of Agronomy, Northwest A&F University, Yangling Shaanxi 712100, China;
2. College of Plant Protection, Northwest A&F University, Yangling Shaanxi 712100, China)

Abstract Oomycetes, especially *Phytophthora*, cause devastating crop diseases such as potato late blight caused by *P. infestans*. To conquer the disease, it is of great significance to explore the plant resistance mechanism and identify novel factors for resistance breeding. Nitroso glutathione reductase 1(GSNOR1) is a highly conserved reductase in plant nitric oxide signaling, which involves in regulating *R*-gene mediated resistance and non-host resistance. However, its potential function and mechanism in resistance against *Phytophthora* remain unclear. In this study, the resistance phenotypic analysis on *Arabidopsis* T-DNA insertion mutant *atgsnor1-3* was used to determine the positive regulation of *AtGSNOR1* in resistance to *P. parasitica*. Furthermore, by exploiting tobacco rattle virus (TRV) for virus-induced gene silencing (VIGS), the *Nicotiana benthamiana* plants with reduced *GSNOR1* homolog levels was used, we demonstrated that silencing of *GSNOR1* homolog in *N. benthamiana* enhances plant susceptibility, accompanied with the suppression of reactive oxygen species(ROS) production, induction of *PR* genes and *MAPK* signaling upon infection by *P. parasitica*. This study revealed the highly conserved function and mechanism of *GSNOR1* in plant resistance against *Phytophthora*, which facilitated further exploration and potential application of *GSNOR1* inbreeding of potatolate blight disease resistance.

Key words GSNOR; *Phytophthora parasitica*; *Nicotiana benthamiana*; *Arabidopsis*; Potato late blight

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Phytophthora, a genus of oomycetes, consists of more than 120 species which cause destructive diseases on agricultural crops, forests and natural ecosystems. For instance, the potato late blight caused by *P. infestans* and tobacco black shank caused by *P. parasitica* threaten the sustainable crop production worldwide[1-2]. Since the virulence variation of *Phytophthora* is rapid, the loss of genotype-specific plant resistance is a key problem in crop production. Take the potato late blight as a typical example, the disease control is highly dependent on pesticides, the outcome of which is the environmental and ecological pollution. Therefore, to identify novel fac-

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First author LIU Xingshao, female, master student. Research area: molecular mechanism of plant disease resistance. E-mail: liuxingshao@nwafu.edu.cn
Corresponding author QIANG Xiaoyu, female, associate professor, master supervisor. Research area: plant disease resistance and plant-microbiota interactions. E-mail: qiangxiaoyu@nwafu.edu.cn

tors those confer durable resistance and decipher their resistance mechanisms are of great importance particularly in accelerating resistance breeding in *Solanaceous* plants and contributing to sustainable agriculture. *P. parasitica*, a model oomycete, is a typical hemibiotrophic pathogen, which has a wide range of hosts[2]. The interacting model systems of *P. parasitica* with *Arabidopsis* as well as *N. benthamiana* provide a powerful tool to establish these mechanistic studies[3].

In natural environment, to avoid pathogenic infection, plants have evolved two levels of innate immune responses against pathogens[4]. The first level is triggered by pathogens-associated molecular pattern (PAMP), which is termed as PAMP-triggered immunity (PTI). The immune response is activated through recognition of PAMPs by plant plasma membrane-localized pattern recognition receptors (PRRs), which typically produce reactive oxygen and nitrogen intermediates (ROIs and RNIs, respectively) and induction of mitogen-activated protein kinase (MAPK) signaling cascade[5]. The second is triggered by pathogen effectors, which is termed as effector-triggered immunity (ETI). The immune response is activated through recognition of effectors by nucleotide binding-leucine rich repeat (NB-LRR) proteins encoded by plant resistance genes (*R* genes)[6].

In the course of evolutionary changes during plant-pathogen interactions, pathogens have developed various invasion strategies, to which the host plant responded with rapid and marked changes in cellular redox status[7]. These changes are mostly triggered by the accumulation of ROIs and RNIs, which are significant features of the plant defense response. Although the enzymes required for apoplastic ROI synthesis are well established[8], the source of RNIs remains elusive[9-10]. Increasing evidence suggests that the redox active, small molecules integrate various plant defense mechanisms. For instance, the signaling function of nitric oxide (NO) is shown during both highly conserved PTI and highly specific ETI, which typically accompanied by the hypersensitive response (HR) at the site of infection[11].

As an endogenous signaling molecule, NO is such kind of free radical gas that can diffuse rapidly through biological membranes[12]. To constitute a relatively stable store of NO bioactivity, S-nitroso glutathione (GSNO) is formed by the covalent attachment of NO to the cysthiol within the antioxidant tripeptide, glutathione (GSH)[13-14]. The addition of an NO moiety to a protein cysteine (Cys) thiol to form an S-nitrosothiol (SNO) has emerged as a prototypic redox-based, post-translational modification, which is termed as S-nitrosylation[15-17]. Cellular GSNO homeostasis is controlled by the enzyme GSNO reductase (GSNOR). This enzyme functions highly conserved among animals, plants and bacteria[18]. *A. thaliana* S-nitroso glutathione reductase 1 (AtGSNOR1), was identified to modulate the extent of cellular SNO formation following nitrosative stress, and alterations in total SNO levels strongly affect signaling transduction and modulate multiple modes of plant immunity[17,19-21].

The phenolic metabolite salicylic acid (SA) is a crucial immune activator in plants. Emerging evidence suggests that the absence of GSNOR1 activity increases cellular GSNO levels and leads to significantly reduced SA accumulation and weak activation of SA-dependent defense genes[19]. *Arabidopsis* plants with compromised GSNOR1 function were shown to be disabled in *R* gene-mediated protection mediated by either the Toll interleukin (TOLL) or coiled-coiled class of NB-LRR proteins, which posse different signaling requirements[22]. Moreover, *atgsnor1-3* plants are disabled in basal resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and the oomycete, *Hyaloperonospora arabidopsidis*. Non-host resistance (NHR) can protect plants against the majority of potential pathogens. However, the underly-

ing molecular mechanisms of NHR are less well understood. Notably, *atgsnor1-3* plants enable the growth of the wheat powdery mildew pathogen, *Blumeria graminis* f. sp. *tritici*, which is a major wheat pathogen but is not adapted for growth on *Arabidopsis*. Besides, *atgsnor1-3* plants are also host for *Pseudomonas syringae* pv. *phaseolicola* and *Pseudomonas fluorescens*; while wild-type *Arabidopsis* plants do not support the growth of these non-adapted bacterial pathogens. Thus, GSNOR1 also regulates the development of non-host resistance (NHR) against both fungaland bacterial pathogens[19]. Collectively, these data imply that GSNOR1 is involved in modulating multiple modes of plant disease resistance.

As a crucial and highly conserved reductase in plant NO signaling, the potential function of GSNOR in plant resistance against *Phytophthora* remains unexplored. In this study, with the benefit of interacting model systems of *P. parasitica* with *Arabidopsis* as well as *N. benthamiana*, we performed set of analyses on the resistance function of *AtGSNOR1* and the *GSNOR1* homolog in *N. benthamiana* against *P. parasitica*. We demonstrate that plant *GSNOR1* might have conserved function in plant resistance against *Phytophthora* through positively regulating ROS production, induction of *PR* genes, and *MAPK* cascade signaling at different stages of attempted infection. This study reveals the conserved mechanism of *GSNOR1* in plant resistance to *Phytophthora*, which facilitate further exploration and potential application of *GSNOR1* on potato late blight disease resistance breeding.

1 Materials and Methods

1.1 Materials

Arabidopsis T-DNA insertion mutants in Col-0 background, *atgsnor1-3* (GABI_315D11) was obtained from the *Arabidopsis* Biological Resource Center (ABRC). *N. benthamiana* was obtained from State Key Laboratory of Crop Stress Biology for Arid Areas of Northwest A&F University.

LB culture medium (500 mL): Trptone 5 g, yeast extract 2.5 g, NaCl 5 g, Agar 4.0 g (Liquid medium without Agar), add to 500 mL with ddH_2O.

5% CA culture medium (500 mL): 5% carrot juice, β-sitosterol 0.02 g, $CaCO_3$ 0.3 g, Agar 4.0 g (Liquid medium without Agar), add to 500 mL with ddH_2O.

Fast pfu DNA polymerase, Easy *Taq* DNA Polymerase, T_4 DNA Ligase, restriction endonuclease (promega): *Eco*R Ⅰ, *Xho* Ⅰ, Antibiotics: Kanamycin, Gentamicin, Rifampin.

1.2 Methods

1.2.1 Plant Materials and Growth Condition For the root inoculation assay, *A. thaliana* seeds were surface-sterilized before being plantedon 1/2 MS medium at 4 ℃ for 3 days and then transferred the seeds to 23 ℃ for about 10 days before infection assays. For other experiments, *A. thaliana* and *N. benthamiana* seeds were surface-sterilized and transferred to soil for about one month. *A. thaliana* and *N. benthamiana* were grown in the same conditions as previously described[23].

1.2.2 Plasmid Constructs To create TRV-based virus induced gene silencing (VIGS) constructs, the full length cDNA sequences of *AtGSNOR1* (AT5G43940) were firstly acquired from TAIR homepage (The *Arabidopsis* Information Resource, https://www.arabidopsis.org/). Thereafter, through BLAST tool from Solanaceae Genomics Network (https://solgenomics.net/tools/blast/), the full length cDNA sequences of *GSNOR1* homolog in *N. benthamiana* were obtained. The sequence of *GSNOR1* homolog was cloned from *N. benthamiana* cDNA and inserted into tobacco rattle virus (TRV) TRV2 vector with *Eco*R Ⅰ and *Xho* Ⅰ sites[24-25]. The coding sequence of *NbGSNOR1* was cloned from *N. benthamiana* cDNA using gene-specific primers (Table 1).

1.2.3 *P. parasitica* Inoculation Assay The

culture and zoospore production of *P. parasitica* strain *Pp016* were conducted as previously described[3]. For the leaf inoculation assay, detached leaves were inoculated on the abaxial leaf surface with a 10 μL droplet containing—200 *P. parasitica* zoospores μL^{-1}. Hyphae in epidermal cells were observed at 48 hpi with fluorescence microscope and *P. parasitica* infected leaf discs were measured at 72 hpi. For the root inoculation assay, 5-day-old *A. thaliana* seedlings grown in 1/2MS medium were inoculated with *P. parasitica*, and resistance phenotype was observed at 10 dpi. Col-0 was used as wild-type control plant in the infection assays. For the inoculation assay on *N. benthamiana* detached leaves, approximately 8 mm diameter of mycelium plugs were cut from the *P. parasitica* strain *Pp016* cultures and inoculated near the infiltrated sites. Lesion diameters were measured at 2 dpi and then stained with trypan blue.

1.2.4 TRV-based VIGS in *N. benthamiana* *Agrobacterium tumefaciens* strain GV3101 harbouring pTRV2:: *NbGSNOR*, pTRV2::*GFP* or pTRV2:: *PDS* construct was mixed with strains carrying pTRV1 vector in a 1∶1 ratio to achieve final concentration of OD_{600} = 0.25 for each component. The largest leaves of 3-week-old plants were chosen for infiltration and the silenced plants were used to inoculate with *P. parasitica* 3 weeks later as previouslydescribed[26]. Three independent experiments were performed.

1.2.5 Gene Expression Analysis Plant total RNA was extracted using TRIzol (Invitrogen) reagent. For qRT-PCR analysis, cDNA was synthesized from 1 μg of total RNA using PrimeScript™ RT reagent Kit (TaKaRa). 20 ng of cDNA was used as template for the amplification of candidate genes using SYBR premix Kit (Roche) according to the manufacturers' instructions. Quantitative analysis for the relative expression level of the tested genes was performed using the SYBR Premix Kit (Roche, Basel, Switzerland) on Q7 Real Time Cycler (Thermo Fisher Scientific). The Ct values of tested genes were normalized to *NbACTIN* in *N. benthamiana*. The primers used are listed in Table 1. Expression fold changes were calculated by the $2^{-\triangle\triangle Ct}$ method.

1.2.6 Trypan Blue Staining Assay Detached *N. benthamiana* leaves were stained with lactophenol-trypan blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol, and 10 mg trypan blue, dissolved in 10 mL of distilled water). After boiling for 2 min in the staining solution and destaining in 2.5 g/mL chloral hydrate, the leaves were mounted in 70% glycerol for microscopic observation.

1.2.7 DAB Staining Assay Detached *N. benthamiana* leaves were blocked in DAB solution (1 g/mL DAB, modulate pH to 3.0 with 0.2 mol/L HCL) supplemented with 0.05% Tween 20 and 10 mmol/L Na_2HPO_4 for 12 h. The leaves were decolorized with decolorizing solution (Ethylalcohol absolute ∶ Acetic Acid ∶ Glycerol = 3 ∶ 1 ∶ 1) and boiled in distilled H_2O for 15 minutes. Thereafter, the leaves were photographed.

1.2.8 Protein Immunoblot Assays Total protein was extracted with GTEN lysis buffer (10% glycerol, 25 mmol/L Tris pH 7.5, 1 mmol/L EDTA, 150 mmol/L NaCl) supplemented with 2% PVPP, 10 mmol/L DTT, 1× protease inhibitor cocktail (Sigma) and 0.1% Tween 20. Proteins were separated by SDS－PAGE and transferred from the gel to a PVDF membrane (Roche) in transfer buffer (25 mmol/L Tris, 200 mmol/L glycine, and 20% methanol). The membrane was then blocked in TBST buffer (Tris-buffered saline with 0.05% Tween 20 [pH 7.2]) containing 10% non-fat dry milk under gentle shaking. The blocked membrane was incubated with anti-MPK6 (Agrisera, Sweden; AS12-2633) dissolved in TBSTM (TBST with 5% non-fat dry milk) at a ratio of 1∶5 000 and incubated at 4 ℃ with shaking at 50 rpm overnight, followed by three washes (10 min each) with TBST. Next, the membrane was incubated

with a secondary antibody HRP goat anti-rabbit IgG(H + L) antibody(# AS014, ABclonal), which was also dissolved in TBST at a ratio of 1∶2 000, at room temperature for 1.5 h with shaking. Thereafter the membrane was washed three times(10 min each) with TBST and one time with TBS, then incubated with ECL(# CW0049S, ComWin) before photographing using a molecular imager(ChemiDoc XRS +, Bio-Rad).

Table 1 Primer sequences used in this study

Primer Name	Primer sequence(5′→3′)
NbGSNOR1-F	CCGGAATTCGTGGAATAAAAAGGAAGTGGAAA
NbGSNOR1-R	CCGCTCGAGTAGGGACGTTCCGTTAATATG
qNbGSNOR1-F	ACTGAGGTTCAGCCAGGAGA
qNbGSNOR1-R	CCGCCCTTACTTTACCACAA
NbACTIN-F	TCCATGCTCAATGGGATACT
NbACTIN -R	TTCAACCCCTTGTCTGTGAT
PR1b-F	TGCCTTCATTTCTTCTTG
PR1b-R	TTAGTATGGACTTTCGCCTCT
PR2-F	CTAATGGCATCAGAAAGA
PR2-R	ATTGGCTAAGAGTGGAAG
PR3-F	AAAGGGATTCTACAGTTAC
PR3-R	AGGATTGTTTAGCAGGT
PR5-F	TGAGGAGGATGAATAGA
PR5-R	AAAGCCTAACAAGTGC
MPK7-F	GAATTGATGCGCTGAGAACA
MPK7-R	GGCTGCGACGACTTAATGAT
MPK8-F	CTTCCCACACCGTCTTTAGC
MPK8-R	GTACTCGGGCTAGGGACCTC

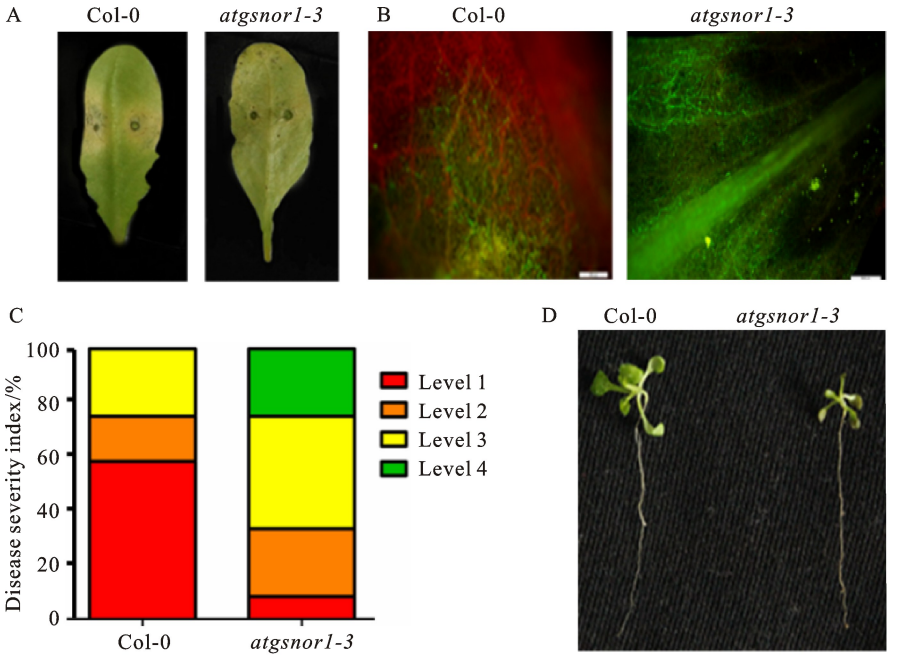
2 Results

2.1 *Atgsnor1* mutants exhibit both leaf and root susceptibility to *P. parasitica*

To explore the potential function of *GSNOR1* in plant resistance to *Phytophthora*, we first examined resistance of *atgsnor1-3* mutant against *P. parasitica*. The detached leaves of six-week-old *atgsnor1-3* mutants as well as WT Col-0 plants were inoculated by *P. parasitica* zoospores. At 72 hpi, leaves of WT Col-0 plants displayed severe water-soaked lesions, indicative of susceptibility against *P. parasitica* infection (Fig. 1-A). In comparison, the water-soaked lesions in *atgsnor1-3* mutant were expanded almost all over the whole leaf, indicating that *atgsnor1-3* mutant is more susceptible to *P. parasitica*(Fig. 1-A). Furthermore, visible GFP expressing hyphae colonized WT Col-0 leaves, following infection with stable GFP-expressing *P. parasitica* transformant. Conversely, *atgsnor1-3* mutant plant leaves showed heavier colonization(Fig. 1-B). Consistently, the disease index statistic for infected leaves indicated that *atgsnor1-3* mutant plants were obviously more susceptible than WT Col-0 plants to *P. parasitica* infection(Fig. 1-C).

To examine whether *GSNOR* functions in root resistance, 2-week-old seedlings were dip inoculated with *P. parasitica* zoospores and incubated for 10 days. Most WT Col-0 seedlings were infected and colonized with *P. parasitica* at 10 dpi, whereas much heavier infection and root wilt symptom were observed in *atgsnor 1-3* mutant seedlings(Fig. 1-D). Taken together, these results implied that *AtGSNOR1* plays an impor-

tant role in plant resistance to *Phytophthora*.



A. Phenotype of detached leaves of wild type Col-0 and *atgsnor1-3* mutant after 3 days inoculation with *P. parasitic*; B. Hypha colonization of GFP expressing *P. parasitica* in detached leaves after 3 days inoculation with *P. parasitic*, Scale bar, 500 μm; C. Disease severity index(DSI) from level 1 to level 4 was recorded at 48 hpi(Level 1:disease symptom is less than 1/3 of whole leaf area; Level 2: disease symptom is between 1/3 and 1/2 of whole leaf area; Level 3: disease symptom is between 1/2 and 2/3 of whole leaf area; Level 4: disease symptom is more than 2/3 of whole leaf area). D. The root phenotype of wild type Col-0 and *atgsnor1-3* mutant after 10 days inoculation with *P. parasitica*.

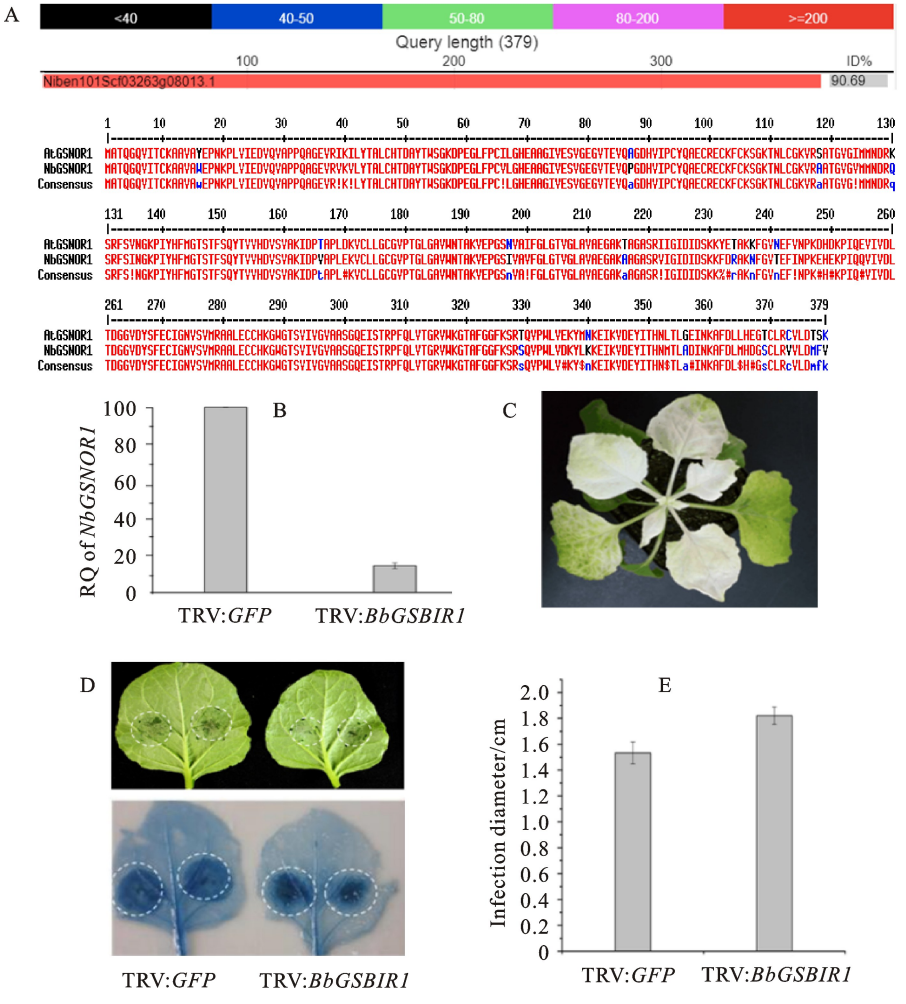
Fig. 1 *atgsnor1-3* mutants exhibit susceptibility to *P. parasitica*

2.2 Silencing of *GSNOR1* homolog in *N. benthamiana* enhances plant susceptibility to *P. parasitica*

To further investigate the resistance function of *GSNOR1* homolog in solanaceous plant to *Phytophthora*, we set out to obtain *N. benthamiana* plants with reduced *GSNOR1* levels by exploiting tobacco rattle virus(TRV) for virus-induced gene silencing(VIGS). For identifying target sequences for VIGS, we first acquired the full length cDNA sequences of *AtGSNOR1* (AT5G43940) from TAIR homepage (The *Arabidopsis* Information Resource, https://www.arabidopsis.org/). Thereafter, through BLAST tool from *Solanaceae* Genomics Network (https://solgenomics.net/tools/blast/), the full length cDNA sequences of *GSNOR1* homolog in *N. benthamiana* were obtained and further sequence alignment showed its high sequence similarity to *AtGSNOR1*, which is 90.69%(Fig. 2-A). Based on this, we generated a VIGS construct that targets *NbGSNOR1*. Quantitative reverse transcription(Q-RT)-PCR analyses showed strongly reduced levels of *NbGSNOR1* mRNA, reaching on average only 12% of the normal level in *N. benthamiana* (Fig. 2-B). Simultaneously, silencing of the endogenous *phytoenedesaturase* (*PDS*) gene, which causes photo bleaching, was used as a control for VIGS efficiency(Fig. 2-C).

To study the role of *NbGSNOR1* in defense against *Phytophthora*, we performed infection assays on leaves detached from *GSNOR1*-silenced *N. benthamiana* plants with *P. parasitica*. The results showed that the water-soaked lesions on *GSNOR1*-silenced plants(TRV:*GSNOR1*) are larger when compared with the lesions on control plants(TRV:*GFP*) at 2 dpi(Fig. 2-D and 2-E). This indicates that in both plant species, *GSNOR1* is required to counteract the

pathogen.



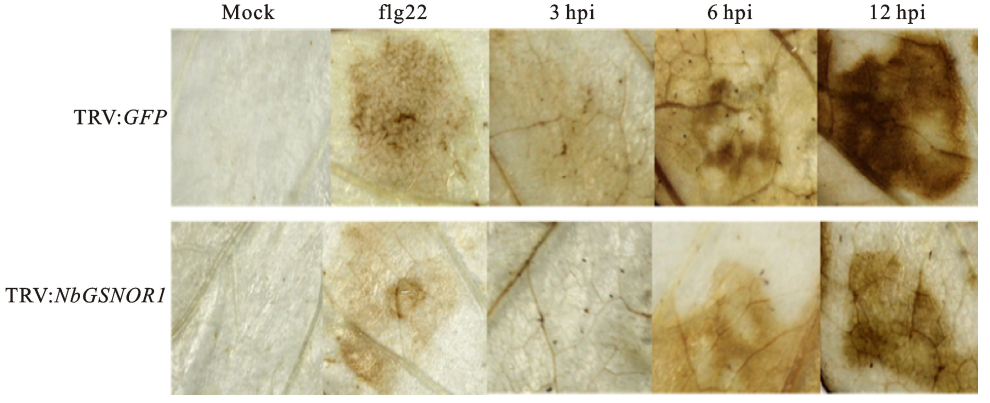
A. *N. benthamiana GSNOR1* homologous sequences were obtained by BLAST sequence alignment; B. Relative quantification(RQ) of *GSNOR1* expression at 21 dpi with TRV constructs in the qRT-PCR assay. *NbACT1N* expression was used for normalization. *GSNOR1* expression in the TRV:*GFP*-treated plant was set at 100%. The experiment was repeated three times. Error bars indicate the SE from three biological replicates; C. silencing of the endogenous phytoenedesaturase(*PDS*) gene, which causes photo bleaching; D. Leaves of *NbGSNOR1* silenced plants and control plants were inoculated by *P. parasitica*, and were strained by trypan blue at 2 dpi. The stained leaves were photographed; E. The lesion diameter statistical analysis of *N. benthamiana* leaves after 2 days inoculation with *P. parasitic*.

Fig. 2 Enhancement of silencing of *GSNOR1* homolog in *N. benthamiana* toplant susceptibility to *P. parasitica*

2.3 Silencing of *NbGSNOR1* suppresses plant reactive oxygen species burst upon infection by *P. parasitica*

Production of reactive oxygen species(ROS) is critical for successful activation of immune responses against pathogen infection[23]. In leaves, the active epitopes of bacterial flagellin (flg22) triggers the oxidative burst and induces defense gene transcription [27-28]. To further elucidate whether *GSNOR1* modulates ROS burst in response to *P. parasitica*, we analyzed the production of H_2O_2, a ROS that can be visualized with 3, 3′-diaminobenzidine- tetrahydrochloride(DAB) staining, in both *GSNOR1*-silenced plants and control plants upon infection by *P. parasitica*. No obvious difference in DAB staining was observed in both *GSNOR1*-silenced plants and control plants at 0 hpi(Fig. 3). As a control assay, clear staining was visible in control plants (TRV: *GFP*) treated with flg22, whereas much weaker staining exhibited in *GSNOR1*-silenced plants treated with flg22(Fig. 3). Similarly, the DAB staining was weaker at 3 hpi, but became gradually stronger from 6 hpi to

12 hpi in control plants, indicating an increase of H_2O_2 production in response to the pathogen. By contrast, the DAB staining was hardly detectable at 3 hpi, and much weaker staining at 6 hpi and 12 hpi was observed in *GSNOR1*-silenced plants (Fig. 3), demonstrating that production of H_2O_2 was clearly reduced. These results suggest that the ROS burst appears to be attenuated in *GSNOR1*-silenced plants upon infection by *P. parasitica*.

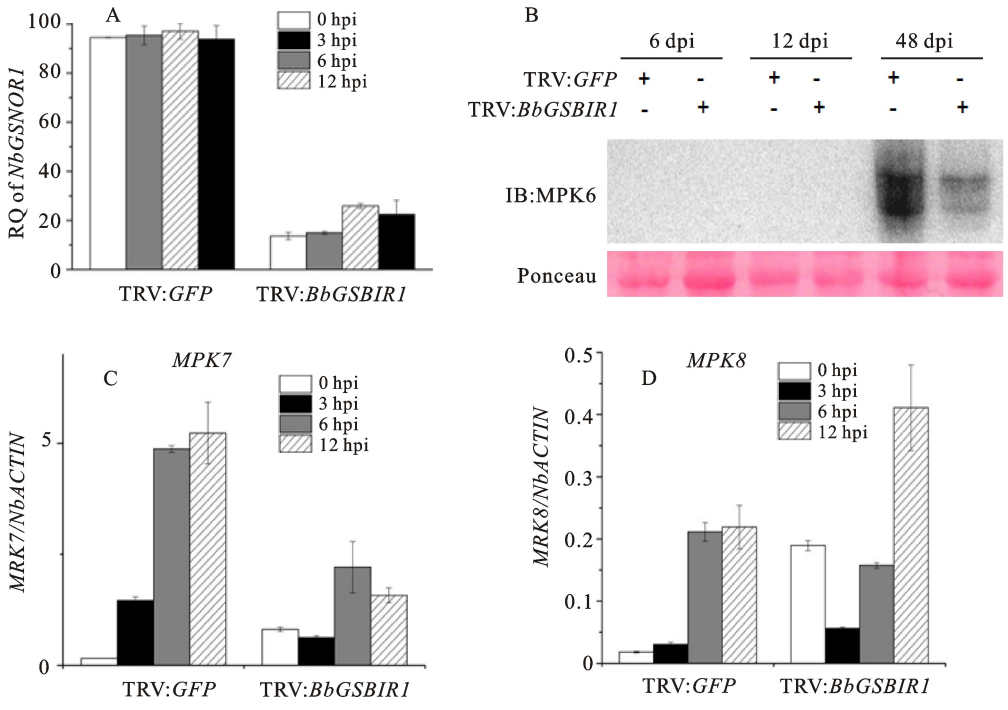


Leaves of *NbGSNOR1* silenced plants and control plants were inoculated by *P. parasitica*, and were strained by DAB at 0 h, 3 h, 6 h and 12 h post inoculation. The DAB staining of 10 mmol/L flg22 injected *N. benthamiana* leaves were used as positive controls. The stained leaves were photographed

Fig. 3 Silencing of *NbGSNOR1* suppresses plant reactive oxygen species burst upon infection by *P. parasitica*

2.4 Silencing of *NbGSNOR1* suppresses induction of *MPK* genes and attenuates accumulation of MPK6 upon infection by *P. parasitica*

The activation of MAPK cascades is one of the marker events for PTI and plays a crucial role in defense response to pathogens in plants[29-30]. In *A. thaliana*, *MPK3*, *MPK4* and *MPK6* are activated by a broad range of biotic stresses[31-33]. Furthermore, *MPK7* and *MPK8* are reported to mediate ROS signaling and regulate *MPK6* in response to jasmonic acid, which is an important phytohormone for activating plant defense response against necrotrophic pathogens[32,34-35]. These prompted us to examine whether *GSNOR1* might involve in the regulation of MAPK signaling in response to the pathogen. The detached leaves of both *GSNOR1*-silenced *N. benthamiana* plants and control plants inoculated with *P. parasitica* were harvested at 3, 6 and 12 hpi. The qRT-PCR analyses showed strongly reduced levels of *NbGSNOR1* mRNA in *GSNOR1*-silenced *N. benthamiana* plants during the infection by *P. parasitica* at indicated time points (Fig. 4-A). Based on this, we first monitored the dynamic transcript levels of *MPK7* and *MPK8*. As expect, both *MPK7* and *MPK8* were highly up-regulated in control plants during the infection, although the *MPK8* showed significant up-regulation from 6 hpi (Fig. 4-C and 4-D). By contrast, in *GSNOR1*-silenced *N. benthamiana* plant, the induction of *MPK7* was significantly reduced during the infection and the induction of *MPK8* was only slightly reduced at 6 hpi (Fig. 4-C and 4-D). Notably, at very early infection stage (3 hpi), *MPK8* showed almost no induction in control plants, whereas the expression of *MPK8* appeared down-regulated compared to that exhibited at 0 hpi in *GSNOR1*-silenced *N. benthamiana* plants (Fig. 4-D). Although the up-regulation of *MPK8* at 12 hpi was detected in *GSNOR1*-silenced *N. benthamiana* plants (Fig. 4-D), it might be due to the attenuated level of *GSNOR1* silencing at indicated time point (Fig. 4-A).



A. Relative quantification(RQ) of *GSNOR1* expression of leaves of *NbGSNOR1* silenced plants which were inoculated by *P. parasitica* at 0 h, 3 h, 6 h and 12 h post inoculation. In the qRT-PCR assay, *NbACT1N* expression was used for normalization. *GSNOR1* expression in the TRV:*GFP*-treated plant was set at 100%. The experiment was repeated three times. Error bars indicate the SE from three biological replicates; B. Protein expression of MPK6. Total proteins were extracted from detached leaves of *NbGSNOR1* silenced plants inoculated by *P. parasitica* at 6 h, 12 h and 48 h post inoculation. The accumulation of MPK6 was detected by immunoblotting using anti-MPK6 antibody. Ponceau staining of the membrane was used to show equal loading; C, D The dynamic expression of *MPK7* and *MPK8* were evaluated by qRT-PCR. Leaves of *NbGSNOR1* -silenced plants and control plants were inoculated by *P. parasitica*. Total RNA was extracted from inoculated leaves at 0h, 3h, 6h and 12h post inoculation. The ratio of candidate gene expression to plant housekeeping gene *NbACT1N* was calculated by the $^{\triangle\triangle}$Ct method. Three independent experiments showed similar result. Error bars indicate the SE from three biological replicates

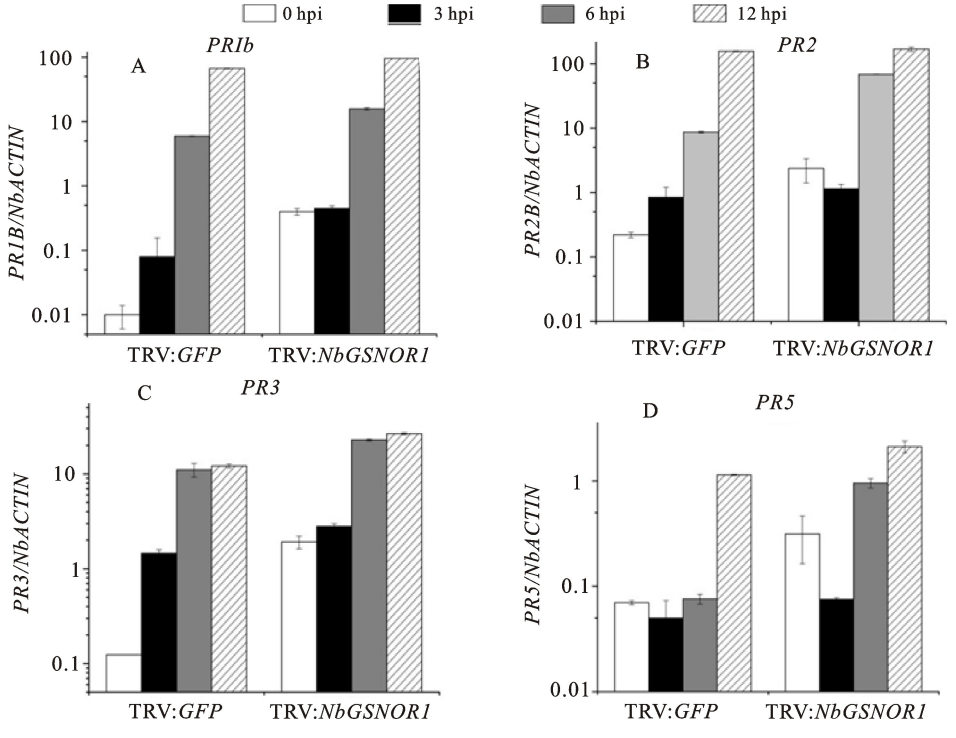
Fig. 4 Silencing of *NbGSNOR1* suppresses induction of MPK genes and attenuates accumulation of MPK6 upon infection by *P. parasitica*

To complement this, we further examined the level of MPK6 protein accumulation in both *GSNOR1*-silenced *N. benthamiana* plants and control plants upon infection by the pathogen. The detached plant leaves were inoculated with *P. parasitica* and harvested at 6, 12 and 48 hpi. Interestingly, we did not detect a clear accumulation of MPK6 protein at early biotrophic infection stage until 48 hpi, which has already developed into necrotrophic infection in either *GSNOR1*-silenced *N. benthamiana* plants or control plants (Fig. 4-B). Notably, at this infection stage, the accumulation of MPK6 was strongly increased in control plants. In comparison, approximately 75% weaker accumulation level of MPK6 was detected in *GSNOR1*-silenced *N. benthamiana* plants(Fig. 4-B). Taken together, these results suggest that silencing of *NbGSNOR1* might attenuate induction of *MPK7* and *MPK8* during early biotrophic infection and suppress the accumulation of MPK6 protein during necrotrophic infection by *P. parasitica*.

2.5 Silencing of *NbGSNOR1* affects induction of *PR* genes upon early infection by *P. parasitica*

The activation of *Pathogenesis-Related* (*PR*) genes is a typical marker event in plant immunity and plays a crucial role in defense response to pathogens in plants[29-30]. Previous research has shown that *Phytophthora* infection induces the expression of *PR1a* [36-37]. These prompted us to further examine, to what extent *GSNOR1* might affect the expression of *PR*

genes in response to *P. parasitica*. The dynamic transcript levels of *PR1b* , *PR2*, *PR3* and *PR5* were further monitored in leaves of both *GSNOR1*-silenced *N. benthamiana* plants and control plants during early infection. The results showed that *PR1b* , *PR2* and *PR3* were highly induced from 3 to 12 hpi, and *PR5* exhibited clear up-regulation at 12 hpi in control plants (Fig. 5). By contrast, in *GSNOR1*-silenced *N. benthamiana* plants, *PR1b* , *PR2* and *PR3* showed resembled expression level at very early infection stage(3 hpi) to that at 0 hpi, whereas expression of *PR5* appeared down-regulated at 3 hpi compared to that exhibited at 0 hpi(Fig. 5). These results implied that silencing of *NbGSNOR1* might attenuate induction of *PR1b* , *PR2* , *PR3* and *PR5* at very early infection phase.



The dynamic expression of *PR1b* , *PR2* , *PR3* and *PR5* were evaluated by qRT-PCR. Leaves of *NbGSNOR1* silenced plants and control plants were inoculated by *P. parasitica*. Total RNA was extracted from inoculated leaves at 0 h, 3 h, 6 h and 12 h post inoculation. The ratio of candidate gene expression to plant housekeeping gene *NbACTIN* was calculated by the $^{\triangle\triangle}$Ct method. Three independent experiments showed similar result. Error bars indicate the SE from three biological replicates

Fig. 5 Silencing of *NbGSNOR1* suppresses induction of *PR* genes infection by *P. parasitica*

3 Discussion

The mechanistic insight into the function of highly conserved reductase GSNOR in plant immunity remains elusive, especially within the plant-*Phytophthora* interacting systems. In this study, we demonstrate that plant *GSNOR1* plays a crucial role in the positive regulation of resistance against *Phytophthora*. Both *Atgsnor1* mutant plants and *GSNOR1*-silenced *N. benthamiana* plants show significantly enhanced susceptibility to *P. parasitica* (Fig. 1 and Fig. 2). This is consistent with previous studies, showing that *Atgsnor1* mutant plants are compromised in basal resistance to bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and non-host resistance against *Blumeria graminis* f. sp. *tritici*[19]. These results indicate that GSNOR1 might be an important and highly conserved candidate, which can be applied to improve broad spectrum resistance in crop plants.

GSNOR1 is the key reductase to participate in regulating protein S-nitrosylation[17], which

has been shown to modulate the activity of a number of key regulators integral to plant immune function including SA binding protein 3 (SABP3)[38], non-expresser of PR1(NPR1)[39], and the basic leucine zipper (bZip) protein TGA1[40]. Furthermore, the NADPH oxidase, RBOHD, an essential enzyme for ROS production in plant defense[41-42], has also been shown to be S-nitrosylated on Cys890[43]. Based on these, we speculate that *GSNOR1* indirectly functions in modulating plant signaling transduction and multiple modes of plant immunity. This might explain its conserved biological function in plant resistance to a broad spectrum of pathogens.

Most *Phytophthora* species, including *P. parasitica*, are hemibiotrophic pathogens, thus these pathogens first establish a biotrophic interaction with host plant and later switch to a destructive necrotrophic lifestyle[44]. As one of the earliest plant immune responses to pathogens, rapid production of ROS is important for resistance against the biotrophic pathogens because it helps drive cell death development[45]. The emerging data suggest that the levels and timing of ROS production are important determinants of cell death in incompatible *P. parasitica* — *N. benthamiana* interactions and correlate with compatible *P. parasitica* proliferation in susceptible plants[46]. Furthermore, *Arabidopsis* root resistance against *P. parasitica* requires an NADPH oxidase mediated oxidative burst[47]. These findings indicate that ROS is important for plant resistance to *Phytophthora* pathogens. Our data suggest that H_2O_2 accumulation is triggered rapidly either by flg22 treatment or *P. parasitica* infection in control plants(Fig. 3). In comparison, the production of H_2O_2 is significantly reduced in *GSNOR1*-silenced plants during the early infection phase(Fig. 3). These results indicate that the timing and level of the oxidative burst are of importance for plant resistance against *Phytophthora* and further that *GSNOR1* plays a key role in regulating these processes.

Plants have developed different defense systems against infections by biotrophic and necrotrophic pathogens. Generally, SA signaling is essential to resist infection from biotrophic pathogens, whereas jasmonic acid/ethylene(JA/ET) signaling is necessary for inhibiting infections from necrotrophic pathogens[48-49]. Interestingly, several reports have shown that both SA and JA/ET signaling are required to defend infection by *Phytophthora* pathogens in both *Arabidopsis* and *N. benthamiana*[36-37, 50]. The induction of *PR* genes is marker event for SA signaling. Particularly, *PR1* has been shown to play an important role in plant defense against *P. capsici*[50]. In addition, *PR1* also participates in *Arabidopsis* root resistance against *P. parasitica*[36] and plays a crucial role in plant resistance to biotrophic pathogens mediated by the novel susceptibility factor *RTP1*[23]. Our kinetic expression analyses on set of PR genes in *N. benthamiana* during early infection by *P. parasitica* showed high induction of *PR1b*, *PR2*, *PR3* and *PR5* in control plants. However, during the very early infection(3 hpi), we did not detect the induction of *PR1b*, *PR2* and *PR3* but the down-regulation of *PR5* in *GSNOR1*-silenced plants(Fig. 5-A). Our findings imply that *GSNOR1* might involve in regulating the induction of *PR* genes during early biotrophic infection by *Phytophthora*, which suggests that *GSNOR1* may affect SA signaling for its function in plant resistance against *Phytophthora*.

In plants, MAPK cascades play essential roles in the transduction of environmental signals, including response to a range of stresses caused by ROS and pathogen infections[51]. Increasing evidence has demonstrated that MAPK cascades have emerged as battlegrounds of plant-pathogen interactions. Activation of MAPKs is one of the earliest signaling events after plant sensing of PAMPs and pathogen effectors[30]. Importantly, hormones such as jasmonic acid (JA) and salicylic acid(SA) are known to influ-

ence signaling through MAPK cascades. For instance, JA involves in regulating the activation of MPK6[34]. Interestingly, out studies showed the protein accumulation of MPK6 in *GSNOR1*-silenced plants was not detected until 48 hpi, representing the necrotrophic infection phase(Fig. 4-B). Moreover, the *GSNOR1*-silenced plants exhibited strong suppression of MPK6 accumulation compared to control plants (Fig. 4-C). These results imply that MPK6 might function during the necrotrophic infection by *Phytophthora* and *GSNOR1* may also affect JA signaling for its function in plant resistance against *Phytophthora*. Furthermore, *MPK7* could be activated by ROS triggered by plant stress stimuli[52-53]. In *Arabidopsis*, through interacting with *MPK8*, LSF2(LIKE SEX FOUR2) functions in the regulation of plant oxidative stress, resulting in modulating ROS homeostasis[54]. The results showed that the induction of *MPK7* and *MPK8* was significantly suppressed in *GSNOR1*-silenced plants during early *P. parasitica* infection(Fig. 4-B). These data indicate that *GSNOR1* may involve in activating MAPK cascade signaling for its resistance function against *Phytophthora*.

In conclusion, this study demonstrated that through positively regulating ROS production, induction of *PR* genes, *MPK7* and *MPK8* at the early biotrophic infection stag, and the accumulation of MPK6 protein at the later necrotrophic infection, *GSNOR1* plays an important role in the positive regulation of plant resistance against *Phytophthora*.

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亚硝基谷胱甘肽还原酶 *GSNOR1* 正调控植物对疫霉菌的抗性

刘杏芍[1]，宋斯雨[2]，高贤贤[1]，单卫星[1]，强晓玉[1]

（1. 西北农林科技大学 农学院，陕西杨凌　712100；2. 西北农林科技大学 植物保护学院，陕西杨凌　712100）

摘　要　疫霉属（*Phytophthora*）卵菌引致马铃薯晚疫病等作物灾难性病害，严重威胁作物的可持续生产。由于病菌毒性变异，导致品种抗病性丧失问题突出。因此，挖掘植物广谱和持久抗病基因，并探索其在抗病育种中的有效利用具有重要的科学意义。亚硝基谷胱甘肽还原酶 1（GSNOR1）是植物氮信号通路中高度保守的关键还原酶，其参与调节 *R* 基因介导的植物抗性和非寄主抗性。然而，*GSNOR1* 参与抗疫霉菌的免疫功能和作用机理尚不清楚。本研究中，借助拟南芥与寄生疫霉菌互作的模式体系，首先发现拟南芥 T-DNA 插入突变体 *gsnor1-3* 对寄生疫霉菌呈现感病表型。进一步利用病毒诱导的基因沉默（VIGS）降低烟草叶片中 *GSNOR1* 同源基因的表达量，在此基础上，通过抗病表型分析以及一系列抗性相关功能的检测，结果表明，沉默本氏烟草*GSNOR1* 同源基因能够在寄生疫霉菌侵染植物的过程中削弱植物体内的活性氧（ROS）迸发、病程相关基因（PR genes）的诱导表达以及 MAPK 信号转导，从而增强了植物对疫霉菌的感病性。本研究揭示了植物*GSNOR1* 对疫霉菌具有高度保守的抗性功能，并初步解析了*GSNOR1* 正调控植物抗疫霉菌的作用机理，为进一步探索*GSNOR1* 在马铃薯抗晚疫病中的功能及其在抗病育种中的有效利用奠定了重要的理论基础。

关键词　亚硝基谷胱甘肽还原酶；寄生疫霉菌；本氏烟草；拟南芥；马铃薯晚疫病

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第一作者：刘杏芍，女，硕士研究生，从事植物抗病分子基础研究。E-mail：liuxingshao@nwafu.edu.cn

通信作者：强晓玉，女，副教授，硕士生导师，主要从事植物抗病性及植物与微生物群体互作研究。E-mail：qiangxiaoyu@nwafu.edu.cn

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