Dual Roles of GSNOR1 in Cell Death and Immunity in Tetraploid *Nicotiana tabacum*

Zhen-Chao Li, Qian-Wei Ren, Yan Guo, Jie Ran, Xiao-Tian Ren, Ni-Ni Wu, Hui-Yang Xu, Xia Liu, and Jian-Zhong Liu

S-nitrosoglutathione reductase 1 (GSNOR1) is the key enzyme that regulates cellular homeostasis of S-nitrosylation. Although extensively studied in *Arabidopsis*, the roles of GSNOR1 in tetraploid *Nicotiana* species have not been investigated previously. To study the function of *Nt*GSNOR1, we knocked out two *Nt*GSNOR1 genes simultaneously in *Nicotiana tabacum* using clustered regularly interspaced short palindromic repeats (CRISPR)/caspase 9 (Cas9) technology. To our surprise, spontaneous cell death occurred on the leaves of the CRISPR/Cas9 lines but not on those of the wild-type (WT) plants, suggesting that *Nt*GSNOR1 negatively regulates cell death. The natural cell death on the CRISPR/Cas9 lines could be a result from interactions between overaccumulated nitric oxide (NO) and hydrogen peroxide (H$_2$O$_2$). This spontaneous cell death phenotype was not affected by knocking out two *Enhanced disease susceptibility* 1 genes (*Nt*EDS11a/1b) and thus was independent of the salicylic acid (SA) pathway. Unexpectedly, we found that the *Nt*GSNOR1a/1b knockout plants displayed a significantly (*p* < 0.001) enhanced resistance to paraquat-induced cell death compared to WT plants, suggesting that *Nt*GSNOR1 functions as a positive regulator of the paraquat-induced cell death. The increased resistance to the paraquat-induced cell death of the *Nt*GSNOR1a/1b knockout plants was correlated with the reduced level of H$_2$O$_2$ accumulation. Interestingly, whereas the *N* gene-mediated resistance to *Tobacco mosaic virus* (TMV) was significantly enhanced (*p* < 0.001), the resistance to *Pseudomonas syringae* pv. *tomato* DC3000 was significantly reduced (*p* < 0.01) in the *Nt*GSNOR1a/1b knockout lines. In summary, our results indicate that *Nt*GSNOR1 functions as both positive and negative regulator of cell death under different conditions and displays distinct effects on resistance against viral and bacterial pathogens.

**Keywords:** cell death, disease resistance, nitric oxide, hypersensitive responses, reactive oxygen species, S-nitrosoglutathione reductase

**INTRODUCTION**

Nitric oxide (NO) is a reactive free radical gas molecule with a plethora of functions in both animals and plants (Wendehenne et al., 2014). In plants, NO is involved in diverse biological processes such as stomatal closure (Desikan et al., 2002; Neill et al., 2008), cell death and disease resistance (Durner et al., 1998; Klessig et al., 2000; Wendehenne et al., 2004; Zeidler et al., 2004; Lin et al., 2012; Wang et al., 2013; Wang and Chu, 2020), abiotic stress (Lee et al., 2008; Xuan et al., 2010), flowering...
Among all these processes, the most significant function of NO is to potentiate the induction of hypersensitive cell death by reactive oxygen species (ROS) (Durner et al., 1998; Delledonne et al., 2001).

S-nitrosylation, attachment of NO moiety to a target protein, is a newly emerged mechanism by which NO regulates the function of various target proteins and thus various biological processes (Hess and Stamler, 2012; Wendehenne et al., 2014). This reversible posttranslational modification is analogous to protein phosphorylation (Stamler et al., 1992; Hess et al., 2005). Hundreds of proteins have been identified as targets of S-nitrosylation whose functions are regulated by this posttranslational modification (Lindermayr et al., 2005; Forrester et al., 2009; Hess and Stamler, 2012; Yang et al., 2015). In plants, the S-nitrosylated cysteine residues of some target proteins have been identified, and the functions of this posttranslational modifications are revealed (Lindermayr et al., 2006, 2010; Romero-Puertas et al., 2007; Tada et al., 2008; Yun et al., 2011; Skelly et al., 2019; Gupta et al., 2020).

S-nitrosoglutathione (GSNO), S-nitrosylated form of glutathione (GSH), functions as a major mediator of protein S-nitrosylation by a process known as transnitrosylation (Hess et al., 2005). S-nitrosoglutathione reductase (GSNOR) is the key enzyme controlling GSNO levels by reducing GSNO to oxidized GSH and NH3 and thus indirectly controlling the levels of protein S-nitrosylation (Liu et al., 2001, 2004; Feechan et al., 2005). In mice, loss of GSNOR1 function results in substantial increases in whole-cell S-nitrosylation, tissue damage, and mortality following endotoxin or bacterial challenge (Liu et al., 2004). In Arabidopsis, the hypersensitive response (HR) cell death triggered by avirulent Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) carrying the AvrB or AvrRPS4 is accelerated in loss of function mutant, gsnor-1-3, but is delayed in overexpressing mutant gsnor1-1 (Yun et al., 2011), suggesting that the function of GSNOR1 is conserved between mice and Arabidopsis in negatively regulating HR cell death in response to biotic stress (Liu et al., 2004; Yun et al., 2011). The cell death in response to incompatible pathogen infections in gsnor1-3 mutant is due to high SNO concentrations and is SA- and hydrogen peroxide (H2O2)-independent (Yun et al., 2011). Interestingly, it has also been revealed that GSNOR1 is a key positive regulator of cell death induced by herbicides (Chen et al., 2009), suggesting that depending on cellular conditions, GSNOR1 could regulate cell death either positively or negatively. NO has been demonstrated to have both proapoptotic and antiapoptotic roles, depending on a variety of factors, including the type of cells involved, redox state of the cell, and the flux and dose of NO (Wang et al., 2013; Wendehenne et al., 2014; Huang et al., 2019). In general, S-nitrosylation resulting from basal low-level NO production in cells has antiapoptotic effects, whereas S-nitrosylation resulting from higher-level stimulated NO production has either proapoptotic effects or serves as a negative feedback mechanism to downregulate apoptotic responses (Mannick, 2007).

NO exerts its effects on cell death mostly through modulating H2O2 production or detoxification. S-nitrosylation of an Arabidopsis cystolic ascorbate peroxidase (cAPX), a key enzyme controlling H2O2 levels in plants, inhibits its enzyme activity, leading to Programmed cell death (PCD) (de Pinto et al., 2013). Loss of H2O2 detoxifying enzyme, catalase in rice, results in both NO and H2O2 overaccumulation and leaf cell death (Lin et al., 2012). Overexpression of rice GSNOR1 alleviates the leaf cell death in noe1 (nitric oxide excess 1) mutant (Lin et al., 2012), suggesting a conserved role of S-nitrosylation in cell death in plants. S-nitrosylation not only can induce cell death but also can function as a negative feedback loop to limit or prevent excessive cell death. Instead of inhibiting the function of a cAPX1, S-nitrosylation of the Arabidopsis cystolic APX1 at Cys32 enhances its enzymatic activity of scavenging H2O2, resulting in an increased resistance to oxidative stress (Yang et al., 2015). NADPH oxidase is the major source of H2O2 production under biotic stress. Arabidopsis AtRBOHD subunit of NADPH oxidase complex is S-nitrosylated at Cys890, and this modification abolishes its ability to synthesize ROS and thus avoids excessive cell death (Yun et al., 2011).

In addition to cell death, GSNOR1 plays a critical role in disease resistance. Loss of GSNOR1 function in Arabidopsis results in compromised basal resistance, R gene-mediated resistance and non-host resistance (NHR) (Feechan et al., 2005). Silencing SGSNOR1 in tomato also compromises basal resistance against Pst DC3000 (Hussain et al., 2019). On the contrary, Arabidopsis and tomato lines that overexpress GSNOR1 lead to enhanced resistance to virulent pathogens (Feechan et al., 2005; Hussain et al., 2019). However, silencing GSNOR1 by antisense approach in Arabidopsis enhances Systemic acquired resistance (SAR) and basal resistance (Rustérucci et al., 2007). Recently, it has been shown that the accelerated cell death observed in gsnor1-3 in response to pathogen infection confers RPP4-mediated resistance to Hyaloperonospora arabidopsidis isolate Emw1 in an SA-independent manner (Yun et al., 2011). These seemingly contradictory results suggest that the roles of GSNOR1 in plant disease resistance are more complicated than we thought. Besides cell death and disease resistance, GSNOR1 is also required for growth/development (Feechan et al., 2005; Lee et al., 2008; Chen et al., 2009; Albertos et al., 2015; Shi et al., 2015; Yun et al., 2016; Kawabe et al., 2018; Gong et al., 2019; Hussain et al., 2019), thermotolerance (Lee et al., 2008), abiotic stress responses (Zhang and Liao, 2019), hypoxia responses (Zhan et al., 2018), cytokinin signaling (Feng et al., 2013), auxin signaling (Terrile et al., 2012; Shi et al., 2015; Ni et al., 2017; Iglesias et al., 2018) and transport (Shi et al., 2015), abscisic acid (ABA)-mediated stomatal closure (Albertos et al., 2015; Wang et al., 2015), nitrogen assimilation (Frungillo et al., 2014), and nodule development (Matamoros et al., 2020).

To investigate the roles of GSNOR1 in tetraploid N. tabacum, the NtGSNOR1a and NtGSNOR1b knockout plants were generated via clustered regularly interspaced short palindromic repeats (CRISPR)/cas9 (Cas9) technology. Unexpectedly, spontaneous cell death was observed in the NtGSNOR1a/1b knockout plants under natural growth conditions, which has not been reported for the Arabidopsis gsnor1 mutant.
previously. This spontaneous cell death phenotype was not rescued by knocking out NtEDS1a/1b and thus was SA-independent. To our surprise, the NtGSNOR1a/1b knockout plants displayed increased resistance to paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride)-induced cell death compared to wild-type (WT) plants, indicating that GSNOR1 in Nicotiana species functions as both positive and negative regulator of cell death under different cellular conditions. Interestingly, whereas the resistance to Tobacco mosaic virus (TMV) was enhanced in the NtGSNOR1a/1b knockout plants, the resistance to Pst DC3000 was significantly reduced ($p < 0.001$), indicating that NtGSNOR1a/1b have opposite effects on viral and bacterial pathogen infections in N. tabacum.

RESULTS

Generation of the CRISPR/Cas9 Lines That Simultaneously Knock Out Two Alleles of NtGSNOR1a and Two Alleles of NtGSNOR1b in Tetraploid N. tabacum

It has been known that NO promotes an increase in ROS-induced cell death (Delledonne et al., 1998; Durner et al., 1998). However, the mechanism behind this NO-promoted cell death is not understood. To investigate the roles of GSNOR1 in cell death and disease resistance in tetraploid N. tabacum, we designed to simultaneously knockout four alleles of two highly related orthologs of Arabidopsis GSNOR1 gene, namely, NtGSNOR1a (LOC107777450) and NtGSNOR1b (LOC107791841) and created NtGSNOR1a/NtGSNOR1b double knockout lines in N. tabacum cv. Samsun, which carries TMV-resistant gene, N, using CRISPR/Cas9 technology. The NtGSNOR1a and NtGSNOR1b share overall 98% identity at the nucleotide level. A consensus 20-bp sequence shared between NtGSNOR1a and NtGSNOR1b within the second exon of NtGSNOR1a/1b was used as a guide to target both genes (Figures 1A,B). The blast searching using the target sequence against the tobacco genome revealed that the target sequence only specifically matched to the tobacco genome with the closest match displayed 4-nt mismatches, which potentially could rule out the possibility of off-target effect. Multiple T0 transgenic lines were generated through Agrobacterium-mediated transformation, and six independent CRISPR/Cas9 lines, in which two alleles of NtGSNOR1a and two alleles of NtGSNOR1b, respectively, were simultaneously knocked out, were identified in the T0, T1, or T2 populations. The types of mutations for each of the four alleles in these six CRISPR/Cas9 lines were summarized in Figure 1C. The sequencing chromatograms showing the insertions and deletions (InDels) in the sequences of NtGSNOR1a and NtGSNOR1b of the CRISPR/Cas9 lines 1 were presented in Figure 1D. The sequencing chromatograms of the CRISPR/Cas9 line 2 to line 6 were presented in Supplementary Figures 1–5. Translational analysis indicated that the mutations (InDels) within all four alleles of NtGSNOR1a/1b in these six CRISPR/Cas9 lines could result in premature stop codons and would generate truncated proteins. Thus, these CRISPR/Cas9 lines can be considered independent knockout lines of both NtGSNOR1a and NtGSNOR1b (NtGSNOR1a/1b).

The Spontaneous Cell Death Occurs on the Leaves of the NtGSNOR1a/1b Knockout Plants

These six CRISPR/Cas9 knockout lines shared a similar morphological phenotype with a smaller overall stature compared to the WT plants through the entire life cycle (Figures 1E, 2). To our surprise, a severe spontaneous cell death phenotype was observed on the leaves of these NtGSNOR1a/1b knockout lines (Figure 1E), which was not observed in the WT plants throughout the entire growth season. The spontaneous cell death was not synchronous even for the same batch of the knockout plants. The cell death showed up earlier on some plants than on the others. The spontaneous cell death was mainly on the fully expanded true leaves independent of developmental stage. It could occur as early as the two-leaf stage (Figure 2A). It usually appeared on the lower leaves and spread progressively to the upper systemic leaves (Figures 1B–E). The cell death was similar to HR cell death, which initially displayed as water-soaked spots and eventually dried out. These results indicate that NtGSNOR1 is a negative regulator of cell death.

Both NO and H$_2$O$_2$ Were Overaccumulated in the NtGSNOR1a/1b Knockout Plants

To investigate the effect of NtGSNOR1a/1b knockout on NO and H$_2$O$_2$ accumulation in tobacco, we compared the NO and H$_2$O$_2$ levels between WT and the CRISPR/Cas9 lines using 4,5-diaminoflorescein diacetate and 3,3′-diaminobenzidine (DAB) staining, respectively. Consistent with the results observed for Arabidopsis hot5-2 (gsnor1-3/par2-1) mutants, NO level was significantly higher ($p < 0.001$) in the roots of the NtGSNOR1a/1b knockout plants of two independent lines compared with that of the WT plants (Figures 3A,B). Contrary to the results observed for gsnor1-3 mutant in Arabidopsis, H$_2$O$_2$ level on the leaves of the NtGSNOR1a/1b knockout lines showing the sign of cell death was also higher than those of WT plants (Figures 3C,D), although little or no H$_2$O$_2$ was detectable on the leaves of the NtGSNOR1a/1b knockout lines without showing any cell death. Together, these results indicate that NO and H$_2$O$_2$ were overaccumulated in the NtGSNOR1a/1b knockout plants and the spontaneous cell death that occurred on the NtGSNOR1a/1b knockout plants could result from synergistic interactions between the overaccumulated NO and H$_2$O$_2$.

The Spontaneous Cell Death on the Leaves of the NtGSNOR1a/1b Knockout Plants Is Not Dependent on the SA Pathway

EDS1 is required for SA accumulation (Rustérucci et al., 2001), and the constitutive cell death of many autoimmune mutants is dependent on EDS1 (Zhou and Zhang, 2020). To examine whether the spontaneous cell death on the NtGSNOR1a/1b knockout plants is SA-dependent, we knocked out four alleles of NtEDS1a/1b and four alleles of NtGSNOR1a/1b simultaneously in N. Samsun (NN) using the CRISPR/Cas9
**FIGURE 1** | Knocking out NtGSNOR1a and NtGSNOR1b simultaneously results in spontaneous cell death on the leaves of *N. tabacum*. (A) Gene model of NtGSNOR1a (LOC10777450) and NtGSNOR1b (LOC107791841). A consensus sequence between NtGSNOR1a and NtGSNOR1b at the second exon (45–65 bp downstream of start codon ATG of the open reading frame) was used as a guide to target both genes simultaneously. The TGG in blue represents PAM sequence. (B) The diagram of the CRISPR/Cas9 construct used for generating transgenic plants. (C) The sequence alignment of the six identified CRISPR/Cas9 knockout lines. The short horizontal lines in red represent the deletions; the TGG in blue represents PAM sequence; and the red letters represent the insertions; // represent some bases were omitted for the purpose of saving space. The mutation types of these six CRISPR/Cas9 lines were shown by the labeling on the right. (D) Comparison of the sequencing chromatograms between the wild type (WT) and the CRISPR/Cas9 knockout line 1 at the edited region. Arrows pointed to the sites of deletions and insertions. + represents insertion, and - represents deletion. The PAMs are marked in blue letters and lines. (E) The spontaneous cell death phenotype is displayed on the leaves of all these six CRISPR/Cas9 lines. Arrows pointed to the regions with cell death.

**FIGURE 2** | The spontaneous cell death phenotype of NtGSNOR1a/1b knockout plants at different developmental stages. (A) The spontaneous cell death phenotype could occur as early as 25 days post germination. (B–F) The cell death was aggravated with the development and progressed to upper systemic leaves. (D–F) Almost all the leaves of the knockout lines eventually died at 90 days post germination. (E,F) The enlarged image in the rectangle area in (E) was shown in (F). Arrows pointed to the dead cells.
Knocking out NtGSNOR1a/1b leads to enhanced accumulation of both NO and H$_2$O$_2$. (A) NO level in the roots of 15-day-old plants was visualized by staining using 4,5-diaminoflorescein diacetate according to He et al. (2004). (B) The fluorescence intensity shown in (A) was quantified by ImageJ. (C) H$_2$O$_2$ was detected by staining the leaves of 40-day-old plants with 3,3'-diaminobenzidine (DAB) as described in section Materials and Methods. Oxidized DAB formed a reddish-brown deposit (examples of these deposits are indicated by the white arrows). (D) The intensity of the reddish-brown deposit shown in (C) was quantified by ImageJ. ** and *** indicate significant differences at 0.01 and 0.001 levels, respectively, by the Student’s t-test.

To further confirm that the NtEDS1a/1b are indeed knocked out in the NtGSNOR1a/1b/NtEDS1a/1b double knockout lines, we transiently overexpressed GmMEKK1 via agro-infiltration on the leaves of the double knockout line, which can trigger SA-dependent cell death (Xu et al., 2018). As expected, the HR cell death induced by overexpressing GmMEKK1 was observed on the leaves of the NtGSNOR1a/1b knockout plants but not on the leaves of the NtGSNOR1a/1b/NtEDS1a/1b double knockout plants (Supplementary Figure 6), indicating that the NtEDS1a/1b is indeed knocked out in the double knockout lines. The fact that the spontaneous cell death was still observed on the NtGSNOR1a/1b/NtEDS1a/1b knockout plants (Figure 4E) indicated that the spontaneous cell death of NtGSNOR1a/1b knockout plants is independent of the SA pathway.

The NtGSNOR1a/1b Knockout Plants Are More Resistant to Paraquat-Induced Cell Death

Paraquat, a non-selective herbicide, can induce cell death in plants (Dodge, 1971; Suntres, 2002). To examine whether knocking out NtGSNOR1a/1b in N. tabacum has a similar paraquat-resistant phenotype observed in Arabidopsis (Chen et al., 2009), 0.25% paraquat was sprayed onto the leaves of both the WT and the NtGSNOR1a/1b knockout plants before showing spontaneous cell death. While the WT plants almost died at 3 days post spraying (dps), two different NtGSNOR1a/1b knockout lines were still alive and relatively healthy (Figure 5A), indicating that the function of GNSOR1 on paraquat-induced cell death is conserved across plant species.
Knocking out NtEDS1a/1b cannot reverse the spontaneous cell death phenotype observed in NtGSNOR1a/1b knockout lines. (A) Gene model of NtEDS1a (LOC107782626) and NtEDS1b (LOC107791841). A consensus sequence between NtEDS1a and NtEDS1b at the first exon (70–92 bp downstream of start codon ATG of the open reading frame) was used as a guide to target both genes simultaneously. The TGG in blue represents PAM sequence. (B) The diagram of the CRISPR/Cas9 construct used for generating transgenic plants that knocks out both NtEDS1a/1b and NtGSNOR1a/1b. (C) Comparison of the sequencing chromatograms near the edited regions between the wild type (WT) and the CRISPR/Cas9 line, in which both NtEDS1a/1b and NtGSNOR1a/1b are knocked out simultaneously. Arrows pointed to the sites of insertions. + represents insertion. The PAM sequences are marked in blue. (D) The sequence alignments of the identified CRISPR/Cas9 double knockout line at both the NtEDS1a/1b and NtGSNOR1a/1b loci. The short horizontal lines in red represent the deletions; the TGG in blue represents PAM sequence; and the red letters represent the insertions; // represents extra space added for the purpose of the perfect sequence alignment. The mutation types of the CRISPR/Cas9 line were presented by the labeling on the right. (E) The spontaneous cell death phenotype is displayed on the leaves of the CRISPR/Cas9 double knockout line, in which both NtEDS1a/1b and NtGSNOR1a/1b are knocked out simultaneously. Arrows pointed to the regions with cell death.

To investigate the resistant mechanism of the NtGSNOR1a/1b knockout plants to the paraquat-induced cell death, we visualized the H<sub>2</sub>O<sub>2</sub> accumulation on the leaves of both WT and the NtGSNOR1a/1b knockout plants after paraquat spraying. Consistent with the result in Arabidopsis (Chen et al., 2009), we found that the level of H<sub>2</sub>O<sub>2</sub> accumulation was much lower on the leaves of the NtGSNOR1a/1b knockout plants than that of the WT plants (Figures 5B,C), indicating that reduced level of paraquat-induced H<sub>2</sub>O<sub>2</sub> production or accumulation could be at least one of the molecular mechanisms behind the resistance of the NtGSNOR1a/1b knockout plants to paraquat-induced cell death.

Knocking Out NtGSNOR1a/1b Has Opposite Effects on Tobacco mosaic virus and P. syringae pv. tomato DC3000 Infections

Contradictory results were obtained regarding the role of Arabidopsis GSNOR1 in disease resistance using gsnor1-3 null mutant (Feechan et al., 2005) and GSNOR1 antisense lines (Rustérucci et al., 2007). However, the role of GSNOR1 in other plant species has not been investigated. To test the effect of NtGSNOR1a/1b knockout on disease resistance in Nicotiana species, we inoculated TMV on both WT N. tabacum Samsun (NN) and the NtGSNOR1a/1b knockout plants. It was found that the sizes of HR lesions triggered by TMV infection on the leaves of the NtGSNOR1a/1b knockout plants were significantly smaller than those on the leaves of N. tabacum cv. Samsun (NN) at 5 days post inoculation (dpi) (Figures 6A,B). Appearance of local HR lesions reflects both replication and cell-to-cell movement of the virus (Meshi et al., 1982). Consistent with this statement, we showed that the transcript level of TMV capsid protein (CP) was much lower on the knockout leaves than on the WT leaves (Supplementary Figure 7). However, we cannot exclude a possibility that the smaller sizes of the HR on the leaves of the knockout plants could also reflect a decreased ability for N protein to trigger the HR upon TMV infection.

To examine the effect of knocking out NtGSNOR1a/1b on bacterial infection, the upper leaves of both WT and NtGSNOR1a/1b knockout plants without spontaneous cell death were infiltrated with Pst DC3000 as the cell death on the lower leaves interferes with the bacterial growth assay. As shown in Figure 6C, Pst DC3000 multiply better on the leaves of the NtGSNOR1a/1b knockout plants than on the leaves of the WT plants (Figure 6C), indicating that NtGSNOR1a/1b positively regulates the basal resistance to a bacterial pathogen. Together, these results indicate that knocking out NtGSNOR1a/1b has opposite effects on resistance to different types of pathogens with different infecting strategies.
Li et al. Roles of NtGSNOR1 in Immunity

FIGURE 5 | The NtGSNOR1a/1b knockout plants exhibited strong resistance to paraquat (PQ)-induced cell death through reducing PQ-induced H\textsubscript{2}O\textsubscript{2} accumulation. (A) Here, 0.25% PQ was sprayed on the leaves of 40-day-old wild type (WT) and the CRISPR/Cas9 knockout lines 1 and 3. The images were taken at 0, 3, and 6 days post spraying. (B) The H\textsubscript{2}O\textsubscript{2} accumulation on the leaves of WT and NtGSNOR1a/1b knockout plants at 0 and 2 days post-paraquat spraying visualized by DAB staining. (C) The intensity of the reddish-brown deposit shown in (B) was quantified by ImageJ. ** and *** indicate significant differences of p-value at 0.01 and 0.001, respectively, by the Student’s t-test.

DISCUSSION

In this report, we found that both anti- and pro-cell death effects were observed in the NtGSNOR1a/1b knockout plants, reinforcing the notion that NO can play either anti- or pro-death roles depending on cellular conditions (Wang et al., 2013; Wendehenne et al., 2014) and the altered NO homeostasis plays key roles in switching its functions during the HR. In addition, we found that knocking out NtGSNOR1a/1b has opposite effects on viral and bacterial resistance.

The cell death phenotype of the NtGSNOR1a/1b knockout plants under natural conditions was not reported for the gsnor1-3/hot5/par2-1 mutants (Feechan et al., 2005; Lee et al., 2008; Chen et al., 2009) or antisense lines of GSNOR1 in Arabidopsis (Rustérucci et al., 2007), although spontaneous cell death was observed on the leaves of GSNOR1-silenced tomato plants by virus-induced gene silencing (VIGS) (Liu et al., 2017) and an accelerated cell death was observed on the leaves of gsnor1-3 mutant upon infections by avirulent bacterial strains or by an avirulent oomycete isolate (Rustérucci et al., 2007; Yun et al., 2011). The generation of both NO and H\text subscript{2}O\textsubscript{2} is simultaneously induced under many different stress conditions (Delledonne et al., 1998; Clarke et al., 2000; Lin et al., 2012; Scheler et al., 2013), indicating that they coexist in most cellular conditions. Both NO and H\textsubscript{2}O\textsubscript{2} was significantly higher in the NtGSNOR1a/1b knockout plants compared to WT plants (Figure 3). The increased H\textsubscript{2}O\textsubscript{2} level in the NtGSNOR1a/1b knockout plants could be induced by the overaccumulation of NO in these plants, since it has been reported previously that NO is essential for generating elicitor- or pathogen-inducible ROS and that ROS likely functions downstream of NO (Rasul et al., 2012; Wang L. et al., 2014; Kulik et al., 2015). It has been reported that efficient activation of hypersensitive cell death required a balance between NO and ROS production (Delledonne et al., 1998, 2001). NO/H\textsubscript{2}O\textsubscript{2} ratio between 0.25 and 2 was effective in inducing cell death. NO/H\textsubscript{2}O\textsubscript{2} ratio during pathogen induction of the HR or during induction of cell death by exogenous NO and artificial oxidative burst falls within this range (Delledonne et al., 2001). Based on this ratio model, we propose the following explanations for the naturally occurring cell death (Figures 1E, 2) and the reduced paraquat-induced cell death observed in the NtGSNOR1a/1b knockout plants (Figure 5A). Under normal growth conditions, NO level is high in the NtGSNOR1a/1b knockout plants, but H\textsubscript{2}O\textsubscript{2} level is relatively low compared to the NO level. As a result, the NO/H\textsubscript{2}O\textsubscript{2} ratio falls above the high end of the optimal ratio range so that cell death is not triggered. At certain development stages or under mild stress conditions, H\textsubscript{2}O\textsubscript{2} is induced gradually. Once the H\textsubscript{2}O\textsubscript{2} level is
Knocking out NtGSNOR1a/1b in N. tabacum results in the enhanced N gene mediated resistance against TMV but reduced resistance against Pseudomonas syringae pv. tomato DC3000 (Pst DC3000). (A) The hypersensitive response (HR) lesions formed on the leaves of the 40-day-old NtGSNOR1a/1b knockout lines (Lines 1 and 3) are reduced in comparison to that of wild-type (WT) Samsun (NN) plants in response to Tobacco mosaic virus (TMV) infection. (B) The sizes of HR lesions shown in (A) were measured under a dissecting microscopy. The lesions on the leaves of three independent plants of each line were measured. Over 30 lesions were measured on each infected leaf. ** represents significant difference at 0.001 level by the Student’s t-test. (C) NtGSNOR1a/1b knockout plants support greater growth of Pst DC3000 compared to WT Samsun (NN) plants. The leaves of 40-day-old plants were infiltrated with Pst DC3000 (OD600 = 0.00001). The numbers of Pst DC3000 were counted at 0-, 2-, 4-, and 6-day post-inoculation and expressed as log10. ** represents significance at p-value 0.01 by the Student’s t-test.
at Cys\textsuperscript{996}, abolishing its ability to synthesize ROS and thus functioning as a negative feedback loop limiting the HR (Yun et al., 2011), raises the possibility that the resistance to paraquat-induced cell death observed in the \textit{NtGSNOR1a/1b} knockout plants is at least partially attributed to the feedback inhibition of H\textsubscript{2}O\textsubscript{2} production by AtRBOHD and APX1. We recently provide evidence that NO may trigger cell death in tomato (\textit{Solanum lycopersicum}) by inhibiting the activity of phosphoinositide-dependent kinase 1 (SIPDK1), a conserved negative regulator of cell death in yeasts, mammals, and plants, \textit{via} S-nitrosylation (Liu et al., 2017). SIPDK1 is primarily S-nitrosylated on Cys\textsuperscript{128} and substitution of Cys\textsuperscript{128} with serine completely abolished SIPDK1 kinase activity, suggesting that S-nitrosylation of Cys\textsuperscript{128} is responsible for SIPDK1 inhibition (Liu et al., 2017). These results establish a potential link between NO-triggered cell death and inhibition of the kinase activity of tomato PDK1. It is possible that the spontaneous cell death observed on the leaves of \textit{NtGSNOR1a/1b} knockout plants is a consequence of inhibition of the NtPDK1 activity.

Even though the naturally occurring spontaneous cell death is not observed in \textit{Arabidopsis gsnor-3} plants, HR was accelerated in both \textit{GSNOR1} antisense plants and in \textit{agsnor-3} mutant plants upon infection by avirulent bacterial strains or by an avirulent oomycete isolate (Rustérucci et al., 2007; Yun et al., 2011), indicating a conserved negative role of GSNOR1 from different plant species in triggering cell death. Interestingly, accelerated cell death is independent of SA and H\textsubscript{2}O\textsubscript{2}, as the cell death in \textit{gsnor-1/sid2} and \textit{gsnor-3/atrhoD}, \textit{gsnor-3/atrhoF} double mutants, and \textit{gsnor-3/atrhoD/atrhoF} triple mutants displays the same degree of cell death as in \textit{gsnor-1} plants, and H\textsubscript{2}O\textsubscript{2} level is less in \textit{gsnor-3} plants than that in Col-0 plants (Yun et al., 2011). Consistent with this result, we found that the spontaneous cell death occurring on the \textit{NtGSNOR1a/1b} knockout plants was not suppressed by knocking out the \textit{NtEDS1a/1b} (Figure 4), strongly indicating that the spontaneous cell death is SA-independent.

The \textit{NtGSNOR1a/1b} knockout plants displayed increased resistance to TMV but reduced resistance to \textit{Pst} DC3000 (Figure 6). The opposite effects of silencing or knocking out \textit{GSNOR1} on the resistance to different types of pathogens were also observed in \textit{Arabidopsis} (Feechan et al., 2005; Rustérucci et al., 2007; Yun et al., 2011). Whereas, the basal resistance to \textit{Pst} DC3000 and Noco2 race of the \textit{H. parasitica}, RPM1- or RPS4-mediated R resistance to \textit{Pst} DC3000 (\textit{avrB}) and \textit{Pst} DC3000 (\textit{avrps4}), and non-host resistance to wheat powdery mildew pathogen \textit{Bgt} are all compromised in \textit{Arabidopsis gsnor-1/3} mutant (Feechan et al., 2005), an enhanced basal resistance to oomycete \textit{Pp} Noco2 and an increased SAR were observed in \textit{Arabidopsis GSNOR1} antisense plants (Rustérucci et al., 2007). Espunya et al. (2012) attributes these opposite phenotypes to the partial reduction (antisense line) vs. the complete loss of function (knockout mutant) in \textit{GSNOR1} activity. Interestingly, contrary to their own report that various types of resistance are compromised in \textit{gsnor-1/3} mutant plant (Feechan et al., 2005), Yun et al. (2011) recently showed that an enhanced resistance to \textit{Hyaloperonospora arabidopsis} isolate Emw1, which is recognized by RPP4, was observed in \textit{gsnor-1/3} mutant in an SA- and H\textsubscript{2}O\textsubscript{2}-independent manner (Yun et al., 2011). This resistance seemed to be attributed solely to increased SNO levels in \textit{gsnor-1-3} mutant (Yun et al., 2011). Thus, the opposing results in resistance to different types of pathogens observed in \textit{gsnor-1-3} mutant cannot be explained solely by the degree of reduction in GSNOR1 activity. Rather, it reflects the complexity and hierarchy of NO signaling events (Wendehenne et al., 2014).

Multiple proteins involved in defense responses could be differentially or simultaneously S-nitrosylated in response to pathogen infections. Depending on the severity of nitrosative stress, the subcellular compartmentalization of both GSNO and its target proteins, GSNO could either promote or inhibit defense responses. As the functions of the positive regulators of SA-dependent pathway, NPR1, TGA1, and SBP3, and the function of a suppressor of negative regulator of defense responses, SRG1, are negatively regulated by S-nitrosylation (Tada et al., 2008; Wang et al., 2009; Lindermayr et al., 2010; Cui et al., 2018), it is reasonable to assume that the compromised resistance of the \textit{NtGSNOR1a/1b} knockout plants to \textit{Pst} DC3000 (Figure 6C) could also be combined results of compromised SA-mediated resistance and de-repression of immunity inhibition. However, Yun et al. (2011) reported that the cell death triggered by incompatible pathogens in \textit{gsnor-1/3} is SA- and H\textsubscript{2}O\textsubscript{2}-independent and the cell death triggered in \textit{gsnor-1/3} is enough to confer the RPP4-mediated resistance to \textit{Hyaloperonospora arabidopsis} isolate Emw1, suggesting that the resistance is SA-independent (Yun et al., 2011). However, our results indicated that the spontaneous cell death on the \textit{NtGSNOR1a/1b} knockout plants (Figures 1, 2) does not confer bacterial resistance (Figure 6B). Intriguingly, contrary to the response to bacterial infection, an enhanced resistance against TMV was observed in the \textit{NtGSNOR1a/1b} knockout plants (Figure 6A). The increased resistance to TMV could be due to inhibitory effects of the overaccumulated Reactive nitrogen species (RNS) and ROS (Figure 3) on virus replication or movement or both. It is also possible that the viral proteins involved in replication or movement is inhibited by S-nitrosylation. Alternatively, the enhanced resistance of the \textit{NtGSNOR1a/1b} knockout plant to TMV could also be due to the inhibition of negative regulators of defense responses by S-nitrosylation. For example, \textit{Arabidopsis} SUMO E2 enzyme, SCE1, plays a negative role in immune responses. S-nitrosylation SCE1 at Cys\textsuperscript{139} inhibits its SUMO-conjugating activities, leading to immune activation by relieving SUMO1/2-mediated suppression (Skelly et al., 2019). Depending on the cell type, subcellular localization, developmental stages, and plant–pathogen interactions, as well as its diffuse and promiscuous nature, NO can exert differential or even opposite effects on cell death and immunity through both S-nitrosylation-dependent and -independent mechanisms, which might explain the opposite effects of \textit{NtGSNOR1a/1b} knocking out on \textit{Pst} DC3000 and TMV resistance.

The CRISPR/Cas9-mediated genome editing tool has been extensively applied in plant biotechnology since its development (Xie and Yang, 2013; Wang Y. et al., 2014; Wang et al., 2015; Char et al., 2017, 2020; Chen et al., 2019; Oliva et al., 2019). Our results strongly indicated that CRISPR/Cas9 is a powerful technology not only in resolving gene redundancy in tetraploid tobacco (Figures 1, 4) but also in studying the functional connections and/or interactions of different genes (Figure 4).
MATERIALS AND METHODS

Plant Materials
Nicotiana tabacum cv. Samsun, carrying a TMV-resistant gene, N, was used in this study. The tobacco plants were maintained in the growth room at 22°C with a photoperiod of 16-h light/8-h dark.

Agro-Infiltration
Agrobacterium infiltration was performed as described (Liu et al., 2005). Agrobacterium GV3101 strain carrying the 35S::GmMEKK1a construct (Xu et al., 2018), which is known to induce HR, was infiltrated into the leaves of the 30–40-day-old N. tabacum plants as indicated. The 35S::GFP construct was included as a negative control. The leaf was photographed at 2 days post infiltration. The experiment was repeated three times (at least three plants each time) with similar results.

CRISPR/Cas9 Constructs and Plant Transformation

Oligos of 20 nucleotides were chosen from the second exon of NtGSNOR1a/1b (CTGGGAACCCCAAGACGCTC, 45–65 bp downstream of the start codon ATG of the open reading frame, was used as a guide to target both genes simultaneously) and first exon of NtEDS1a/1b (GAAGCTCACAGTTTGTCTTC, 70–92 bp downstream of the start codon ATG) (Figures 1A, 4B). Blast analysis indicated these two guides only perfectly matched the target sequences of NtGSNOR1a/1b and NtEDS1a/1b, respectively, but not any other sequences in the N. tabacum genome. The closest sequences in the genome to both guides displayed at least 4-nt mismatches. Thus, the off-target effects could potentially be ruled out with great probability. Both the sense and antisense strands of the chosen oligos were synthesized with ATTG and AAAC that are compatible to Bsa I sticky ends attached to the sense and antisense strands, respectively. The sense and antisense strands of the synthesized oligos were mixed in TE buffer and annealed to each other by heating at 98°C for 5 min followed by cooling to room temperature. The annealed double-strand oligos were subsequently ligated into the intermediate AtU6-26-sgRNA-SK vector (Yan et al., 2015) predigested with Bsa I. The constructed plasmid was double digested with Nhe I and Spe I (Nhe I and Spe I are isocaudomers), and the fragment of 642 bp containing the guide sequence was ligated into the destination vector pCAMBIA-1300-pYAO:Cas9 predigested with Spe I and treated with alkaline phosphatase (Yan et al., 2015). The authenticity of the final construct was confirmed by sequencing.

For double targets (NtGSNOR1a/1b + NtEDS1a/1b) construct, the Nhe I and Spe I digested fragment from the intermediate vector containing NtEDS1a/1b guide was ligated into the destination vector containing NtGSNOR1a/1b guide digested with Spe I. The authenticity of the final construct was confirmed by sequencing.

The oligos used for target guides are:

NtGSNOR1a/1b-F: 5’-ATTGCTGGGAACCCCAAGACGCTC-3’;
NtGSNOR1a/1b-R: 5’-AAACGAGGCTTTGTTGGTCCAG-3’;
NtEDS1a/1b-F: 5’-ATTGGAAGCTCACAGTTTGTCTTC-3’;
NtEDS1a/1b-R: 5’-AAACGAAGACAAACTGTGAGCTTC-3’.

The CRISPR/Cas9 constructs were transformed into N. tabacum cv. Samsun (NN) as described previously (Horsch et al., 1985). Briefly, fully expanded leaves from 3-week-old tobacco plants were surface-sterilized with 70% ethanol and 0.5–2% NaClO3, respectively. Leaf discs of 0.5 cm in diameter were precultivated in MS medium for 3 days followed by incubating in Agrobacterium (GV3101) suspension solution for 20–30 min. Agrobacterium-infected leaf discs were cultivated in MS medium containing 1 mg/L Indole-3-acetic acid (IAA), 1 mg/L 6-BA in the growth room for 2 days, and then transferred to MS medium supplemented with 1 mg/L IAA, 1 mg/L 6-BA, 50 mg/L Hygromycin, and 25 mg/L rifampicin. Shoots were transferred to rooting medium containing IAA (1 mg/L), 50 mg/L Hygromycin, and 25 mg/L Timetin. The rooted transformants were finally transferred to soil and grown in a growth room.

Screening of the Homozygous CRISPR/Cas9 Lines

The transgenic CRISPR/Cas9 plants generated were characterized by genomic PCR with specific primers listed below and followed by DNA sequencing. DNA was extracted from leaf tissue with the DNA extraction kit (Invitrogen). Sequence results were aligned against the wild type sequence to identify insertions and deletions (InDels). The sequencing chromatograms contained no double peaks but contained InDels indicating homozygous mutations. The double peaks in the chromatograms indicate a heterozygous insertion/deletion or WT/InDels. In this case, the genomic PCR products were cloned into the PMD19-T vector (Takara), and multiple clones were subjected to sequencing to determine the exact nature of InDels. The primers used for the genomic PCR or for sequencing positive clones by colony PCR are:

NtGSNOR1a/1b-F: 5’-CACTACTAGTATACACACTTA-3’
NtGSNOR1a-R: 5’-GTGGTTAAGTTATGAAAATTTTG-3’
NtGSNOR1b-R: 5’-TTAGAAACTCATAAGCCGG-3’
NtEDS1a-F: 5’-CCATCTTTGAGCTGATCAAACTA-3’
NtEDS1a-R: 5’-GGGAACATATTCTTGGGACATG-3’
NtEDS1b-F: 5’-CCATCTTTGAGCTGCTGACAT-3’
NtEDS1b-R: 5’-GGAATTAAGCTGATCAAATG-3’

Tobacco mosaic virus Infection and P. syringae pv. Tomato DC3000 Growth Assay

The properly diluted sap extracted from the TMV (U1 strain) infected leaves was rub-inoculated on the upper surface of the N. tabacum cv. Samsun (NN) leaves, as described previously (Whitham et al., 1994). The HR normally is visible at 2 dpi, and the photos were taken at 4 dpi. The primers used for qRT-PCR quantification of the TMV CP are:

CP-F: GCTCTCGAAAGAGCTCCGAT
CP-R: TTTATCGGCGTCTTTATGGC
The primers for endogenous reference gene NtActin are:

\textit{NtActin-F, TGGCATCACACTTTTCAAA}
\textit{NtActin-R, CCACGTGACCAATAATT}

The Pst DC3000 of OD_{600} = 0.00001 was infiltrated into the leaves of respective tobacco lines, as described previously (Qi et al., 2018). The leaf discs of 0.5 cm in diameter were punched from the infiltrated areas and ground in the 10 mM MgSO\textsubscript{4} buffer. The saps were diluted 10, 100, and 1,000 times and spread on KB agar plates. Colony-forming unit (cfu) was obtained by counting the number of the colonies on a serial of plates.

**H\textsubscript{2}O\textsubscript{2} Detection by 3,3\textquotesingle-Diaminobenzidine Staining**

H\textsubscript{2}O\textsubscript{2} was detected by an endogenous peroxidase-dependent \textit{in situ} histochemical staining procedure using DAB (Sigma-Aldrich; Ren et al., 2002). Leaves of 30–40-day-old plants were detached and placed in a solution containing 1 mg/ml DAB (pH 5.5) for 2 h. The leaves were cleared by boiling in ethanol (96%) for 10 min and then stored in 96% ethanol. H\textsubscript{2}O\textsubscript{2} production was visualized as a reddish-brown precipitate in cleared leaves (Thordal-Christensen et al., 1997; Ren et al., 2002).

**NO Detection by DAF-2DA Staining**

NO detection was performed by DAF-FM DA staining as described (He et al., 2004). Briefly, leaf discs and 15-day-old seedlings were preincubated for 2 h at room temperature in a solution of 0.1 mM CaCl\textsubscript{2}, 10 mM KCl, 10 mM MES-Tris, pH 5, and then stained with 10 \mu M DAF-FM DA for 45 min. After rinsing with water, the sample was observed and photographed with an inverted Axio phot microscope (Zeiss) equipped with a digital camera (Diagnostic Instruments).

**Accession Numbers**

The accession numbers for NtGSNOR1a, NtGSNOR1b, NtEDS1a, and NtEDS1b are LOC107777450, LOC107791841, LOC107782626, and LOC107791841, respectively.

**REFERENCES**

Albertos, P., Romero-Puertas, M. C., Tatematsu, K., Mateos, I., Sánchez-Vicente, I., Nambara, E., et al. (2015). N-sitrolylation triggers AB15 degradation to promote seed germination and seedling growth. \textit{Nat. Commun.} 6:8669. doi: 10.1038/ncomms9669

Char, S. N., Neelakandan, A. K., Nahampun, H., Frame, B., Main, M., Spalding, M. H., et al. (2017). An Agrobacterium-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. \textit{Plant Biotechnol. J.} 15, 257–268. doi: 10.1111/pbi.12611

Char, S. N., Wei, J., Mu, Q., Li, X., Zhang, Z. J., Yu, J., et al. (2020). An Agrobacterium-delivered CRISPR/Cas9 system for targeted mutagenesis in sorghum. \textit{Plant Biotechnol. J.} 18, 319–321. doi: 10.1111/pbi.13229

Chen, K., Wang, Y., Zhanh, R., Zhang, H., and Gao, C. (2019). CRISPR/cas genome editing and precision plant breeding in agriculture. \textit{Annu. Rev. Plant Biol.} 70, 667–697. doi: 10.1146/annurev-arplant-050718-100049

Chen, R., Sun, S., Wang, C., Li, Y., Liang, Y., An, F., et al. (2009). The \textit{Arabidopsis} PARARQUIT RESISTANT2 gene encodes an S-nitrosoglutathione reductase that is a key regulator of cell death. \textit{Cell Res.} 19, 1377–1387. doi: 10.1038/cr.2009.117

Clarke, A., Desikan, R., Hurst, R. D., Hancock, J. T., and Neill, S. J. (2000). NO way back: nitric oxide and programmed cell death in \textit{Arabidopsis} thaliana suspension cultures. \textit{Plant J.} 24, 667–677. doi: 10.1046/j.1365-313x.2000.00911.x

Cui, B., Pan, Q., Clarke, D., Villarreal, M. O., Umbreen, S., Yuan, B., et al. (2018). S-nitrosylation of the zinc finger protein SRG1 regulates plant immunity. \textit{Nat. Commun.} 9:4226. doi: 10.1038/s41467-018-06578-3

de Pinto, M. C., Locato, V., Gogbab, A., Romero-Puertas, M. D. C., Gadaleta, C., Delledonne, M., et al. (2013). S-nitrosylation of ascorbate peroxidase is part of programmed cell death signaling in tobacco bright yellow-2 cells. \textit{Plant Physiol.} 163, 1766–1775. doi: 10.1104/pp.113.222703

Delledonne, M., Xia, Y., Dixon, R. A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. \textit{Nature} 394, 585–588. doi: 10.1038/29087

Delledonne, M., Zeier, J., Marocco, A., and Lamb, C. (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. \textit{Proc. Natl. Acad. Sci. U.S.A.} 98, 13454–13459. doi: 10.1073/pnas.231178298

Desikan, R., Griffiths, R., Hancock, J., and Neill, S. (2002). A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for...
Lamattina, L., Garcia-Mata, C., Graziano, M., and Pagnussat, G. (2012). S-Nitrosoglutathione is a component of wound- and salicylic acid-induced systemic responses in Arabidopsis thaliana. J. Exp. Bot. 63, 3219–3227. doi:10.1093/jxb/erq043

Lee, U., Wie, C., Fernandez, B. O., Feelsich, M., and Vierling, E. (2008). Modulation of nitrosative stress by S-nitrosoglutathione reductase is critical for thermotolerance and plant growth in Arabidopsis. Plant Cell 20, 786–802. doi:10.1105/tpc.107.052647

Lin, A., Wang, Y., Tang, J., Xue, P., Li, C., Liu, L., et al. (2012). Nitric oxide and protein S-nitrosylation are integral to hydrogen peroxide-induced leaf cell death in rice. Plant Physiol. 158, 451–464. doi:10.1104/pp.111.184531

Lindermayr, C., Saalbach, G., Bahnweg, G., and Durner, J. (2006). Differential inhibition of Arabidopsis methionine adenosyltransferases by protein S-nitrosylation. J. Biol. Chem. 281, 4285–4291. doi:10.1074/jbc.M515163200

Lindermayr, C., Saalbach, G., and Durner, J. (2005). Proteomic identification of S-nitrosylated proteins in Arabidopsis. Plant Physiol. 137, 921–930. doi:10.1104/pp.104.058719

Liu, J. Z., Bluancclor, E. B., and Nelson, R. S. (2005). The tobacco mosaic virus 126-kilodalton protein, a constituent of the virus replication complex, alone or within the complex aligns with and traffics along microfilaments. Plant Physiol. 138, 1853–1865. doi:10.1105/tpc.104.058719

Mannick, J. B. (2007). Regulation of apoptosis by protein S-nitrosylation. Amino Acids 32, 523–526. doi:10.1007/s00726-006-0427-6

Matamoros, M. A., Cutròn, M. C., Wienkoop, S., Begara-Morales, J. C., Sandal, N., Orella, I., et al. (2020). Altered plant and nodule development and protein S-nitrosylation in Lotus japonicus mutants deficient in S-nitrosothiol reductases. Plant Cell Physiol. 61, 105–117. doi:10.1093/pcp/pca182

Nieminen, T., Takamatsu, N., Ohno, T., and Okada, Y. (1982). Molecular cloning of the complementary DNA copies of the common and cowpea strains of tobacco mosaic virus RNA. Virology 118, 4–75. doi:10.1016/0042-6822(82)90320-8

Neill, S., Barros, R., Bright, J., et al. (2008). Nitric oxide, stomatal closure, and abiotic stress. J. Exp. Bot. 59, 165–176. doi:10.1093/jxb/erm293

Ni, M., Zhang, L., Shi, Y. F., Wang, C., Lu, Y., Pan, J., et al. (2017). Excessive cellular S-nitrosothiol impair endocytosis of auxin efflux transporter PIN2. Front. Plant Sci. 8:1988. doi:10.3389/fpls.2017.01988

Oliva, R., Ji, C., Atienza-Grande, G., Huguet-Tapia, J. C., Perez-Quintero, A. I., Li, T., et al. (2019). Broad-spectrum resistance to bacterial blight in rice using genome editing. Nat. Biotechnol. 37, 1344–1350. doi:10.1038/s41587-019-0267-z

Ortega-Galisteo, A. P., Rodriguez-Serrano, M., Pazmiño, D. M., Gupta, D., Sandalio, L. M., and Romero-Puertas, M. C. (2012). S-Nitrosylated proteins in Pea (Pisum Sativum L.) leaf peroxisomes: changes under abiotic stress. J. Exp. Bot. 63, 2089–2103. doi:10.1093/jxb/err414

Qi, T., Seong, K., Thomazella, D. P. T., Kim, J. R., Pham, J., Seo, E., et al. (2019). NRGI functions downstream of EDS1 to regulate TIR-NLR-mediated plant immunity in Nicotiana benthamiana. Proc. Natl. Acad. Sci. U.S.A. 115, E19079–E19087. doi:10.1073/pnas.1814561115

Rasul, S., Wendehenne, D., and Jeandrout, S. (2012). Study of oligogalacturonides-triggered nitric oxide (NO) production provokes new questioning about the origin of NO biosynthesis in plants. Plant Signal. Behav. 7, 1031–1033. doi:10.4161/psb.20658

Ren, D., Yang, H., and Zhang, S. (2002). Cell death mediated by MAPK is associated with hydrogen peroxide production in Arabidopsis. J. Biol. Chem. 277, 559–565. doi:10.1074/jbc.M109495200

Romero-Puertas, M. C., Lacra, M., Matté, A., Zanninotto, F., Finkemeier, I., Jones, A. M., et al. (2007). S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. Plant Cell 19, 4120–4130. doi:10.1105/tpc.107.059561

Rustuccii, C., Espunya, M. C., Díaz, M., Chabannes, M., and Martinez, M. C. (2007). S-nitrosoglutathione reductase affords protection against pathogens.
Wang, Y., and Chu, C. (2020). S-Nitrosylation control of ROS and RNS.

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., et al. (2014). Wendehenne, D., Durner, J., and Astier, J. (2013). Nitric oxide and reactive oxygen species in plant biotic interactions. Curr. Opin. Plant Biol. 16, 534–539. doi: 10.1016/j.pbi.2013.06.020

Shi, Y. F., Wang, D. L., Wang, C., Culler, A. H., Kreiser, M. A., Suresh, J., et al. (2015). Loss of GSNOR1 function leads to compromised auxin signaling and polar auxin transport. Mol. Plant 8, 1350–1365. doi: 10.1016/j.molp.2015.04.008

Skelly, M. J., Malik, S. L., Le, B. T., Bo, Y., Jiang, J., Spoel, S. H., et al. (2019). A role for S-nitrosylation of the SUMO-conjugating enzyme SCU1 in plant immunity. Proc. Natl. Acad. Sci. U.S.A. 116, 17990–17995. doi: 10.1073/pnas.1900652116

Stamler, J. S., Simon, D. L., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., et al. (1992). S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc. Natl. Acad. Sci. U.S.A. 89, 444–448. doi: 10.1073/pnas.89.1.444

Suntres, Z. E. (2002). Role of antioxidants in paraquat toxicity. Toxicology 180, 65–77. doi: 10.1016/S0300-488X(02)00382-7

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. Science 321, 952–956. doi: 10.1126/science.1156970

Terrile, M. C., Parri’s, R., Caldero’ n-Villalobos, L. I., Iglesias, M. I., Lamattina, L., Estelle, M., et al. (2012). Nitric oxide influences auxin signaling through S-nitrosylation of the Arabidopsis TRANSPORT INHIBITOR RESPONSE 1 auxin receptor. Plant J. 70, 492–500. doi: 10.1111/j.1365-313X.2011.04885.x

Thordal-Christensen, H., Zhang, Z. G., Wei, Y. D., and Collinge, D. B. (1997). Subcellular localization of H$_2$O$_2$ in plants: H$_2$O$_2$ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J. 11, 1187–1194. doi: 10.1046/j.1365-313X.1997.11061187.x

Wang, L., Guo,Y., Jia, L., Chu, H., Zhou, S., Chen, K., et al. (2014). Hydrogen peroxide acts upstream of nitric oxide in the heat shock pathway in Arabidopsis seedlings. Plant Physiol. 164, 2184–2196. doi: 10.1104/pp.113.229369

Wang, P., Du, Y., Hou, Y. J., Zhao, Y., Hsu, C. C., Yuan, F., et al. (2015). Nitric oxide negatively regulates abscisic acid signaling in guard cells by S-nitrosylation of OST1. Proc. Natl. Acad. Sci. U.S.A. 112, 613–618. doi: 10.1073/pnas.1423481112

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., et al. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol. 32, 947–951. doi: 10.1038/nbt.2969

Wang, Y., and Chu, C. (2020). S-Nitrosylation control of ROS and RNS homeostasis in plants: the switching function of catalase. Mol. Plant 13, 946–948. doi: 10.1016/j.molp.2020.05.013

Wang, Y., Y. make, G. J., and Chu, C. (2013). Cross-talk of nitric oxide and reactive oxygen species in plant programmed cell death. Front. Plant Sci. 4:314. doi: 10.3389/fpls.2013.00314

Wang, Y., Feechan, A., Yun, B. W., Shafii rei, R., Hofmann, A., Taylor, P., et al. (2009). S-Nitrosylation of AtSAPB3 antagonizes the expression of plant immunity. J. Biol. Chem. 284, 2131–2137. doi: 10.1074/jbc.M806782200

Wendehenne, D., Durner, J., and Klessig, D. F. (2004). Nitric oxide: a new player in plant signaling and defense responses. Curr. Opin. Plant Biol. 7, 449–455. doi: 10.1016/j.pbi.2004.04.002

Wendehenne, D., Gao, Q. M., Kachroo, A., and Kachroo, P. (2014). Free radical-mediated systemic immunity in plants. Curr. Opin. Plant Biol. 20C, 127–134. doi: 10.1016/j.xplb.2014.05.012

Xie, K., and Yang, Y. (2013). RNA-guided genome editing in plants using a CRISPR–Cas9 system. Mol. Plant 6, 975–983. doi: 10.1010/mp/s1119

Xu, H. Y., Zhang, C., Li, Z. C., Wang, Z. R., Jiang, X. X., Shi, Y. F., et al. (2018). The MAPK kinase kinase GmMkk1 regulates cell death and defense responses. Plant Physiol. 178, 907–922. doi: 10.1104/pp.18.00903

Xu, Y., Zhou, S., Wang, L., Cheng, Y., and Zhao, L. (2010). Nitric oxide functions as a signal and acts upstream of AtCaM3 in thermotolerance in Arabidopsis seedlings. Plant Physiol. 153, 1895–1906. doi: 10.1104/pp.110.160424

Yan, L., Wei, S., Yu, Y., Hu, R., Li, H., Yang, W., et al. (2015). High-efficiency genome editing in Arabidopsis using YAO promoter-driven CRISPR/Cas9 system. Mol. Plant 8, 1820–1823. doi: 10.1016/j.molp.2015.10.004

Yun, H., Mu, J., Chen, L., Feng, J., Hu, J., Li, L., et al. (2015). S-Nitrosylation positively regulates ascorbate peroxidase activity during plant stress responses. Plant Physiol. 167, 1604–1615. doi: 10.1104/pp.115.255216

Yun, B. W., Feechan, A., Yin, M., Saidi, N. B., Le Bihan, T., Yu, M., et al. (2011). S-Nitrosylation of NADPH oxidase regulates cell death in plant immunity. Nature 478, 264–268. doi: 10.1038/nature10427

Yun, B. W., Skelly, M. J., Yin, M., Yu, M., Mun, B. G., Lee, S. U., et al. (2016). Nitric oxide and S-nitrosogluthatione function additively during plant immunity. New Phytol. 211, 516–526. doi: 10.1111/nph.13903

Zaninotto, F., Camera, S. L., Polverari, A., and Delledonne, M. (2006). Cross talk between reactive nitrogen and oxygen species during the hypersensitive disease resistance response. Plant Physiol. 141, 379–383. doi: 10.1104/pp.106.078857

Zeidler, D., Za hringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., et al. (2004). Innate immunity in Arabidopsis thaliana: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc. Natl. Acad. Sci. U.S.A. 101, 15811–15816. doi: 10.1073/pnas.040536101

Zhan, N., Wang, C., Chen, L., Yang, H., Feng, J., Gong, X., et al. (2018). S-nitrosylation targets GSNO reductase for selective autophagy during hypoxia responses in plants. Molecular. Cell. 71, 142–154. doi: 10.1016/j.molcel.2018.05.024

Zhang, J., and Liu, W. (2019). Protein S-nitrosylation in plant abiotic stresses. Funct. Plant Biol. 47, 1–10. doi: 10.1071/FP19071

Zhou, J., and Zhang, Y. (2020). Plant immunity: danger perception and signaling. Cell. 18, 978–989. doi: 10.1016/j.cell.2020.04.028

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Li, Ren, Guo, Ran, Ren, Wu, Xu, Liu and Liu. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.