The Role of Hydrogen Peroxide and Nitric Oxide in the Induction of Plant-Encoded RNA-Dependent RNA Polymerase 1 in the Basal Defense against *Tobacco Mosaic Virus*

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Abstract

Plant RNA-dependent RNA Polymerase 1 (RDR1) is an important element of the RNA silencing pathway in the plant defense against viruses. RDR1 expression can be elicited by viral infection and salicylic acid (SA), but the mechanisms of signaling during this process remains undefined. The involvement of hydrogen peroxide (H_2O_2) and nitric oxide (NO) in RDR1 induction in the compatible interactions between *Tobacco mosaic tobamovirus* (TMV) and *Nicotiana tabacum*, *Nicotiana benthamiana*, and *Arabidopsis thaliana* was examined. TMV inoculation onto the lower leaves of *N. tabacum* induced the rapid accumulation of H_2O_2 and NO followed by the increased accumulation of *RDR1* transcripts in the non-inoculated upper leaves. Pretreatment with exogenous H_2O_2 and NO on upper leaf led to increased *RDR1* expression and systemic TMV resistance. Conversely, dimethylthiourea (an H_2O_2 scavenger) and 2-(4-carboxyphenyl)- 4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (an NO scavenger) partly blocked TMV- and SA-induced *RDR1* expression and increased TMV susceptibility, whereas pretreatment with exogenous H_2O_2 and NO failed to diminish TMV infection in *N. benthamiana* plants with naturally occurring RDR1 loss-of-function. Furthermore, in *N. tabacum* and *A. thaliana*, TMV-induced H_2O_2 accumulation was NO-dependent, whereas NO generation was not affected by H_2O_2. These results suggest that, in response to TMV infection, H_2O_2 acts downstream of NO to mediate induction of RDR1, which plays a critical role in strengthening RNA silencing to restrict systemic viral infection.

Introduction

Most eukaryotes possess an RNA silencing system as a gene regulation and host defense mechanism. This system is activated in cells by double-stranded (ds) RNA, followed by the cleavage of the inducing RNA into short (21- to 24-nucleotide) fragments. These fragments, in turn, mediate multiple regulatory and defense functions in the cells [1,2]. During this process, RNA-dependent RNA polymerases (RDRs) synthesize dsRNA intermediates, which can be cleaved by different Dicer-like proteins to produce different small interfering RNAs (siRNAs) [3]. Three functionally distinct RDRs have been characterized in tomato, tobacco, and *Arabidopsis* [4–6]. RDR1 has been found to be an element of the RNA silencing pathway in the plant defense against viruses and herbivores [6–9]. Several recent studies profiled virus-specific small interfering RNAs (vsRNAs) using next-generation sequencing platforms and compellingly implicated plant RDR1 in vsRNA

biogenesis and vsRNA-mediated antiviral defense [10–12]. RDR1 activity and gene expression in *Arabidopsis* (AtRDR1) and *Nicotiana tabacum* (NtRDR1, the ortholog of AtRDR1) are elicited by viral infection and by salicylic acid (SA) treatment [7,8]. After infection with a strain of *Potato virus X* (PVX), which does not spread in wild-type tobacco plants, transgenic *NtRDR1* antisense plants accumulated the virus and developed symptoms locally in inoculated lower leaves and systemically in non-inoculated upper leaves [7]. A follow-up study also found that a T-DNA insertion AtRDR1 mutant exhibited markedly enhanced susceptibility to *Tobacco mosaic tobamovirus* (TMV) and *Tobacco rattle virus* [8]. *Nicotiana benthamiana* possesses an *RDR1* gene that contains a 72-nucleotide insert with consecutive in-frame stop codons in the open reading frame and lacks active virus- and SA-inducible function [6]. This natural loss-of-function mutation was named *NbRDR1m* and was suggested to account for the plant's hypersusceptibility to viruses. However, *N. benthamiana* transformed with the SA-inducible *RDR1*

gene from *Medicago truncatula* (*MtRDR1*) stilled showed enhanced resistance to tobamoviruses (e.g., TMV, *Turnip vein-clearing virus*, and *Sunn hemp mosaic virus*) but not to viruses outside of the tobamovirus genus (e.g., *Cucumber mosaic virus* (CMV) and PVX) [6]. These results strongly suggest that virus- and SA-inducible RDR1 plays an important role in the plant antiviral defense. However, the details of RDR1 induction and signal transduction during this process remain largely unknown.

In plants, one of the earliest host plant responses to pathogen invasion is an oxidative burst, in which the levels of reactive oxygen species (ROS) increase rapidly [13,14]. ROS are believed to perform multiple roles during plant defense responses to microbial attack by acting directly in the initial defense and, possibly, serving as central cellular signaling molecules [15]. In particular, hydrogen peroxide (H_2O_2) is the most attractive candidate for a ROS signal because of its relatively long half-life and high permeability across membranes [16]. H_2O_2 may also play a role in the signaling cascade of virus- and SA-induced RDR1 activation in plants. This idea is supported by several lines of evidence. First, SA application and virus infection resulted in increased H_2O_2 concentration, and both are well known RDR1 elicitors [15,17,18]. Second, the first identified SA binding protein was a catalase (CAT, EC 1.11.1.6) from tobacco. CAT activity was inhibited by SA in vitro and in vivo, resulting in the generation of H_2O_2, which mediated the SA-induced defense response, whereas several antioxidants suppressed the SA-mediated activation of *PR-1* expression and plant defense [15,19]. Third, a recent study in *Nicotiana glutinosa* plants showed that exogenous H_2O_2 induced a significant increase in *NgRDR1* transcripts [13].

In addition to H_2O_2, other reactive compounds are also involved in signaling in plants. Nitric oxide (NO) is an emerging essential regulatory molecule in plant immunity and has been well studied. Often, the production of NO and H_2O_2 overlaps both spatially and developmentally [20,21]. Importantly, H_2O_2 and NO can react with each other and may influence the activities of enzymes that alter each other's levels. NO donors caused rapid H_2O_2 accumulation in *N. tabacum* [22]. In contrast, a NO accumulation mutant *noe1* was also identified that has a defect in a functional rice CAT, resulting in increased H_2O_2 in the leaves, which consequently promoted NO production via activation of nitrate reductase [23]. Similarly, in guard cells of *Arabidopsis* plants, NO synthesis and stomata closure in response to abscisic acid (ABA) were severely reduced in the H_2O_2-generation-related NADPH oxidase double mutant *atrbohD/F* [24]. Furthermore, a previous study also showed that virus infection and SA activated NO synthesis [25,26]. Collectively, these studies suggest that H_2O_2 and NO generation may be related to virus- and SA-induced RDR1 induction in plants. However, the potential cross talk between H_2O_2 and NO, as well as its function in plant antiviral processes, has not yet been resolved.

In the present study, using pharmacological and biochemical approaches, we investigated the potential role of H_2O_2 and NO in virus- and SA-induced RDR1 activation and RDR1 function in the compatible interactions between TMV and the model host plants *N. tabacum*, *N. benthamiana*, and *Arabidopsis*. We found that RDR1 expression was elevated by H_2O_2 and NO donors, whereas TMV- and SA-induced RDR1 transcript levels were compromised by H_2O_2 and NO scavengers in all three plant species. Employing *Arabidopsis* mutants that were defective in H_2O_2 or NO synthesis and transgenic *N. benthamiana* plants transformed with SA-inducible *MtRDR1* from *M. truncatula* [6], we showed that H_2O_2 may function downstream of NO and may mediate the induction of RDR1 in response to TMV challenge, thereby limiting the systemic infection and accumulation of the virus. These results

provide initial insights into the signaling mechanisms underpinning virus- and SA-induced *RDR1* activation in host plants.

Results

Changes of *NtRDR1* expression and H_2O_2 and NO accumulation in *N. tabacum* after TMV inoculation

NtRDR1 relative expression and the quantity of H_2O_2 and NO were determined in the compatible interactions between TMV and *N. tabacum* plants (Figure 1). In non-inoculated upper leaves, *NtRDR1* relative expression began to increase 2 days post inoculation (dpi) and reached a maximum induction of 3.7-fold at 4 dpi. Concomitant with the induction of *NtRDR1* expression, we observed a rapid increase in the levels of H_2O_2 and NO signaling compounds, which resulted in significant differences over mock-treated plants at 1 and 0.5 dpi, respectively. Furthermore, the increased relative expression of *NtRDR1* and increased concentration of H_2O_2 and NO resulting from TMV inoculation remained high until the end of the experiment. At 7 dpi, the relative expression of *NtRDR1* and the concentration of H_2O_2 and NO were 2.2-, 1.0-, and 1.1-fold higher than those in mock-inoculated plants, respectively.
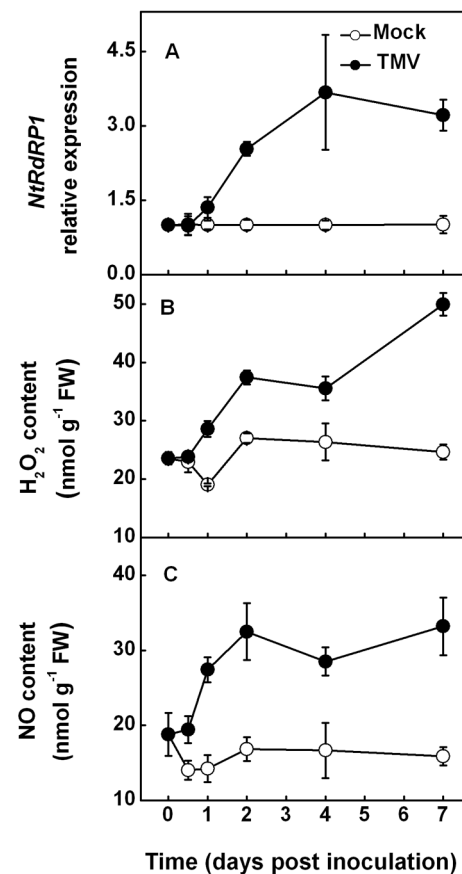
Figure 1. Time course changes of *NtRDR1* transcripts and concentrations of H_2O_2 and NO. Changes in the transcript levels of *Nicotiana tabacum* RNA-dependent RNA Polymerase 1 (*NtRDR1*, **A**), hydrogen peroxide (H_2O_2, **B**), and nitric oxide (NO, **C**) over time in the non-inoculated upper leaves of *Nicotiana tabacum* plants after TMV inoculation of the lower leaves. The results are expressed as the mean ± SD, *n* = 4.
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TMV- and SA-induced *RDR1* induction is enhanced by exogenous H$_2$O$_2$ and NO, and is reversed by scavengers

To explore the possible involvement of H$_2$O$_2$ and NO in *RDR1* induction, we pretreated non-inoculated upper leaves of *N. tabacum* plants with dimethylthiourea (DMTU, a H$_2$O$_2$ and OH· scavenger) and 2-(4-carboxyphenyl)-4,4,5,5- tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, a NO scavenger) 12 hours before TMV inoculation. TMV-induced *NtRDR1* expression was compromised by DMTU and cPTIO application, showing a reduction of approximately 50% with both chemical modulators at 4 dpi (Figure 2A). Next, we applied the exogenous H$_2$O$_2$, the NO chemical donor sodium nitroprusside (SNP), and SA to *N. tabacum* leaves, and *NtRDR1* expression was analyzed throughout a 2-day experiment. Exogenous H$_2$O$_2$ and SNP induced a significant increase in *NtRDR1* transcript levels (Figure 2B). Furthermore, the SA-induced increase in *NtRDR1* transcript levels was substantially suppressed by elimination of H$_2$O$_2$ and NO.

To determine the involvement of H$_2$O$_2$ and NO in TMV- and SA-induced *RDR1* in other plants, the same experiments were also performed with *N. benthamiana* and *Arabidopsis* plants. Similar to the results obtained with *N. tabacum*, DMTU and cPTIO significantly reduced TMV- and SA-induced *NbRDR1m* and *AtRDR1* relative expression (Figure 3), whereas exogenous H$_2$O$_2$ and SNP application resulted in increased *NbRDR1m* and *AtRDR1* transcript levels.

TMV-induced H$_2$O$_2$ synthesis and associated *RDR1* expression are reduced by NO elimination and reversed by exogenous NO application, but H$_2$O$_2$ does not influence TMV-induced NO evolution

To study the relationship between H$_2$O$_2$ and NO in *RDR1* induction under TMV inoculation conditions, we treated the non-inoculated upper leaves of *N. tabacum* plants with DMTU and cPTIO, followed by inoculation with TMV inoculum on the lower leaves. At 2 dpi, a confocal laser scanning microscope (CLSM) was used to detect changes in H$_2$O$_2$ in the leaves. There was an increase in the green fluorescence H$_2$O$_2$ signal in TMV-inoculated plants compared to mock-inoculated plants (Figure 4A). The TMV-induced H$_2$O$_2$ accumulation was greatly suppressed by its scavenger DMTU. Interestingly, the NO scavenger cPTIO also completely inhibited H$_2$O$_2$ accumulation (Figure 4A). In contrast, NO quantification using Griess reagent showed that NO

generation from TMV attack was only http://dict.cnki.net/dict _result.aspx? searchword = %e5%bd%bb%e5%ba%95&tjType = sentence&style = &t = thoroughlyblocked by cPTIO and not by H$_2$O$_2$ elimination (Figure 4B).

To establish a more direct relationship between H$_2$O$_2$ and NO in TMV-induced *RDR1* expression, we collected the *Arabidopsis* H$_2$O$_2$ synthetic enzymatic mutant *atrbohD*, the mutant *atnoa1* that is indirectly impaired in NO synthesis, and the wild-type Columbia (Col-0) ecotype. We pretreated the plants with exogenous H$_2$O$_2$ and SNP on the non-inoculated upper leaves followed by TMV inoculation on the lower leaves. The *in planta* concentration of H$_2$O$_2$ and NO as well as *RDR1* transcript levels in the non-inoculated upper leaves were analyzed at 2 dpi. In wild type Col-0 plants, pretreatment with either H$_2$O$_2$ or the NO donor SNP resulted in a significant increase in H$_2$O$_2$ histochemical staining compared to untreated plants (Figure 5), whereas the NO concentration was elevated by SNP pretreatment but not by exogenous H$_2$O$_2$. As expected, TMV-induced H$_2$O$_2$ accumulation was abolished in *atrbohD* http://www.arabidopsis.org/ servlets/TairObject?id = 132871&type = locusplants and was increased when exogenous H$_2$O$_2$ was applied. However, this effect was not observed in SNP-pretreated plants. At the same time, the NO concentration in *atrbohD* http://www.arabidopsis.org/ servlets/TairObject?id = 132871&type = locusplants was comparable to that in Col-0 plants, and no significant increase was observed after exogenous H$_2$O$_2$ treatment. In contrast, the H$_2$O$_2$ concentration in *atnoa1* http://www.arabidopsis.org/servlets/ TairObject?id = 40002&type = locusplants was greatly reduced compared to that in Col-0 plants. However, the H$_2$O$_2$ concentration increased when either exogenous H$_2$O$_2$ or SNP was applied. The impaired NO accumulation in *atnoa1* plants recovered to levels observed in the Col-0 control level after SNP application but showed no significant changes in response to exogenous H$_2$O$_2$ treatment. Furthermore, the changes of *in planta* H$_2$O$_2$ and NO concentration were closely related to *RDR1* induction. In contrast to the further increase in TMV-induced *AtRDR1* expression by exogenous H$_2$O$_2$ and SNP in Col-0 plants, *AtRDR1* transcript levels in the leaves of *atrbohD* http://www. arabidopsis.org/servlets/TairObject?id = 132871&type = locusand *atnoa1* http://www.arabidopsis.org/servlets/TairObject?id = 40002&type = locusplants showed a decrease of approximately 70% compared to the levels in Col-0 leaves. The lower *AtRDR1* transcript levels
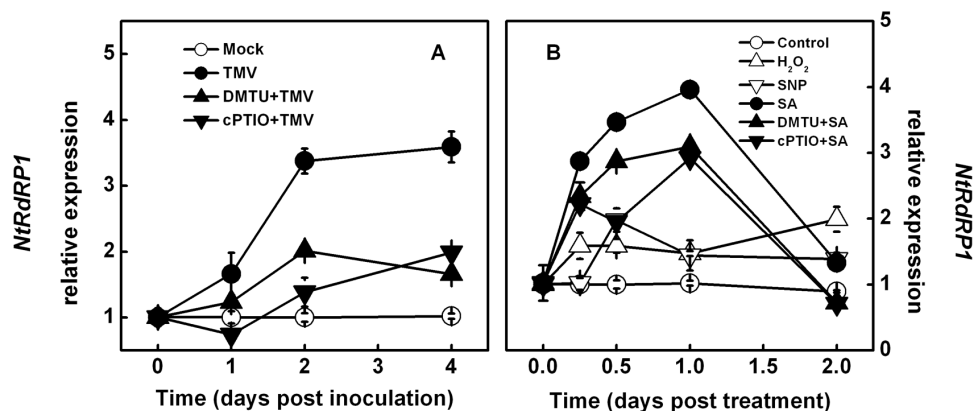

Figure 2. Effects of TMV infection, SA, and different chemical pretreatments on *NtRDR1* transcripts. A. Changes in TMV-induced *NtRDR1* transcript levels over time in non-inoculated upper leaves induced by hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO) scavengers. The non-inoculated upper leaves were subjected to DMTU (H$_2$O$_2$ scavenger) or cPTIO (NO scavenger) pretreatment 12 hours before TMV inoculation. **B**. Changes in SA-induced *NtRDR1* transcript levels over time caused by exogenous H$_2$O$_2$, NO, and their scavengers. The results are expressed as the mean ± SD, *n* = 4.
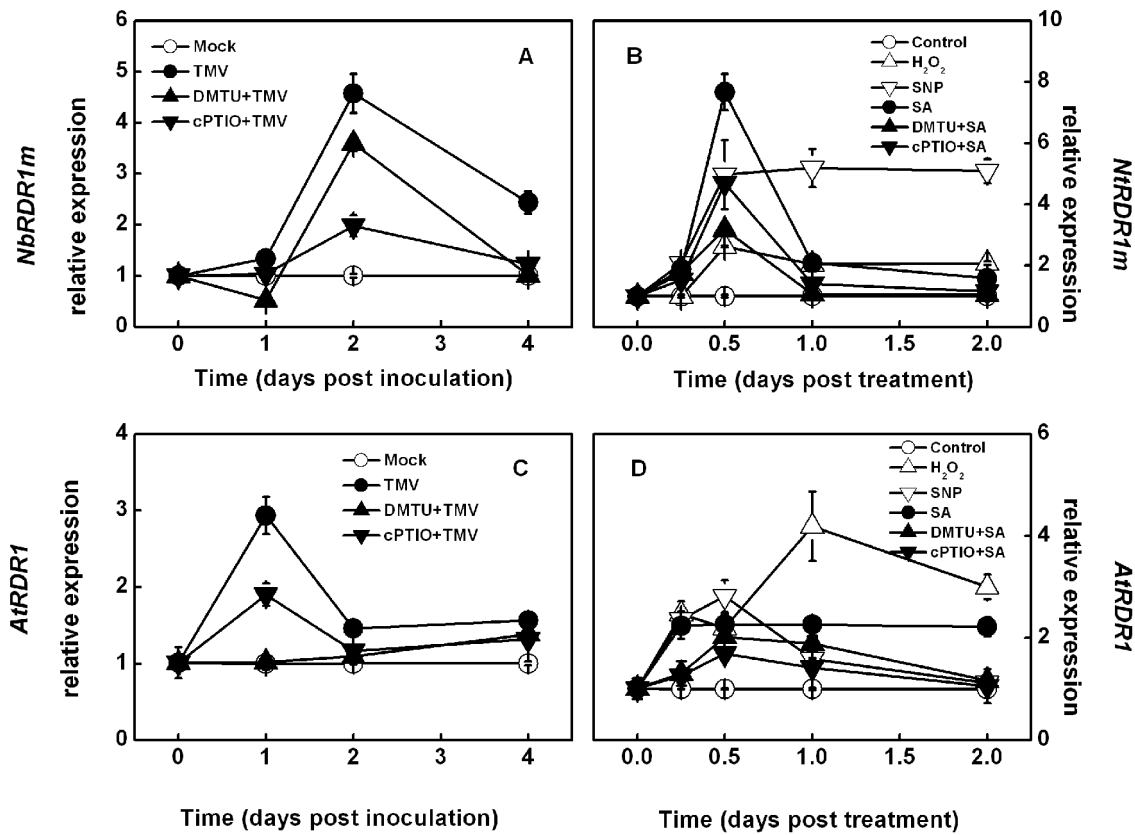doi:10.1371/journal.pone.0076090.g002

Figure 3. Effects of TMV infection, SA, and different chemical pretreatment on transcripts of *NbRDR1m* and *AtRDR1*. A and **C**. Changes in TMV-induced *NbRDR1m* (**A**) and *AtRDR1* (**C**) transcript levels over time in non-inoculated upper leaves induced by scavengers of hydrogen peroxide (H_2O_2) and nitric oxide (NO). The non-inoculated upper leaves were subjected to DMTU (H_2O_2 scavenger) or cPTIO (NO scavenger) pretreatment 12 hours before TMV inoculation. **B** and **D**. Changes in SA-induced *NbRDR1m* (**B**) and *AtRDR1* (**D**) transcript levels over time induced by exogenous H_2O_2, NO, and their scavengers. The results are expressed as the mean $\pm$ SD, $n = 4$.
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in these mutants were greatly increased with exogenous H_2O_2 treatment, whereas SNP pretreatment only increased *AtRDR1* transcript levels in *atnoa1*http://www.arabidopsis.org/servlets/TairObject?id = 40002&type = locusplants and not *atrbohD* http://www.arabidopsis.org/servlets/TairObject?id = 132871&type = locus plants.

Function of H₂O₂- and NO-associated *RDR1* induction in the basal antiviral defense

We then tested whether the H_2O_2- and NO-associated *RDR1* induction functions in the basal defense against TMV inoculation. Exogenous H_2O_2 and NO chemical modulators were applied on the non-inoculated upper leaves of *N. tabacum* plants 12 hours prior to TMV inoculation. The symptoms of systemic leaves were then photographed at 27 dpi (Figure 6A). The TMV infection clearly resulted in visible damage with necrotic lesions and crinkling of leaves. Pretreatment with H_2O_2 or SNP significantly decreased TMV susceptibility, and these leaves did not show any visible systemic symptoms. In contrast, DMTU or cPTIO pretreatment substantially increased the TMV susceptibility and led to the development of enhanced necrotic lesions and crinkling of leaves. Moreover, the levels of *TMV-CP* mRNA in non-inoculated upper leaves analyzed by RT-PCR at 7 dpi correlated highly with the observed symptoms (Figure 6B). Compared to plants that received the TMV challenge alone, H_2O_2 and SNP pretreatment reduced *TMV-CP* mRNA by 75% and 98%,

respectively, whereas DMTU and cPTIO pretreatment markedly increased these levels.

To test whether H_2O_2- and NO-associated RDR1 has a general function in different species, we also applied exogenous H_2O_2 and NO to transgenic *N. benthamiana* plants transformed with the SA-inducible *MtRDR1* gene from *M. truncatula* or an empty vector (EV) as a control. A chlorophyll fluorescence imaging method was used to analyze the response of the maximum photochemical efficiency of PSII in the dark-adapted state (Fv/Fm) to the H_2O_2 and NO chemical modulators in TMV-infected *N. benthamiana* plants. At 7 dpi, TMV inoculation resulted in a significantly lower Fv/Fm in the central leaves of EV control plants relative to their *MtRDR1* transgenic counterpart (Figure 7A), which indicated that TMV damaged EV plants more severely than it damaged *MtRDR1*-transformed plants. Furthermore, there were no evident effects of H_2O_2 or NO on TMV susceptibility in EV and transgenic plants. *TMV-CP* transcripts assayed at the same time showed the same pattern as the chlorophyll fluorescence imaging (Figure 7B), which showed that without the significant effects of H_2O_2 or SNP, the *TMV-CP* mRNA level in non-inoculated upper leaves of EV plants was approximately 2-fold higher than that in leaves of *MtRDR1* transformed plant.

Wild-type Col-0 *Arabidopsis* was also pretreated with H_2O_2 and SNP followed by inoculation with crucifer tobamovirus, which is related to TMV (TMV-cg, a strain of *Turnipvein-clearing virus*). As shown in Figure 8, in non-inoculated upper leaves, H_2O_2 and SNP pretreatment greatly reduced TMV-cg mRNA levels at 4 and 6
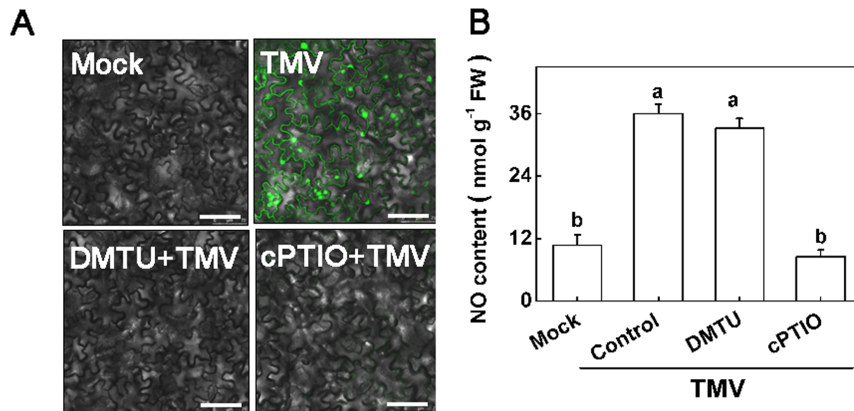
Figure 4. Effects of scavengers of H₂O₂ and NO on TMV-induced H₂O₂ and NO accumulation in *Nicotiana tabacum*. The non-inoculated upper leaves were subjected to DMTU (H₂O₂ scavenger) or cPTIO (NO scavenger) pretreatment 12 hours before TMV inoculation. **A**. Representative H₂O₂ visualization using H₂DCF-DA and a confocal microscope in which H₂O₂ exhibited green fluorescence. All images are at the same magnification and the white scale bar indicates 75 μm. **B**. NO assayed with Griess reagent. The results are expressed as the mean ± SD, *n* = 4. The letters indicate significant differences between the treatments (*P*<0.05).
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dpi compared to untreated plants that received TMV inoculation alone.

Discussion

RDR1, a key enzyme in viral resistance, is known to be induced by viruses and SA [6,8,27,28]. In the present study, we showed that *RDR1* transcript activation was accompanied by H₂O₂ and NO accumulation after TMV challenge in *N. tabacum*, *N. benthamiana*, and *Arabidopsis* plants. Chemical scavenging of H₂O₂ and NO partly blocked TMV- and SA-induced *RDR1* expression and increased TMV susceptibility. Furthermore, TMV-induced H₂O₂ synthesis and the associated *RDR1* expression were reduced by NO elimination and reversed by exogenous NO application,
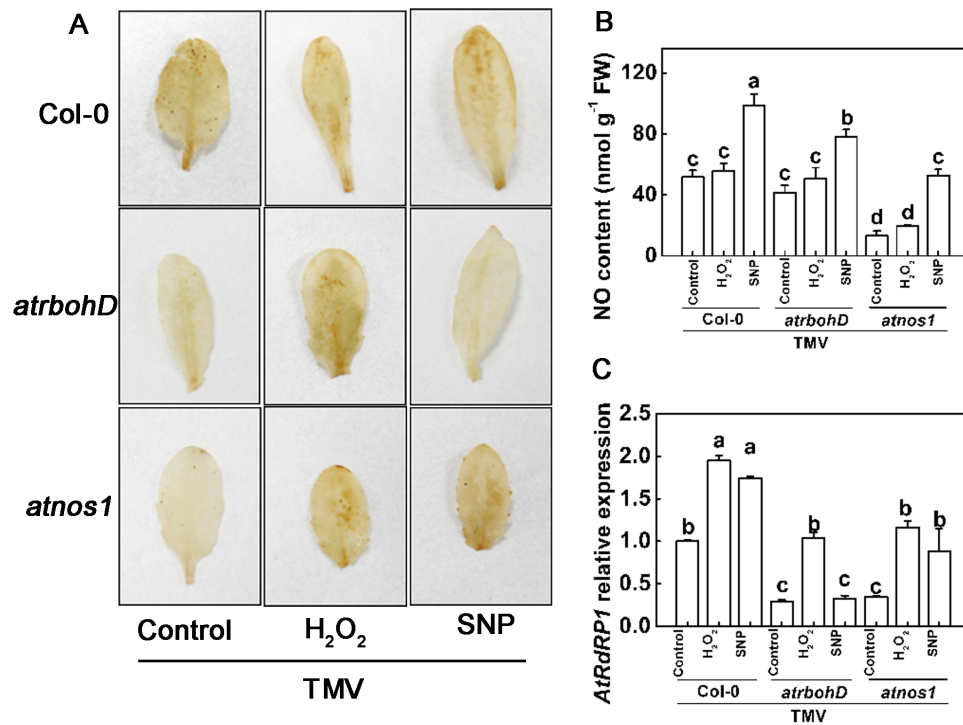

Figure 5. Relationship of H₂O₂, NO, and *AtRDR1* transcripts in wild-type and mutant *Arabidopsis* plants. Effects of TMV inoculation and exogenous application of hydrogen peroxide (H₂O₂) and nitric oxide (NO) on endogenous H₂O₂ and NO concentrations and on transcript levels of *RNA-dependent RNA Polymerase 1* in non-inoculated upper leaves of wild-type and mutant *Arabidopsis* plants (*AtRDR1*). The non-inoculated upper leaves were pretreated with H₂O₂ or SNP 12 hours before TMV inoculation, and leaf samples were collected at 2 days post inoculation. **A**. H₂O₂ detection using DAB histochemical staining. **B**. NO concentration. **C**. *AtRDR1* transcripts. The results are expressed as the mean ± SD, *n* = 4. The letters indicate significant differences between the treatments (*P*< 0.05).
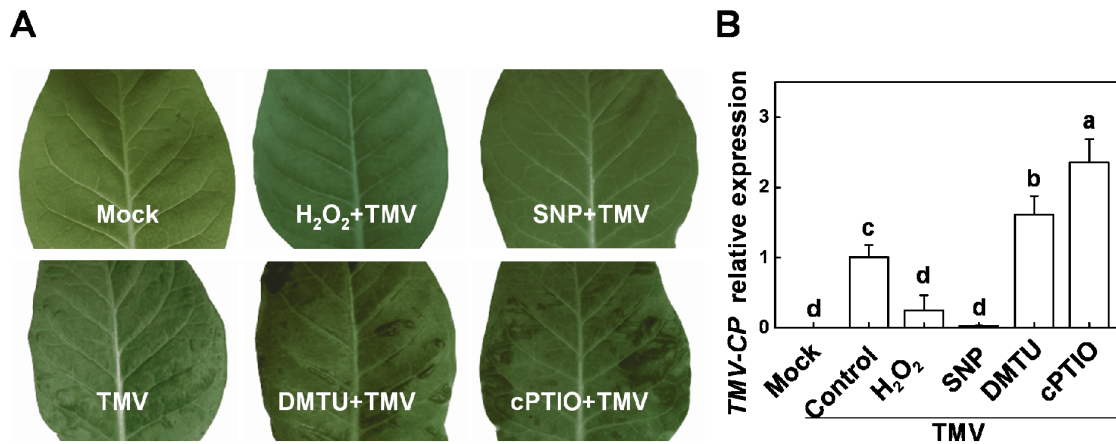doi:10.1371/journal.pone.0076090.g005

Figure 6. Effects of chemical pretreatments on TMV infection in *Nicotiana tabacum* plants. Effects of different chemical pretreatments and TMV infection on TMV symptom development and *TMV-CP* transcription in the non-inoculated upper leaves of *Nicotiana tabacum* plants. The non-inoculated upper leaves were subjected to application of hydrogen peroxide (H_2O_2) or nitric oxide (NO) chemical modulators 12 hours before TMV inoculation. **A**. Symptoms were photographed at 27 days after inoculation. **B**. *TMV-CP* transcript levels were analyzed by real-time reverse-transcription polymerase chain reaction at 7 days after inoculation. The results are expressed as the mean $\pm$ SD, $n = 4$. The letters indicate significant differences between the treatments ($P < 0.05$).
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but H_2O_2 removal had no effect on TMV-induced NO evolution. Therefore, our results indicate that H_2O_2 may act downstream of NO to up-regulate *RDR1*, which contributes to the restriction of systemic infection and virus accumulation. This study provides initial insights into the signaling mechanisms underpinning virus- and SA-induced RDR1 activation in host plants.

Involvement of H_2O_2 and NO in TMV- and SA-induced *RDR1* induction

H_2O_2 and NO are secondary molecules induced by pathogen invasion to activate defense expression and mediate SA defense responses in processes called oxidative bursting and NO bursting,

respectively [18,20,29]. In this study, we observed a rapid and significant accumulation of H_2O_2 and NO in *N. tabacum* plants (Figure 1). Interestingly, this TMV-induced generation of NO and H_2O_2 was followed by *NtRDR1* transcript induction in short succession (Figure 1). These results indicate a possible connection between TMV-induced H_2O_2 and NO production and the induction of *RDR1* expression. Furthermore, in agreement with a previous study on *N. glutinosa* plants where exogenous H_2O_2 induced transcript expression of *NgRDR1* [13], we observed that exogenous H_2O_2 and NO induced *NtRDR1* transcript expression in *N. tabacum*, and TMV-induced *NtRDR1* expression was strongly suppressed by DMTU and cPTIO application. As the important
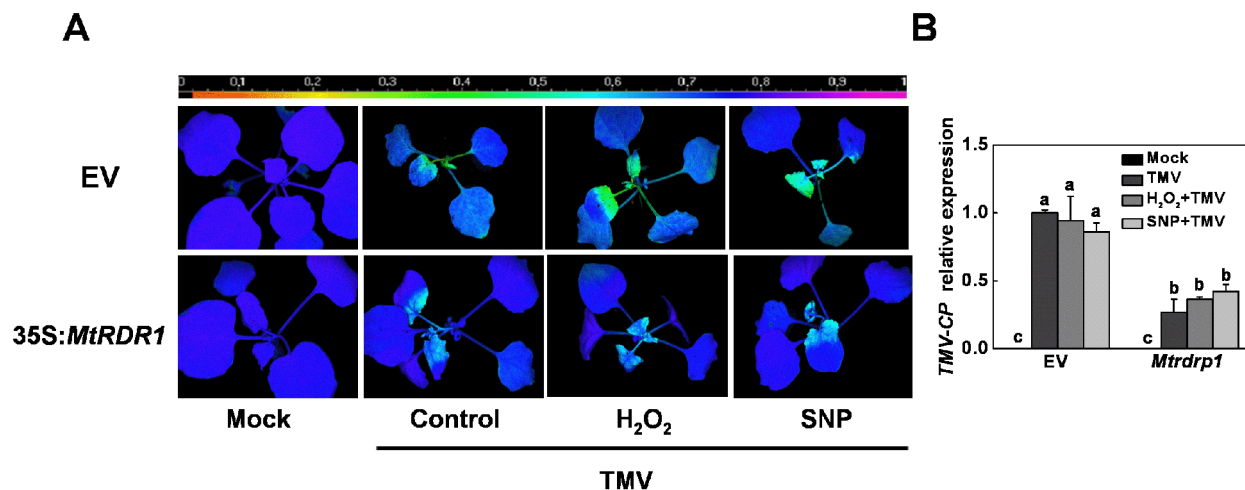

Figure 7. Effects of exogenous H_2O_2 and NO on TMV infection in *Nicotiana benthamiana* plants. Effects of exogenous application of hydrogen peroxide (H_2O_2) and nitric oxide (NO) and TMV inoculation on the maximum photochemical efficiency of photo system II (Fv/Fm) and *TMV-CP* gene transcription in the non-inoculated upper leaves of empty vector (EV)-transformed and *MtRDR1*-transformed *Nicotiana benthamiana* plants. The non-inoculated upper leaves were pretreated with hydrogen peroxide (H_2O_2) or nitric oxide (NO) 12 hours before TMV inoculation. **A**. Chlorophyll fluorescence images of the maximum quantum yield of PSII (Fv/Fm) at 7 days post inoculation. The color gradient scale at the top indicates the magnitude of the fluorescence signal represented by each color. **B**. *TMV-CP* transcript levels analyzed by real-time reverse-transcription polymerase chain reaction at 7 days post inoculation. The results are expressed as the mean $\pm$ SD, $n = 4$. The letters indicate significant differences between the treatments ($P < 0.05$).
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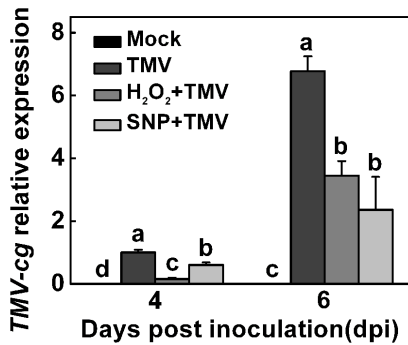
Figure 8. Effects of exogenous H$_2$O$_2$ and NO on TMV infection in *Arabidopsis* plants. Effects of exogenous application of hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO) and TMV inoculation on *TMV-cg* transcription in non-inoculated upper leaves of *Arabidopsis* plants. The non-inoculated upper leaves were pretreated with H$_2$O$_2$ or SNP 12 hours before TMV inoculation. *TMV-cg* transcript levels were analyzed by real-time reverse-transcription polymerase chain reaction at 4 and 6 days post inoculation (dpi). The results are expressed as the mean ± SD, n = 4. The letters indicate significant differences between the treatments ($P < 0.05$).
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plant signaling molecule involved in defense responses to pathogen attack, SA has also been found to elicit plant *RDR1* expression [6–8], and in this study, the expression of SA-induced *NtRDR1* was also compromised by H$_2$O$_2$ and NO scavengers. These results highlight the indispensable roles of H$_2$O$_2$ and NO in TMV- and SA-induced *RDR1* induction in *N. tabacum* plants. However, *NtRDR1* induction was not fully abolished by DMTU or cPTIO treatment (Figure 2); thus, additional unknown H$_2$O$_2$- and NO-independent pathways in the RDR1 signaling pathway may also exist.

The characteristics of H$_2$O$_2$ and NO involvement in TMV- and SA-induced *NtRDR1* expression in *N. tabacum* are similar in *N. benthamiana* and *Arabidopsis* plants (Figure 3), although the magnitude of induction/suppression by exogenous H$_2$O$_2$, NO, and their scavengers differed among species. In *Arabidopsis*, *AtRDR1* expression in *atrbohD* and *atnoa1* mutants was only 20% of the expression in Col-0 plants in response to TMV challenge (Figure 5C), which further emphasizes the role of H$_2$O$_2$ and NO in *RDR1* induction. Collectively, H$_2$O$_2$ and NO mediate TMV- and SA-induced *RDR1* activation in all three model host plants, and this may be a general mechanism in all plants.

Relationship between H$_2$O$_2$ and NO in the *RDR1* induction signaling pathway

In *N. tabacum*, the NO scavenger cPTIO completely inhibited H$_2$O$_2$ accumulation by TMV inoculation (Figure 4). Conversely, H$_2$O$_2$ elimination did not affect TMV-induced NO generation. These observations suggest a possible crosstalk between H$_2$O$_2$ and NO, and NO may act upstream of H$_2$O$_2$ in modulating TMV-induced *RDR1* activation. This possibility was also supported by time-course data showing that NO generation preceded H$_2$O$_2$ induction in *N. tabacum* plants in response to TMV infection (Figure 1). This signaling pathway also seems to exist in *Arabidopsis* plants, as H$_2$O$_2$ accumulation was sensitive to NO, whereas NO generation was not affected by H$_2$O$_2$ under TMV-inoculated conditions using http://www.arabidopsis.org/servlets/TairObject?id = 40002&type = locusatrbohD and *atnoa1* http://www.arabidopsis.org/servlets/TairObject?id = 132871&type = locusmutants (Figure 5). Furthermore, *in planta* H$_2$O$_2$ concentration changes were closely related to *AtRDR1* induction, and lower *AtRDR1*

transcript levels in both *atrbohD* http://www.arabidopsis.org/servlets/TairObject?id = 132871&type = locusand *atnoa1* mutants were greatly increased by exogenous H$_2$O$_2$ treatment. In contrast, SNP pretreatment only increased *AtRDR1* transcript levels in *atnoa1*http://www.arabidopsis.org/servlets/TairObject?id = 40002&type = locus plants and not in *atrbohD* plants. Therefore, TMV-induced NO likely triggers H$_2$O$_2$ accumulation, which plays a role in inducing plant RDR1 activation.

Exactly how NO cooperates with H$_2$O$_2$ to induce RDR1 remains unknown. NO inhibits CAT and ascorbate peroxidase (APX) activity to increase the level of H$_2$O$_2$ [30]. It has been proposed that periodic H$_2$O$_2$ accumulation can be attributed to the inhibition of CAT and APX by a strong early NO burst, which is accompanied by a following wave of secondary NO generation to enhance defense responses to pathogen stress [31]. H$_2$O$_2$ may function as a downstream amplifier to spread the signal and induce the defense response. Thus far, it is not known whether a direct interaction between H$_2$O$_2$ and *RDR1* occurs. Alternatively, H$_2$O$_2$ may activate downstream signal transduction such as the mitogen-activated protein kinase (MAPK) cascade and transcription factors to modify *RDR1* expression [32,33]. Nevertheless, H$_2$O$_2$ and NO have been reported to interact in a variety of patterns, and although some studies have shown that NO treatment can induce the production of H$_2$O$_2$ [22], other studies have shown opposite results. For example, in cucumber plants, NO acted downstream of H$_2$O$_2$ in brassinosteroid-induced abiotic stress tolerance [21]. Thus, the relationship between H$_2$O$_2$ and NO in signal transduction may be more complicated than the simple linear manner in which H$_2$O$_2$ induces NO or vice versa [21]. Thus, the relationship between H$_2$O$_2$ and NO in signal transduction may be more complicated than a simple linear relationship in which H$_2$O$_2$ induces NO or vice versa [21]. In addition, H$_2$O$_2$ and NO may crosstalk differently under different stresses, species, and plant status. Further experiments are still needed to fully establish the relationship between H$_2$O$_2$ and NO and its possible function in RDR1 induction in different plants.
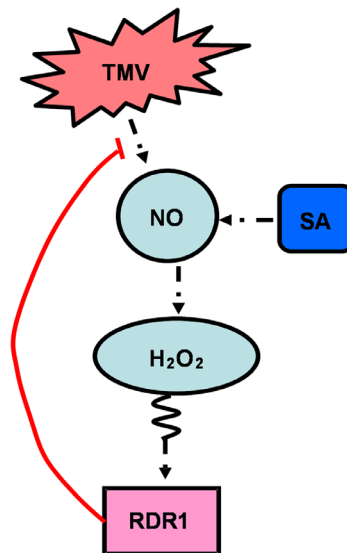

Figure 9. A hypothetical model for the role of H$_2$O$_2$ and NO in the induction of RDR1. Black dashed lines indicate positive interactions; red solid line indicates negative regulation.
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H₂O₂- and NO-associated *RDR1* induction functions in the basal defense against TMV infection

In this study, H_2O_2 and NO assisted in defending against viral attack in *N. tabacum* and *Arabidopsis*, and DMTU and cPTIO significantly increased TMV susceptibility in *N. tabacum* (Figures 6 and 8). These results imply that H_2O_2- and NO-associated RDR1 induction may play a key role in the anti-TMV defense. Furthermore, TMV attack damaged EV plants more severely than it damaged *MtRDR1*-transformed *N. benthamiana* plants (Figure 7), and the natural loss of RDR1 function in *N. benthamiana* did not allow H_2O_2 and NO to diminish TMV infection in these plants, even though *NbRDR1m* was induced by exogenous H_2O_2 and NO. There were also no evident effects of H_2O_2 and NO on TMV susceptibility in transgenic *N. benthamiana* plants, as the *MtRDR1* gene was constitutively expressed under the 35S promoter in the transgenic plants (Figure 7). These results highlight the primary function of H_2O_2- and NO-associated RDR1 in the antiviral mechanism, which is contingent on functional RDR1. RDR1 was found to be involved in separate but overlapping viral resistance and post-transcriptional gene silencing mechanisms in plants [34]. A recent study using siRNA deep sequencing revealed that *rdr1-1* plants contained dramatically fewer viral siRNAs in a short time after the inoculation of TMV-cg, which clearly suggests that RDR1 contributes to the biosynthesis of TMV siRNAs [11]. AtRDR1-dependent production of CMV siRNA has also been observed when the silencing suppressor of CMV is absent [10]. In the present study, H_2O_2 and NO pretreatment induced a higher level of the initial *RDR1* transcript, which may have induced stronger RNA silencing and contributed to the basal antiviral defense in *N. tabacum* and *Arabidopsis*. Furthermore, *N. benthamiana* naturally loses RDR1 function to maintain a higher level of RDR6-dependent antiviral defense [34]. However, in this study, *MtRDR1*-transformed plants showed a higher constant resistance to TMV than EV *N. benthamiana* plants (Figure 7), and this compensation mechanism may not be sufficiently effective to compensate for the loss of RDR1.

In summary, we have addressed the role of H_2O_2, NO, and the relationship between them in TMV-induced RDR1 activation and the antiviral defense response in the model host plants *N. tabacum*, *N. benthamiana*, and *Arabidopsis*. The results strongly imply that TMV-induced NO accumulation functions upstream of H_2O_2 to mediate RDR1 induction, which plays a critical role in strengthening RNA silencing to restrict virus systemic infection and accumulation (Figure 9). This signaling pathway may be a general pathway common to plants.

Materials and Methods

Plants, viruses, and chemical treatments

Transgenic homozygous *N. benthamiana* plants with the binary vector pCAMBIA 2300 containing the *RDR1* gene from *Medicago truncatula* (R15-1) or empty vector (EV) not containing the *RDR1* transgene (V16-2) were generously provided by Richard S. Nelson (Samuel Robert Noble Foundation, Ardmore, OK). The leaf disc method was used for *Agrobacterium tumefaciens*-mediated transformation of *N. benthamiana* harboring the 35S: *MtRDR1* transgene, which was detailed in a previous study [6]. These *N. benthamiana* and *N. tabacum* plants were cultivated in a controlled growth chamber at 24°C with a 16-hour photoperiod and 50% humidity. For *Arabidopsis*, seeds of wild-type ecotype Col-0 and mutants *atrbohD* (AT5G47910) and *atnoa1* (AT3G47450) were sown on autoclaved soil and vernalized at 4°C for 3 days. Then, the plants were germinated and grown at 23 to 25°C with 16

hours of light and 8 hours of darkness. Approximately 3 to 4 weeks after germination, the *N. tabacum*, *N. benthamiana*, and *Arabidopsis* plants were chemically treated in the experiments. The plants leaves were sprayed with water or fresh solutions of 5 mM H_2O_2, 0.2 mM SNP, 5 mM DMTU, 0.2 mM cPTIO, 2 mM SA, 5 mM DMTU plus 2 mM SA, or 0.2 mM cPTIO plus 2 mM SA. In the experiments with a combination treatment of chemical modulator and TMV inoculation, chemical pretreatments were only applied on the upper leaves, and the lower 2–3 fully developed leaves were used for inoculation with TMV 12 hours after chemical pretreatment. Specifically, leaves of *N. tabacum* and *N. benthamiana* were inoculated with TMV (U1 strain) suspensions using cotton tips on adaxial surfaces previously dusted with carborundum powder. The viral inocula (prepared in 5 mM sodium phosphate, pH 7.5) of *N. tabacum* and *N. benthamiana* were applied at concentrations of 10 μg/mL and 1 μg/mL, respectively. The inoculation of *Arabidopsis* plants was performed using a fine sable paintbrush to apply a suspension of the TMV-cg strain at 5 μg/mL in 5 mM sodium phosphate, pH 7.5 onto leaves sprinkled with carborundum. Mock inoculations were performed with phosphate buffer only. Plants were randomly assigned to the treatments, each with four replicates. The upper, newly developed systemic leaves were collected at different time points as indicated for physiological and molecular measurements. One biological sample was obtained by pooling the leaves from three to four plants, and four biological repeats were analyzed for each treatment. The experiments were independently performed three times.

H₂O₂ and NO detection and quantification

H_2O_2 detection using a CLSM system was conducted as described in previous studies with minor modifications [35]. Leaf sections (0.5 by 0.5 cm) were placed into a loading buffer with 50 mM Tris-KCl (pH 7.2) containing 100 μM of H_2DCF-DA. Before further experiments were performed, the peels were preincubated in the dark for 1 hour and immediately examined under a CLSM system (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany). The sections were excited using the 488-nm line of an argon laser, and dye emissions were recorded using a 505- to 530-nm band-pass filter.

H_2O_2 *in situ* detection was performed using a 3,3-diaminobenzidine (DAB) staining method [36]. H_2O_2 quantification was performed using a spectrophotometric assay at OD412 [37,38]. The minor modifications to these experiments have been previously described [39]. The NO concentration was determined using Griess reagent (Sigma-Aldrich) [40].

RNA extraction and transcript level estimation by real-time quantitative PCR

One biological sample was obtained by pooling the leaves from five plants, and four biological repeats were analyzed. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. Genomic DNA was removed using a purifying column. Total RNA (1 μg) was reverse-transcribed using 0.5 mg of oligo(dT)₁₂₋₁₈ (Invitrogen, Carlsbad, CA, USA) and 200 units of Superscript II (Invitrogen) following the manufacturer's instruction. Gene-specific primers were designed based on the mRNA sequence for analyzing transcript levels of *RDR1* in each species. TMV-specific primers were also designed according to the sequence encoding the TMV-CP (U1 strain) and TMV genome (cg strain). The primers used are listed in Table S1.

Quantitative real time-PCR was performed using an iCycler IQTM Real-Time PCR Detection System (Bio-Rad, Hercules,

CA, USA). PCR was performed using SYBR Green PCR Master Mix. PCR cycling conditions were as follows: 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 30 seconds. Fluorescent signals were collected during the 58°C step. To verify the amplification of a single product, a dissociation curve was generated at the end of the PCR cycles using software provided with the iCycler iQTM real-time PCR detection system. The *NtActin*, *NbActin*, and *AtActin* genes were used as internal controls, relative gene expression was calculated [41].

Chlorophyll fluorescence imaging

Chlorophyll fluorescence was determined using PAM imaging (IMAG-MAXI, Heinz Walz, Effeltrich, Germany). Plants were placed in darkness for 30 s to measure Fv/Fm. Minimal fluorescence (F_0) was measured during the weak measuring pulses, and maximal fluorescence (Fm) was measured by a 0.8-second pulse of light at approximately 4000 μmol/(m²s).

Statistical methods

Four independent replicates were applied in each determination. The data were statistically analyzed using analysis of variance and tested for significance ($P<0.05$) using Tukey's test.

Supporting Information

Table S1 Primers used for real time reverse-transcription polymerase chain reaction assays. F: forward; R: reverse. (DOCX)

Author Contributions

Conceived and designed the experiments: KS ZXC JQY. Performed the experiments: YWKL ZHS XL GQZ. Analyzed the data: YWKL KS. Contributed reagents/materials/analysis tools: XJX YHZ. Wrote the paper: YWKL KS.

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