Host-Mediated S-Nitrosylation Disarms the Bacterial Effector HopAI1 to Reestablish Immunity

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Pathogens deliver effectors into plant cells to suppress immunity-related signaling. However, effector recognition by the host elicits a hypersensitive response (HR) that overcomes the inhibition of host signaling networks, restoring disease resistance. Signaling components are shared between the pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity, and it is unclear how plants inactivate these effectors to execute the HR. Here, we report that, in Arabidopsis thaliana, during the onset of the HR, the bacterial effector HopAI1 is S-nitrosylated and that this modification inhibits its phosphothreonine lyase activity. HopAI1 targets and suppresses mitogen-activated protein kinases (MAPKs). The S-nitrosylation of HopAI1 restores MAPK signaling and is required during the HR for activation of the associated cell death. S-nitrosylation is therefore revealed here as a nitric oxide-dependent host strategy involved in plant immunity that works by directly disarming effector proteins.

INTRODUCTION

Plants have two different strategies to detect microbial pathogens and activate defense mechanisms (Chisholm et al., 2006; Jones and Dangl, 2006). The first detection strategy is mediated at the plasma membrane by pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), i.e., conserved microbial molecules such as bacterial flagellin or translation elongation factor Tu, and establish PAMP-triggered immunity (PTI) (Boller and Felix, 2009). The second detection mechanism is mediated by plant Resistance (R) proteins that sense specific pathogen effectors secreted inside the plant cell, also known as avirulence proteins. Their secretion is a pivotal strategy used by bacterial, fungal, and oomycete pathogens to suppress PTI and promote virulence (Boller and He, 2009; Jones and Dangl, 2006). The recognition of these effectors by plants induces effector-triggered immunity (ETI), a stronger defense pathway sharing some components with PTI, but specifically associated with the plant hypersensitive response (HR) that ultimately restricts the proliferation and spreading of the pathogen (Jones and Dangl, 2006). The model pathogen Pseudomonas syringae pv tomato DC3000 (Pst DC3000), a bacterium pathogenic to tomato (Solanum lycopersicum) and Arabidopsis thaliana, injects ~30 such effectors into the host during infection (Schechter et al., 2006) using the type-III secretion system (TTSS). Where effectors target defense signaling components also involved in ETI, they should also suppress the HR. However, the HR occurs despite the presence of such effectors by mechanisms that are still largely unknown. In principle, plants could restore the activity of components targeted by such effectors during the HR by modifying the targets or inactivating the effectors, or they could bypass the pathogen-suppressed components altogether by activating alternative defense pathways. In line with the restoration option, a host mechanism for effector inhibition based on phosphorylation has been reported: The inhibition of AvrPtoB, which directly targets a protein involved in ETI, by host target-specific Pto kinase-mediated phosphorylation, restores Prf-mediated ETI signaling and the HR (Ntoukakis et al., 2009). The characterization of additional effectors targeting further signaling components involved in ETI confirms that plants can rely on the restoration of targeted immunity components to fulfill ETI. Indeed, the plant blocks HopM1-triggered degradation of the immunity-associated vesicle traffic regulator AtMIN7, which is required for ETI (Nomura et al., 2011), but the underlying mechanism has not been determined. Similarly, the delivery of effectors targeting mitogen-activated protein kinase (MAPK) signaling cascades, which are active in both PTI and ETI (Asai et al., 2002; Meng and Zhang, 2013; Underwood et al., 2007), does not prevent the typical ETI-associated sustained MAPK activation (Tsuda et al., 2013), and HR occurs when these effectors are present (Almeida et al., 2009).

Alternatively, pathogen-mediated suppression of resistance components can be bypassed through activation of alternative defense pathways, as recently shown for disruption of AtMPK4 signaling, which is guarded through SUMM2 protein leading to autoimmunity and alternative plant resistance (Zhang et al., 2012). Among the effectors targeting MAPK signaling, the P. syringae bacterial effector HopAI1, member of a widely conserved bacterial TTSS effector family (Li et al., 2005), catalyzes the irreversible dephosphorylation of MAPK through a phosphothreonine lyase activity that cleaves the C–OP bond of the phosphothreonine residue in the pThr-X-pTyr motif (Li et al., 2007). HopAI1 activity in plants has been characterized in detail. Its expression in Arabidopsis showed that it directly targets and irreversibly dephosphorylates AtMPK3 and AtMPK6 in vivo to suppress PTI.
responses induced by the PAMP flg22 (Zhang et al., 2007) as well as AtMPK4 (Zhang et al., 2012). However, infection with Pst DC3000 carrying HopAI1 fused to AvrRpt2101-255 reporter did not compromise the establishment of AvrRpt2-induced HR-cell death in Arabidopsis (Vinatzer et al., 2005). In the same way, the T1 isolate of P. syringae pv tomato, which carries and expresses HopAI1, induced nonhost resistance in Arabidopsis involving a HR-like cell death (Almeida et al., 2009). All these reports suggest that HopAI1 is somehow inhibited during the HR.

One of the earliest events following effector recognition during the HR is the rapid accumulation of nitric oxide (NO) (Delledonne et al., 1998; Durner et al., 1998), a free radical product of metabolism that plays diverse and important roles in the regulation of cellular activity (Yu et al., 2014). Key regulators of plant immunity, including the transcriptional coactivator of broad-spectrum plant immunity (NPR1) and its interaction partner TGA1, undergo S-nitrosylation, a form of NO-mediated protein modification that results in the covalent binding of a NO group to a cysteine residue, causing the direct modulation of protein activity or a change in protein localization or interaction with other proteins (Scheler et al., 2013; Trapet et al., 2015; Bellin et al., 2013). Interestingly, in animals, host-mediated S-nitrosylation has been recognized as a mechanism for the direct inactivation also of pathogen virulence proteins as toxins (Badorff et al., 2000; Saura et al., 1999; Savidge et al., 2011).

Given the temporal features of NO accumulation in plants (Chen et al., 2014) and the crucial role of S-nitrosylation in the modulation of protein functions, we hypothesized that the activity of bacterial effector proteins could also be directly modulated by S-nitrosylation during the HR to restore defense signaling. Taking advantage of the well-characterized activity of the bacterial effector HopAI1 in Arabidopsis plants, we demonstrate here that this effector is indeed inhibited by host-mediated S-nitrosylation during the HR to restore MAPK signaling functions.

RESULTS

HopAI1 Is Susceptible to S-Nitrosylation

The amino acid sequence of the bacterial effector HopAI1 from Pst DC3000 includes a single cysteine residue at position 138, which is widely conserved across P. syringae species (Supplemental Figure 1).

To assess whether this bacterial effector is targeted by S-nitrosylation, the recombinant HopAI1 and the cysteine-to-serine substitution mutant HopAI1CS were incubated with 0.5 to 1 mM of the nitrosylating agent S-nitrosothiolane (GSNO) and then subjected to the biotin-switch assay, which allows detection of S-nitrosylated proteins through conversion of nitrosylated cysteines to biotinylated cysteines followed by immunoblotting (Jaffrey and Snyder, 2001). GSNO treatment resulted in the S-nitrosylation of HopAI1; the reaction could be reversed by incubation with the reductant DTT, consistent with a reversible thiol modification (Figure 1A). In contrast, no S-nitrosylation was observed for the mutant form HopAI1CS. Similar data were obtained using the 2,3-diaminonaphthalene (DAN) assay (Gu et al., 2002) (Figure 1B).

NO Inhibits HopAI1 Activity

We investigated whether S-nitrosylation could affect the activity of HopAI1. The phosphothreonine lyase activity of the recombinant protein was inhibited in a dose-dependent manner by GSNO (Figure 1C) as well as by the alternative NO donors CysNO, SNAP, NOC-12, and diethylamine NONOate (Supplemental Table 1). On the other hand, the S-nitrosylation-insensitive mutant HopAI1CS was not affected by NO treatment. These results clearly demonstrate that S-nitrosylation is a mechanism for the direct inactivation also of pathogen virulence proteins as toxins (Badorff et al., 2000; Saura et al., 1999; Savidge et al., 2011).

![Figure 1](image)

**Figure 1.** The S-Nitrosylation of Recombinant HopAI1 Inhibits Its Phosphothreonine Lyase Activity.

(A) Biotin switch assay of HopAI1 and HopAI1CS. Recombinant HopAI1 and HopAI1CS proteins were preincubated with the indicated amount of the nitrosylating agent GSNO followed by a biotin-switch assay to detect S-nitrosylated proteins. As a control, the biotin-switch assay was performed in the absence of ascorbate (Asc) or on proteins pretreated with DTT. Equal protein loading was evaluated by staining the membrane with Ponceau S staining after protein transfer.

(B) DAN assay of HopAI1 and HopAI1CS. Recombinant HopAI1 and HopAI1CS proteins were preincubated with 0.5 mM GSNO and then analyzed for NO release by measuring fluorometric NAT (2,3-naphthyltriazole), which is produced by stoichiometric conversion of DAN by NO. (A) and (B) were repeated three times on independent batches of recombinant protein with similar results.

(C) Phosphothreonine lyase activity assay of HopAI1 and HopAI1CS. Recombinant HopAI1 and HopAI1CS proteins were preincubated with the indicated amount of GSNO before the activity assay. Enzyme activity is represented as the amount of inorganic phosphate released from a synthetic phosphopeptide. The data are means of technical replicates n = 3, ± so. The experiment was performed twice on independent samples with similar results.
activity was not affected by GSNO, confirming the NO-mediated protein activity inhibition by S-nitrosylation at Cys-138.

HopAI1 activity could also be inhibited by NO in planta. Transient expression of the constitutively active forms of Arabidopsis MKK4 (AtMKK4EE) or MKK5 (AtMKK5EE) in tobacco (Nicotiana tabacum cv SR1) leaves causes the activation of the two tobacco MAPK orthologs of AtMPK3/6, namely, stress-induced protein kinase and wound-induced protein kinase, ultimately triggering HR-like cell death (Ren et al., 2002). The transient coexpression of either HopAI1 or HopAI1CS clearly prevented MKK-induced cell death in tobacco and Nicotiana benthamiana (Supplemental Figure 2; Figure 2) compared with the coexpression of the empty vector or of the phosphatase dead mutant HopAI1HA, in which the catalytically active residue His102 (Zhang et al., 2007) was mutagenized into alanine (Figure 2). This indicated that both HopAI1 and HopAI1CS can similarly target and block tobacco stress-induced protein kinase and wound-induced protein kinase and that the HopAI1CS protein level in planta was sufficient to compensate for its reduced activity observed in vitro. We also experimentally demonstrated that FLAG tag length did not significantly affect HopAI1CS activity (Figure 2). Together, these results justify the use of the HopAI1CS mutant as a reliable control in testing NO-mediated effect on HopAI1 activity in planta.

We found that exposure of tobacco plants to NO gas (50 ppm) restored the cell death induced by AtMKK5EE in the presence of HopAI1 but not in the presence of HopAI1CS (either tagged with 1xFLAG or 3xFLAG). Thus, HopAI1CS still actively prevented MKK-induced cell death, compared with the phosphatase dead HopAI1HA under NO (Figure 2). In line with the cell death phenotype, plant exposure to NO gas partially compromised the dephosphorylation of tobacco MAPks by HopAI1, which was particularly evident for one of the two activated MAPks (Figure 2). Conversely, HopAI1CS (either tagged with 1xFLAG or 3xFLAG) still strongly suppressed MAPk activity compared with HopAI1HA to nearly background levels, demonstrating that NO inhibits HopAI1 activity in planta and this relies on its unique Cys residue (Figure 2).

NO Inhibits HopAI1 Activity by S-Nitrosylation during the HR

Pst DC3000 carrying the avirulence gene AvrRpt2 (Pst AvrRpt2) triggers HR in Arabidopsis through the recognition of AvrRpt2 by the R protein RPS2 (Kunkel et al., 1993). This induces the sustained activation of AtMPK3 and AtMPK6 (Tsuda et al., 2013; Underwood et al., 2007), which is required for full mounting of HR-cell death (Supplemental Figure 3). To understand whether NO endogenously produced during the HR can also inhibit HopAI1 activity by S-nitrosylation, we used two independent experimental approaches. In the first approach, HopAI1 and HopAI1CS were stably expressed (Guo et al., 2009) in the avirulent Pst AvrRpt2 to be delivered into the plant during infection through the bacterial type III secretion system. In the second approach, the transgenic Arabidopsis plants stably expressing HopAI1 or HopAI1CS (Supplemental Figures 4A to 4C) were infected with Pst AvrRpt2. HR-cell death was observed both in wild-type Arabidopsis plants challenged with Pst AvrRpt2 expressing HopAI1 and in transgenic Arabidopsis plants expressing HopAI1 following a challenge with Pst AvrRpt2. When HopAI1CS was present instead of HopAI1, either through pathogen delivery or by direct expression in planta, the HR-cell death was much weaker (Figures 3A to 3C; Supplemental Figure 4D) and resembled that observed in mpk6 and mpk3 mutants and in the M KK5 upstream kinase knockdown line produced by RNA interference (Supplemental Figure 3), suggesting that MAPK signaling was impaired by the effector. A similar phenotype was observed in the transgenic HopAI1CS lines challenged with the avirulent Pst AvrB strain, which triggers HR through the recognition of AvrB by the
resistance protein RPM1 (Mackey et al., 2002) (Supplemental Figure 5). Conversely, in the atnoa1 mutant line, which accumulates lower NO levels (Moreau et al., 2010; Zemojtel et al., 2006), HopAI1 partially impaired the HR-related electrolyte leakage from infected leaves similarly to HopAI1CS, indicating a compromised HR-cell death (Figure 3D) (Mackey et al., 2003). These data further support that the inhibition of HopAI1 allows a normal cell death phenotype in wild-type plants and is indeed mediated by the NO produced endogenously during the HR.

As expected, HopAI1 was found to be modified specifically through S-nitrosylation at its Cys residue 8 h postinfection (hpi), whereas no S-nitrosylation was detected in the lines expressing HopAI1CS (Supplemental Figure 6; Figure 3E), despite normal HR-associated NO accumulation (Supplemental Figure 7).

**Figure 3.** HopAI1 Is S-Nitrosylated during the HR in Arabidopsis to Allow HR-Cell Death Establishment.

(A) HR-induced cell death in the presence of HopAI1 or HopAI1CS observed as macroscopic symptoms or by staining dead cells with trypan blue. Half-leaves were infiltrated with avirulent Pst AvrRpt2 bacterial strains using the plant/pathogen combinations indicated in each panel. Plants/bacteria expressing empty vector (EV) are used as control. Transgenic lines were preinduced with β-estradiol 24 h before infection. Pictures were taken at 24 hpi.

(B) Electrolyte leakage from leaf discs of wild-type Arabidopsis plants infiltrated with avirulent Pst AvrRpt2 bacterial strains expressing either HopAI1 or HopAI1CS (1 × 10^7 cfu/mL), or with MgCl₂ as control. The values are means ± so, n = 2, and different letters indicate significant differences (P < 0.01, two-way ANOVA, Fisher’s test). ANOVA test is reported in Supplemental File 1.

(C) Electrolytes leakage from leaf discs of transgenic Arabidopsis plants expressing HopAI1 or HopAI1CS. Plants were preinduced with β-estradiol 24 h before infection and infiltrated with Pst AvrRpt2 (1 × 10^7 cfu/mL) or MgCl₂ as a control. The data are means ± so, n = 2, and different letters indicate significant differences (P < 0.01, two-way ANOVA, Fisher’s test). The ANOVA tables are provided in Supplemental File 1.

(D) Electrolyte leakage from leaf discs of Atnoa1 Arabidopsis mutant plants infiltrated with avirulent Pst AvrRpt2 bacterial strains expressing either HopAI1 or HopAI1CS (1 × 10^7 cfu/mL) or with MgCl₂ as control. The values are means ± so, n = 3, and different letters indicate significant differences (P < 0.01, two-way ANOVA, Fisher’s test). The ANOVA tables are provided in Supplemental File 1.

(E) S-nitrosylation of HopAI1 during the HR. Transgenic Arabidopsis plants expressing HopAI1 or HopAI1CS were preinduced with β-estradiol 24 h before infection and infiltrated with Pst AvrRpt2 (1 × 10^7 cfu/mL). Proteins were extracted from leaves harvested at the indicated times after infiltration. Following a biotin-switch assay, the proteins were immunoprecipitated with NeutrAvidin and analyzed by immunoblot using an anti-FLAG antibody (output). A comparative immunoblot of samples before immunoprecipitation is presented as effector loading control (input) and protein loading is shown as Ponceau S staining. Differences in protein mobility between HopAI1 and HopAI1CS reflect the difference in FLAG tag length.

All experiments were repeated twice with similar results.
specificity of this modification was demonstrated by its ascorbate dependence (Supplemental Figure 6). During the HR, the NO accumulation starts around 6 to 8 h following pathogen infection to reach its maximum after 12 h (Supplemental Figure 7). Accordingly, HopAI1 was also S-nitrosylated 12 hpi (Figure 3E).

**Exogenous NO Inhibits PTI Suppression Mediated by HopAI1**

HopAI1 actively promotes *P. syringae* strain 0288-9 virulence in tomato plants (Zhang et al., 2007) and expression of HopAI1 in Arabidopsis transgenic plants abolishes PTI triggered by flg22 pretreatment, leading to increased susceptibility to *Pst* DC3000 infection, compared with pretreated wild-type plants (Li et al., 2005; Zhang et al., 2007).

To further confirm the effect of NO on HopAI1 activity, we evaluated both flg22-triggered MAPK activation status and kinetics of bacterial growth in the transgenic lines expressing HopAI1 or HopAI1CS in the presence/absence of exogenous NO. As expected, flg22-induced phosphorylation of AtMPK3 and AtMPK6 was prevented in plants expressing either HopAI1 or HopAI1CS. By contrast, treatment of plants with NO gas (50 ppm) significantly restored MAPK phosphorylation in the line expressing HopAI1 but not in the line expressing HopAI1CS (Figure 4A; Supplemental Figure 8 and Supplemental Table 2). Accordingly, the exposure of plants to NO gas inhibited HopAI1-mediated suppression of PTI, restoring flg22-induced resistance. By contrast, HopAI1CS retained its suppression activity of PTI even in presence of NO (Figure 4B). These data confirmed, by administrating exogenous NO, the NO-mediated inhibition of HopAI1 activity in planta through S-nitrosylation, as previously observed during the endogenous NO burst associated with the HR.

**S-Nitrosylation of HopAI1 during the HR Restores MAPK Signaling**

The effect on MAPK activation and signaling of HopAI1 S-nitrosylation during the HR was further studied by monitoring the activity and the phosphorylation status of MAPK as well as the

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**Figure 4. NO Inhibits PTI Suppression Mediated by HopAI1 but Not HopAI1CS.**

(A) MAPK activation in response to flg22 in Arabidopsis transgenic plants expressing HopAI1 or the mutated HopAI1CS in absence or presence of NO. Plants expressing the empty vector (EV) are used as control. Plants were preinduced with β-estradiol 12 h before 1 μM flg22 treatment and immediately subjected to air or NO fumigation (50 ppm). Samples were harvested 15 min after flg22 treatment by pooling leaves from three plants. Ponceau S staining of the Rubisco large subunit (Rubisco LS) is shown as loading control and anti-FLAG immunoblot as control for protein expression. The result of a replicated independent experiment is provided as Supplemental Figure 8. Image quantification for this image is provided in Supplemental Figure 8, and relative statistical analysis is provided in Supplemental Table 2.

(B) PTI activation in response to flg22 in Arabidopsis transgenic plants expressing HopAI1 or the mutated HopAI1CS in the absence or presence of NO. Plants were preinduced with β-estradiol 12 h before 1 μM flg22 treatment and immediately subjected to air or NO fumigation (50 ppm). One day after flg22 treatment, plants were infiltrated with 5 × 10⁵ cfu/mL *Pst* DC3000, and bacterial population density was determined at the indicated times. The data are means ± so, n = 3, and different letters indicate significant differences (P < 0.01, one-way ANOVA, Fisher’s test). The experiment was repeated twice on independent samples with similar results. The ANOVA tables are provided in Supplemental File 1.
expression of some MAPK-dependent genes in the above-mentioned transgenic Arabidopsis plants challenged with the avirulent Pst AvrRpt2. MAPK activity increased significantly in wild-type plants at 8 hpi but was inhibited in transgenic plants expressing either HopAI1 or HopAI1\textsuperscript{CS} (Figure 5A). However, at 12 hpi, when the HR-associated NO burst reaches its highest level, MAPK activity inhibition was only observed in transgenic plants expressing HopAI1\textsuperscript{CS} (Figure 5A), confirming that the wild-type HopAI1 activity, differently from the NO insensitive effector, is suppressed when NO accumulates during the HR thus allowing MAPK signaling. The expression of AtMPK3/6-dependent genes, such as \textit{ERF1}, \textit{POD62}, and \textit{Trxh8} (Supplemental Table 3), in HopAI1-expressing plants infected with Pst AvrRpt2 showed an activation profile more comparable to the expression observed in infected EV control plants, whereas the induction of these genes was strongly suppressed in the presence of HopAI1\textsuperscript{CS} (Figure 5B). By contrast, the additional AtMPK3/6-dependent genes \textit{PAD3} and \textit{SAG13} were unexpectedly induced in the transgenic plants expressing HopAI1\textsuperscript{CS} (Figure 5C). This may reflect the modulation of the expression of these genes in an opposite manner by AtMPK4, also a target of HopAI1 (Zhang et al., 2012), which works as an apparent negative regulator in the fine-tuning of defense gene expression (Li et al., 2015; Zhang et al., 2012). This hypothesis is further reinforced by the induction of these genes in the \textit{mpk4} mutant (Supplemental Table 3).

NO-Insensitive HopAI1\textsuperscript{CS} Activity in HR Is Guarded by SUMM2

In agreement with its inactivation by S-nitrosylation, the bacterial effector HopAI1 did not promote Pst AvrRpt2 virulence, and plant resistance was unaffected when it was expressed either by the bacterial strain or directly in transgenic plants. However, plants were unexpectedly more resistant to pathogen infection in the presence of HopAI1\textsuperscript{CS} (Supplemental Figure 9A).

The disruption of AtMPK4 activity is known to be guarded by SUMM2, which promotes autoimmunity featured by the expression of the defense marker gene \textit{PATHOGENESIS-RELATED1} (\textit{PR1}) (Zhang et al., 2012; Eschen-Lippold et al., 2016). Accordingly, we found that \textit{PR1} expression was strongly induced in HopAI1 as well as in HopAI1\textsuperscript{CS} transgenic lines in the absence of the pathogen, thus reflecting the activation of this AtMPK4-mediated autoimmunity pathway (Supplemental Figure 9B). In line with this, following avirulent pathogen infection, this autoimmunity-related strong \textit{PR1} induction was strongly reduced in HopAI1 transgenic line, likely because of the inhibition of HopAI1 activity.

**Figure 5.** S-Nitrosylation Inhibits HopAI1 Activity during the HR Restoring MAPK Signaling for Plant Resistance.

(A) In-gel MAPK activity assay and immunoblot analysis of MAPK phosphorylation in transgenic Arabidopsis plants expressing HopAI1 or HopAI1\textsuperscript{CS} and infiltrated with Pst AvrRpt2 (1 \times 10\textsuperscript{7} cfu/mL). Plants expressing the empty vector (EV) were used as control. Plants were preinduced with \textit{β}-estradiol 24 h before infection. Proteins were extracted from leaf samples pooled from three plants at the indicated times after infection and analyzed by in-gel MAPK assay and immunoblot to detect MAPK activation with an anti-phospho-ERK1/2 antibody. Immunoblot using \textit{α}-AtMPK3, \textit{α}-AtMPK4, and \textit{α}-AtMPK6 is provided as control for MPKs expression. Ponceau S staining of the Rubisco large subunit (Rubisco LS) is shown as gel loading control.

(B) Expression of AtMPK3/MPK6-dependent genes normalized to \textit{ACTIN2} presenting the expected profiles in the different lines under the same conditions described in (A). Statistics was performed using one way ANOVA, Scheffe test, \textit{P} < 0.05, and different letters indicate significant differences. The data are means of technical replicates \pm SD, \textit{n} = 2. The ANOVA tables are provided in Supplemental File 1.

(C) Expression normalized to \textit{ACTIN2} of AtMPK3/MPK6-dependent genes presenting unexpected profiles in the different lines under the same conditions described in (A). Statistics was performed using one way ANOVA, Scheffe test, \textit{P} < 0.05, and different letters indicate significant differences. The data are means of technical replicates \pm SD, \textit{n} = 2. The ANOVA tables are provided in Supplemental File 1.

All experiments were performed for three biological replicates with similar results.
DISCUSSION

The experiments described in this work show that plants can use host-mediated S-nitrosylation as a mechanism to target and disarm pathogen bacterial effectors. Exogenously applied NO inhibited the HopAI1 activity in tobacco plants and suppressed HopAI1 virulence promoting activity during PTI. Moreover, we showed that NO produced endogenously by plants at the onset of the HR inhibits the phosphothreonine lyase activity of this bacterial effector by S-nitrosylation, restoring the sustained MAPK signaling (Figure 5) typical of ETI (Tsuda et al., 2013). MAPK signaling is required to induce one of the immune subsector pathways contributing to ETI, leading to the ETI-associated HR-cell death (Supplemental Figure 3). Accordingly, constitutive activation of AtMPK3 was recently shown to be associated to cell death (Genot et al., 2017). This highlights the biological relevance of the mechanism we have found and reveals a novel function for NO in plant defense.

In animals, host-based S-nitrosylation mediated by NO was also shown as a mechanism for inactivation of non-effector virulence pathogen proteins. Coxsackievirus cysteine proteases as well as the microbial exotoxin TcdA secreted by Clostridium difficile, another cysteine protease, were both reported to be S-nitrosylated and inhibited through this modification (Saura et al., 1999; Badorff et al., 2000; Savidge et al., 2011). Our data are somehow partially reminiscent of these findings but reveal that TTSS bacterial effectors are also targeted and inhibited through S-nitrosylation by the host.

Virulence factors can also be inactivated in plants by other forms of posttranslational modification, e.g., the E3 ubiquitin ligase activity of AvrPtoB is inhibited by a specific host enzyme, the Pto kinase, which specifically phosphorylates and inactivates its catalytic domain (Ntoukakis et al., 2009). Unlike AvrPtoB, the inactivation of HopAI1 by S-nitrosylation targets the noncatalytic Cys-138 residue, likely affecting indirectly HopAI1 phosphothreonine lyase activity, possibly by modifying protein conformation. Other S-nitrosylated enzymes showed reduced substrate binding affinity due to conformational changes (Lindermayr et al., 2005; Park et al., 2004). Further study will allow us to further clarify the structural basis for this activity inhibition. In any case, cysteine residues are widely distributed in TTSS effector proteins, and the accumulation of NO during the HR (Chen et al., 2014) may represent a flexible strategy suitable for the modulation of many different effectors.

However, in line with data showing that HopAI1 activity in plants is also indirectly guarded by the R protein SUMM2 (Zhang et al., 2012), the overexpression of an S-nitrosylation-insensitive mutant (HopAI1CS) triggered autoimmunity and failed to promote the anticipated susceptibility to the pathogen in addition to impairment of HR-cell death. The activation of this alternative defense mechanism, which relies on sensing the overexpression of a typically repressed AtMPK4 gene, AtMEKK2, and requires the accumulation of an unphosphorylated AtMPK4 target, CRCK3, due to effector activity (Su et al., 2013; Zhang et al., 2017) is indeed responsible for overcoming the anticipated susceptibility (Supplemental Figures 9 and 10) and confirms that plants adopt multiple regulatory mechanisms to overcome the effector-mediated HR suppression. The existence of multiple mechanisms to inhibit or overcome HopAI1 activity explains the lack of strong selection among P. syringae bacteria for the development of HopAI1 allelic variants that are insensitive to S-nitrosylation. Consistent with these findings, the comparative sequencing of 19 P. syringae isolates and the analysis of their effector repertoires revealed the preferential disruption/truncation of this effector over the evolution of novel alleles, a phenomenon shared by multiple strains of the bacterium in different clades (Baltrus et al., 2011). On the other hand, we can speculate that the selective pressure for effector disruption has reduced the likelihood that HopAI1 allelic variants insensitive to S-nitrosylation will emerge, preserving the cysteine target residue and allowing the identification of this NO-mediated mechanism for the direct inhibition of effector activity and restoration of MAPK signaling during the HR.

Overall, the finding that S-nitrosylation can disarm pathogenic effector proteins highlights a novel function for NO in plant immunity that enhances and further enriches our current understanding of plant-pathogen interactions.

METHODS

Bacterial Strains and Plant Materials

The bacterial strain expressing HopAI1-3XFLAG was obtained by amplifying the full-length sequence from Pseudomonas syringae pv tomato DC3000 (Pst DC3000) with primers FP1/RP1 (Supplemental Table 4) and cloning the products in pENTR/D-Topo (Thermo Fisher Scientific). The mutant HopAI1CS-1XFLAG and HopAI1CS-3XFLAG sequences were generated by overlapping extension PCR (Higuchi et al., 1988) with primer pairs FP1/RmP1 and FP1/RP1 (Supplemental Table 4). The pENTR constructs were recombined with destination vector pLN615, derived from pML123 (Underwood et al., 2007), using LR clonase (Thermo Fisher Scientific) and were introduced into Pst DC3000 AvrRpt2 (Pst AvrRpt2), Broad-host-range cloning vector pML123 was introduced into Pst AvrRpt2 as an empty vector control.

We used the wild-type Arabidopsis thaliana line Col-0 as a control, as well as an estradiol-inducible Arabidopsis transgenic line expressing HopAI1-3XFLAG (Li et al., 2005), the T-DNA insertion mutants mpk3-1 and mpk6-2 (SALK_151594 and SALK_073907) (Galletti et al., 2011), and the fast neutron deletion mutant mpk3-DG (Miles et al., 2005). To generate the β-estradiol-inducible constructs for transient expression of HopAI1CS-1XFLAG or HopAI1CS-3XFLAG or for producing the stable transgenic line expressing HopAI1CS-1XFLAG, the pENTR constructs described above were recombined with the pMDC7 vector (Curtis and Grossniklaus, 2003). The β-estradiol-inducible pMDC7 construct for transient expression of HopAI1CS-3XFLAG was produced through in vitro mutagenesis from HopAI1-3XFLAG (Li et al., 2005) (QuikChange Lightning site-directed mutagenesis kit; Agilent Technologies) using primers included in Supplemental Table 4. A pMDC7 empty vector control was generated by removing the Gateway cassette using restriction enzyme Sall. The tandem
RNAi construct for the silencing of AtMKKS was kindly provided by S. Zhang (Wang et al., 2007). Constructs were introduced into Agrobacterium tumefaciens GV3101. Transgenic plants were produced by the floral dip method (Clough and Bent, 1998), and homozygous lines were selected. Arabidopsis plants were grown in a growth chamber for 6 weeks under controlled conditions (8-h-light/16-h-dark photoperiod, 24°C/21.5°C, 70% relative humidity) and watered weekly. Light was provided by warm fluorescent tubes, 120 to 160 μmol photons m⁻² s⁻¹. Tobacco plants (Nicotiana tabacum cv SR1) were also grown in growth chambers at controlled conditions (14-h-light/19-h-dark photoperiod and constant 22°C). Transgene expression was induced by spraying the plants with 40 μM β-estradiol at indicated time before treatments and tested by immunoblotting using anti-FLAG polyclonal antibody from rabbit (Sigma-Aldrich; F7425) diluted 1:2000.

Production of Recombinant HopAI1/HopAI1CS

The core HopAI1.2-245 sequence previously shown to be functionally sufficient (Zhang et al., 2007) was amplified from Pst DC3000 using the primers as listed in Supplemental Table 4, transferred to the pENTR-SD/D-Tope vector, and then recombined with the destination vector pDEST17 (Thermo Fisher Scientific). The pDEST17-HopAI1CS construct was obtained by site-directed mutagenesis using the QuikChange II XL kit (Agilent Technologies). Protein expression in Escherichia coli BL21 was induced with 0.2 mM isopropylthio-β-galactoside at 37°C for 6 h and tested by anti-His immunoblotting. His-tagged recombinant HopAI1/HopAI1CS was purified from inclusion bodies under denaturing conditions (8 M urea) using His GraviTrap columns (GE Healthcare Biosciences) and refolded by dialysis (0.2 mg/L in 20 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA-Na₂, pH 7.4) using a multiple-step gradient of urea.

Biotin Switch and DAN Assays

S-nitrosylated HopAI1/HopAI1CS proteins were detected using an in vitro biotin switch assay (5 μg protein) and an in vitro DAN assay (15 μg protein) as previously described (Ghelardoni et al., 2003; Romero-Puertas et al., 2007). In each case, the protein was incubated with GSNO (Cayman) in the dark for 30 min, and excess GSNO was removed by cold acetone precipitation. Briefly, for biotin switch three volumes of blocking buffer (HEN buffer, 2.5% SDS, and 20 mM methyl methanethiosulfonate) were added to 5 μg of recombinant protein. After incubation at 50°C for 20 min, MMTS was removed by cold acetone precipitation and air-dry pellet resuspended in HEN buffer. Na-ascorbate and biotin-HPD were added to the samples to the final concentration of 1 mM, and samples were incubated room temperature in the dark for 60 min. S-nitrosylation was detected by immunoblotting of samples with mouse antibiotin monoclonal antibody 1:5000 (Sigma-Aldrich; clone BN-34, B7653). In vivo biotin switch assay was performed as described by Romero-Puertas et al. (2007) and Tada et al. (2008), with few modifications. Preincubated (24 h before infection) transgenic Arabidopsis lines were infected with Pst AvrRpt2 (1 × 10⁷ cfu/ mL) for the indicated times. Leaves sampled from ~10 independent infected plants were homogenized in 1 mL HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM N-ethylmaleimide, and Sigma-Aldrich Protease Inhibitor cocktail). Extracts were centrifuged 14,000g for 25 min. Protein concentration of the supernatant was measured by Bradford assay and 1 mg of protein extract diluted to 0.8 μg/mL was then subjected to blocking of free SH-groups by adding 3 volumes of blocking buffer (HEN buffer, 2.5% SDS, 20 mM S-methylmethionithiosulfinate) and incubating at 50°C for 20 min. Excess S-methylmethionithiosulfinate was removed by precipitation with one volume cold acetone and repeated washing with 50% acetone. Dried pellets were dissolved in 880 μL HEN buffer plus 1% SDS followed by addition of 20 μL of freshly prepared 1 M sodium ascorbate and 100 μL of 10 mM biotin-HPD (Thermo Fisher Scientific) for biotin labeling. No sodium ascorbate was added in control samples. After labeling for 90 min at 37°C in dark, samples were precipitated with 1 volume acetonitrile, washed twice with 70% acetonitrile (one volume and half volume), and dissolved in suspension buffer (25 mM HEPES, pH 7.7, 1 mM EDTA, and 1% SDS). After further precipitation with 50% acetonitrile pellets were finally resuspended in 250 μL resuspension buffer plus 750 μL neutralization buffer (25 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.05% Triton X-100). Prior immunoprecipitation samples were tested for HopAI1/HopAI1CS protein content by immunoblotting with polyclonal anti-FLAG produced in rabbit (Sigma-Aldrich; F7425) diluted 1:2000 and for successful switching by immunoblotting with monoclonal antibody produced in mouse (Sigma-Aldrich; clone BN-34, B7653) diluted 1:5000. Sample volumes were eventually adjusted to achieve equal immunoprecipitation protein loading (input samples). After centrifugation at 10,000g for 5 min, 40 μL NeutrAvidin Agarose resin (Thermo Fisher Scientific) was added to supernatants and samples were incubated overnight at 4°C. Beads were washed three times with washing buffer (25 mM HEPES, pH 7.7, 600 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) and once in neutralization buffer and then eluted with 40 μL elution buffer (25 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 100 mM DTT) at 50°C for 30 min. After 12% PAGE samples were immunoblotted by anti-FLAG polyclonal antibody produced in rabbit (Sigma-Aldrich; F7425) diluted 1:2000 to test for recovery of S-nitrosylated immunoprecipitated proteins (output samples).

Phosphothreonine Lyase Activity Assay

Phosphothreonine lyase activity was detected as previously described with some modifications (Zhang et al., 2007). Synthetic MAPK phosphopeptide SESDFM-pTE-pYVVTR (Sanogen) was incubated with 1 μg HopAI1/HopAI1CS in 40 μL reaction buffer (10 mM HEPES, 150 mM NaCl, and 1 mM EDTA, pH 7.4) at 30°C for 10 min. Released phosphate was detected with Malachite green dye (Millipore) according to the manufacturer’s instructions. Recombinant HopAI1/HopAI1CS was S-nitrosylated with the NO donors GSNO (various amounts), in-house CysNO, SNAP, NOC-12, or diethylenetriamine NONOate (all 100 μM) for 30 min before the assay. Excess NO donors were removed by dialysis against refolding buffer using Slide-A-Lyzer MINI dialysis devices (Pierce).

Transient Expression of Effectors/MKK in Tobacco

Transient expression (Lee and Yang, 2006) was performed using the pER8-HopAI1-3XFLAG construct (Li et al., 2005) and the previously described pMDC7-HopAI1CS-3XFLAG, and pMDC7-HopAI1-3XFLAG constructs (Lee and Yang, 2006) was triggered using a construct achieved by recombining constructs pENTR-AtMKK4DE-1XFLAG and pENTR-AtMKK5EE-1XFLAG with pMDC7 destination vectors. Agrobacterium GV3101 suspensions (OD₆₀₀ = 0.8) were mixed in equal amounts and infiltrated into the leaves of 6-week-old tobacco (N. tabacum cv SR1) and Nicotiana benthamiana plants. Transgene expression was induced with 40 μM β-estradiol applied 48 h after infiltration and tested by immunoblotting with anti-FLAG polyclonal antibody produced in rabbit (Sigma-Aldrich; F7425) diluted 1:2000.

Plant Fumigation with NO

Tobacco and Arabidopsis plants were placed into transparent NO fumigation closed chambers (40 μmol m⁻² s⁻¹ constant light) supplied with 50 ppm NO in an air gas mixture at 300 mL/min at indicated times after infiltration/induction and for the indicated times. The NO concentration at the chamber inlet was checked using a CLD70 NO detector (Ecophysics). Air was used as the control gas.
MKK-Induced Cell Death and HR-Cell Death Assessment

MKK-induced cell death was visually assessed in Agrobacterium-infiltrated tobacco leaves 48 h after induction of protein expression. In Arabidopsis, half-leaves from 6-week-old wild-type (Col-0) or transgenic plants were infiltrated with avirulent bacterial suspensions (1 × 10^7 cfu/mL, unless otherwise specified). One day after infiltration, cell death symptoms were observed and dead cells visualized by trypan blue staining (Choi et al., 2007). HR electrolyte leakage assays were performed as previously reported (Mackey et al., 2003). Briefly, leaf discs (8-mm diameter) were excised from six independent plants, vacuum-infiltrated with avirulent bacterial suspensions (1 × 10^7 cfu/mL), rinsed in water for 30 min, and then transferred to Petri dishes containing 6 mL Milli-Q water. Conductance was measured for 24 h using the B-173 compact conductivity meter (Horiba). Average values from three replicates for each sample were plotted against time. One representative experiment of two to three independent experiments is shown.

MAPK Assays

Leaf disc samples were collected from infiltrated area of tobacco plants one day after protein induction. In Arabidopsis, to test for PTI-associated MAPK activation, samples were collected 15 min after 1 μM flg222 treatment by pooling leaves from three independent plants for each line. Similarly, to test for ETI-associated MAPK activation in the different lines, each sample was harvested by pooling leaves from three independent plants at indicated times after infection with the Pst AvrRpt2 avirulent bacteria (1 × 10^7 cfu/mL). Protein extracts (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 1 mM Na2VO4, 20% glycerol, and 1% Triton plus protease inhibitors) from Arabidopsis or tobacco leaves were analyzed by immunodetection of the dual phosphorylated MAPK activation loop (pTEpY) using the phospho-p44/42 MAPK (Erk1/2) (Thr-202/Tyr-204) (D13.14.4E) XP rabbit monoclonal antibody (Cell Signaling; 4370) according to the manufacturer's instructions or an in-gel kinase assay as previously described (Zhang and Klessig, 1997). Anti-AtMPK3/4/6 antibodies were used as control to test for MAPK content in Arabidopsis extracts according to the previously described protocol (Menke et al., 2004).

Bacterial Growth

Kinetics of bacterial growth were tested as described by Katagiri et al. (2002). To test PTI resistance in the different Arabidopsis lines, bacterial growth was estimated by inverting plants 24 h after 1 μM flg22 or water (mock) treatment with Pst DC3000 bacterial suspensions (5 × 10^6 cfu/mL). Samples were harvested at the indicated times post infection. To test HR resistance, Arabidopsis lines were infiltrated with Pst AvrRpt2 bacterial suspension (5 × 10^8 cfu/mL) and leaf samples harvested at the indicated times. Each sample consisted of leaf discs from three plants and was tested in triplicate. Two/three dilutions were plated for each sample to carefully estimate plant bacterial content. A representative plot of bacterial growth against time for one of two to three (as indicated in each figure legend) independent experiments is shown.

Expression Analysis

Total RNA was extracted for each Arabidopsis line from leaves pooled form at least three plants using Trizol reagent (Invitrogen) and was treated with the TURBO DNA-free kit (Ambion). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Thermo Fisher Scientific). Real-time PCR was performed in triplicate for each sample using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher Scientific) with the ACTIN2 housekeeping gene for normalization (GenBank accession number AF428330.1). All primers are listed in Supplemental Table 4.

Gas Phase Plant-Produced NO Detection

NO measurement during pathogen infections was performed by infiltrating whole root-cut plants with Pst AvrRpt2 avirulent bacterial suspension (1 × 10^8 cfu/mL) as described by Chen et al. (2014) using a chemiluminescence detector (ECO Physics CLD 88Bt). Average NO emission in 18 h was estimated for each Arabidopsis line from three experiments.

Bioinformatics and Statistical Analysis

HopAI1 sequences from P. syringae strains were aligned using MEGA v6 (Tamura et al., 2013), and conservation profiles were visualized using Weblogo v3.4 (Crooks et al., 2004). Fold change and P value data for gene expression were extracted from E-MEXP-173/174, ArrayExpress, and EMB-EBI and were analyzed using Multiexperiment Viewer (MeV) with Linear Models for Microarray Data (LIMMA) module (Saeed et al., 2003). Statistical significance analyses for experimental data based on one-way or two-way ANOVA according to the experimental setup with Fisher’s or Sheffe test as referred in figure legends were performed using Origin 9/2015 or SPSS v13.0 (IBM) as well as grouping.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: HopAI1 (NC_004578) and ACTIN2 (AF428330.1). Additional accession numbers can be found in Supplemental Table 3.

Supplemental Data

Supplemental Figure 1. Conservation profile surrounding the unique cysteine residue of Pseudomonas syringae DC3000 in 47 sequences from Pseudomonas HopAI1.

Supplemental Figure 2. Transient expression of HopAI1 or HopAI1CS suppresses HR-like cell death induced by AtMKK4DE or AtMKK5EE.

Supplemental Figure 3. MPK3 and MPK6 are involved in HR-cell death.

Supplemental Figure 4. Expression analysis and phenotypes of transgenic Arabidopsis lines expressing HopAI1 or HopAI1CS.

Supplemental Figure 5. Pst AvrB triggered HR-cell death in transgenic Arabidopsis expressing HopAI1 or HopAI1CS.

Supplemental Figure 6. Specific in vivo HopAI1 S-nitrosylation during the HR.

Supplemental Figure 7. HR-associated NO production in transgenic Arabidopsis expressing HopAI1 or HopAI1CS.

Supplemental Figure 8. MAPK activation in response to flg22 in Arabidopsis plants expressing HopAI1/HopAI1CS in presence/absence of NO (second replicate for Figure 4A).

Supplemental Figure 9. Plant resistance is not affected by HopAI1 expression but is enhanced by HopAI1CS expression.

Supplemental Figure 10. SUMM2-8-triggered autoimmunity by HopAI1CS contributes to bacterial resistance.

Supplemental Table 1. Inhibition of HopAI1 activity by NO.

Supplemental Table 2. MAPK activation in response to flg22 in Arabidopsis plants expressing HopAI1/HopAI1CS in presence/absence of NO.

Supplemental Table 3. Gene expression in wild-type Arabidopsis plants and the mpk4 mutant.

Supplemental Table 4. Primers used in this study.

Supplemental File 1. ANOVA tables.
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AUTHOR CONTRIBUTIONS

D.B., T.L., E.V., and M.D. designed and conceived the experiments. T.L., D.B., E.V., and Z.I. performed the experiments. D.B., T.L., E.V., and M.D. wrote the manuscript. All authors commented and agreed on the manuscript before submission.

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