Nitric Oxide Regulates the *Ralstonia solanacearum* Type 3 Secretion System

Connor G. Hendrich¹, Alicia N. Truchon¹, Beth L. Dalsing¹,², and Caitlyn Allen¹

¹ Department of Plant Pathology, University of Wisconsin – Madison, Madison, Wisconsin, USA

² Present address: Syngenta, Durham, NC

* Corresponding author: Caitlyn Allen, E-mail: callen@wisc.edu

**Keywords:** *Ralstonia solanacearum*, Type III Secretion, nitric oxide, bacterial wilt, vascular wilt, tomato

Funding: National Sciences Foundation grant IOS 1258082.
Abstract

*Ralstonia solanacearum* causes bacterial wilt disease on diverse plant hosts. *R. solanacearum* cells enter a host from soil or infested water through the roots, then multiply and spread in the water-transporting xylem vessels. Despite the low nutrient content of xylem sap, *R. solanacearum* grows very well inside the host, using denitrification to respire in this hypoxic environment. *R. solanacearum* growth *in planta* also depends on the successful deployment of protein effectors into host cells via a Type III Secretion System (T3SS). The T3SS is absolutely required for *R. solanacearum* virulence, but it is metabolically costly and can trigger host defenses. Thus, the pathogen’s success depends on optimized regulation of the T3SS. We found that a byproduct of denitrification, the toxic free-radical nitric oxide (NO), positively regulates the *R. solanacearum* T3SS both *in vitro* and *in planta*. Using chemical treatments and *R. solanacearum* mutants with altered NO levels, we show that the expression of a key T3SS regulator is induced by NO in culture. Analyzing the transcriptome of *R. solanacearum* responding to varying levels of NO both in culture and *in planta* revealed that the T3SS and effectors were broadly upregulated with increasing levels of NO. This regulation was specific to the T3SS and was not shared by other stressors. Our results suggest that *R. solanacearum* experiences an NO-rich environment in the plant host and may use this NO as a signal to activate T3SS during infection.
Introduction

*Ralstonia solanacearum* (*Rs*) is a Gram-negative soil-borne betaproteobacterium that causes bacterial wilt disease on a wide range of plant hosts, including important crops like tomato, potato, and banana (Elphinstone 2005). The bacteria survive in and are spread through infected soil and water, infecting host roots through wounds and natural openings (Vasse et al. 1994). Once inside, *R. solanacearum* cells move to the xylem, where they can grow and spread throughout the plant. Although this bacterium is slow growing and sensitive to stress when grown *in vitro*, in plant xylem vessels *R. solanacearum* is a formidable force. *R. solanacearum* quickly grows to high cell densities in xylem even though this habitat is low in oxygen and nutrients, and accessible to host defenses (Genin and Denny 2012). As it grows, *R. solanacearum* produces an arsenal of virulence factors, including highly mucoid extracellular polymeric substances (EPS), a consortium of cell wall degrading enzymes, and dozens of protein effectors that are injected into host cells via the Type III Secretion System (T3SS) (Genin and Denny 2012). Water transport in infected plants is eventually blocked by a combination of host-produced gels and tyloses, degradation of vessel walls, EPS, and the sheer mass of bacterial cells. Without sufficient water, plants wilt and die while *R. solanacearum* cells escape back into the soil, where they can find another host (Swanson et al. 2007).
The lethality of bacterial wilt disease combined with its wide host range and a lack of effective treatments make *R. solanacearum* an important agricultural problem around the world (Yuliar et al. 2015; Elphinstone 2005). Successful disease management depends on understanding *R. solanacearum* biology, but the pathogen behaves very differently in culture than it does in its natural environment (Jacobs et al. 2012). In particular, the T3SS, which is absolutely essential for virulence and subject to a complex regulatory cascade, is affected by the plant environment in ways that remain poorly understood (Poueymiro and Genin 2009). The PhcA quorum sensing system upregulates T3 secretion in culture, but not *in planta* (Génin et al. 2005; Jacobs et al. 2012; Monteiro et al. 2012a; Khokhani et al. 2017). An unidentified ‘diffusible factor’ increases T3SS expression *in planta* via the HrpG global regulator (Genin and Denny 2012).

We have discovered that the diffusible free radical nitric oxide (NO) plays a role in *R. solanacearum* life inside plants. Xylem sap is rich in nitrate, which *R. solanacearum* uses as an alternate electron acceptor to respire in the hypoxic xylem environment (Dalsing et al. 2015). The *R. solanacearum* strain GMI1000 reduces nitrate completely to dinitrogen gas by means of denitrifying respiration. The genes required for this process are highly expressed during infection (Jacobs et al. 2012; Khokhani et al. 2017). However, denitrifying respiration produces NO as an intermediate. In large quantities, NO is toxic and strongly inhibits *R. solanacearum* growth. The pathogen manages this threat by detoxifying NO with the nitric oxide reductase NorB and the flavohemoglobin HmpX, which are both highly expressed *in planta* and required for full
Plants use NO extensively for both signaling and defense, and can produce NO through multiple different pathways (Scheler et al. 2013; Delledonne et al. 1998; Simontacchi et al. 2015; Wilson et al. 2008; Leitner et al. 2009). Thus, *R. solanacearum* likely encounters NO during plant pathogenesis.

In this study, we identify a link between NO and regulation of the *R. solanacearum* T3SS. We first show that T3SS gene expression increases when *R. solanacearum* is actively denitrifying. We then show that this effect depends specifically on the presence of NO. Based on these results, we conducted a transcriptomics experiment to determine the effects of NO on *R. solanacearum* gene expression in culture and *in planta* and show that levels of many transcripts, including 107 T3SS-related genes, are altered by changes in NO levels.

**Results**

**Nitric oxide changes expression of the *R. solanacearum* T3SS**

Regulation of the T3SS in *R. solanacearum* is multi-layered and incompletely understood (Fig 1.A). Because *R. solanacearum* actively denitrifies *in planta*, we wondered if this pathway or its products affect T3SS expression during plant infection (Dalsing et al. 2015; Jacobs et al. 2012). To test this hypothesis, we used an *R. solanacearum* reporter strain carrying a fusion of the *lux* operon to the promoter of *hrpB*, which encodes a key T3SS regulator (Genin and Denny 2012). Transcription of *hrpB* increased >3-fold when the bacterium was denitrifying in culture (Fig. 1B).
An intermediate product of denitrification is the highly reactive free radical NO. Because NO is a common signaling molecule in eukaryotes, we decided to determine if it was responsible for hrpB activation observed during denitrification. To block the effects of NO, we used the NO-scavenging chemical cPTIO (Pfeiffer et al. 1997). While cPTIO is commonly used to both detect and deplete NO from solutions, it can also be reduced or inactivated by factors other than NO (D'Alessandro et al. 2013). To mitigate cPTIO effects unrelated to NO, we tested its ability to affect hrpB expression in two conditions. First, we incubated R. solanacearum in denitrification-promoting conditions with and without cPTIO. We measured hrpB expression in RNA extracted from these cultures at 16 hpi, near the peak of hrpB::lux expression. In these conditions, NO scavenging by cPTIO slightly but significantly reduced hrpB expression (Fig. 1C). We then tested the effect of a host on the interaction between hrpB expression and NO. We incubated a cell suspension of R. solanacearum hrpB::lux with tomato seedlings for 24 hours with or without cPTIO. As expected, the presence of a host plant activated hrpB gene expression. However, adding cPTIO to the mixture completely reversed this effect (Fig. 1D). Together, these results indicated that the denitrifying respiration-dependent increase in hrpB expression observed in culture is specifically triggered by NO. Further, this result suggests that NO could be the unknown soluble signal that increases hrp regulon expression in planta.
Fig. 1. Nitric oxide affects expression of the *R. solanacearum* T3SS. **A**, Summary of known T3SS regulation in *R. solanacearum* strain GMI1000. Arrows indicate positive regulation, barred lines indicate repression, dashed lines indicate an unknown or uncharacterized mechanism, green text indicates plant-derived signals, and blue text indicates metabolites. T3SS genes, such as the T3 effector *ripX*, are controlled by the transcriptional regulator HrpB. HrpB is transcriptionally activated by HrpG and PrhG, which are themselves controlled by the quorum-sensing regulator PhcA, but only *in vitro*. PrhG activity also depends on three uncharacterized proteins, PrhK, PrhL, and PrhM, through an unknown mechanism. HrpG is activated by the contact-dependent
plant cell wall sensor PrhA as well as by an unknown soluble signal present during plant
infection. 

B, Expression of a bacterial luciferase transcriptional reporter of HrpB. *R. solanacearum* strain GMI1000 hrpB::lux was grown in denitrification-inducing conditions with or without 30 mM nitrate. Both culture density (OD$_{600}$) and luminescence were measured every 30 min, and luminescence was normalized to culture density. Error bars represent standard error of the mean. Cultures grown with nitrate had significantly increased hrpB expression (2-way ANOVA, *P*=0.0003). 

C, The NO scavenger cPTIO reduces hrpB expression in *R. solanacearum* cells grown in denitrifying conditions. Cultures were grown as in B with and without 1 mM cPTIO and harvested at 16 hpi. hrpB expression was measured using qRT-PCR. Expression of hrpB was reduced in cPTIO-treated cultures (*P*<0.0001, students T test) Error bars represent standard error of the mean. 

D, The NO scavenger cPTIO decreased T3SS activity in the presence of a host. Luminescence of *R. solanacearum* hrpB::lux was measured after 24 h incubation in water with or without five day-old tomato seedlings, and with or without 1 mM cPTIO, an NO-scavenger. Exposure to tomato seedlings induced hrpB::lux expression (*P*=0.004, students T test). *R. solanacearum* cells exposed to both tomato seedlings and cPTIO had luminescence indistinguishable from that of cells without tomato seedlings. Error bars represent standard error of the mean.

Denitrifying metabolism causes broad changes in *R. solanacearum* gene expression.
While the above results showed that NO can affect hrpB expression, many other genes are involved in regulation, biosynthesis, and function of the *R. solanacearum* T3SS. To better understand the broader effects of NO on pathogen biology and T3SS gene expression, we sequenced the transcriptomes of *R. solanacearum* cells grown with varying levels of NO both in culture and in infected tomato plants (Fig. 2A). In culture, we grew the cells in 0.1% O\textsubscript{2} in a modified Van den Mooter medium (VDM), which promotes denitrifying respiration (Dalsing et al. 2015). We manipulated intracellular *R. solanacearum* NO levels genetically by using mutants lacking either the NarG nitrate reductase (the ∆narG mutant cannot denitrify and does not produce NO), or the NorB nitric oxide reductase (the ∆norB mutant accumulates NO, see Supplementary Fig 1). Exogenous NO levels were manipulated by treating cells with CysNO, a nitrosothiol NO donor. Because high NO levels are toxic to *R. solanacearum*, we controlled for general stress responses by also profiling transcriptomes of cultures treated with H\textsubscript{2}O\textsubscript{2}, a non-NO source of stress. After about 18 h, growth of the ∆norB mutant was inhibited by accumulating NO, so we sampled cultures 16 hpi, when ∆narG, ∆norB, and the wild-type strain were at similar densities (Supplementary Fig 1). CysNO or H\textsubscript{2}O\textsubscript{2} treatments were added 4 h before sampling, at the highest concentrations that did not significantly alter culture growth (Supplementary Fig 2A). Total RNA was extracted from bacteria *in planta* 3 days after tomato plants were inoculated with ~2000 CFU of *R. solanacearum* through a cut petiole (Khokhani et al. 2017). This method synchronized the infection process better than root inoculation. Bacterial colonization in sampled tissue was standardized by extracting RNA only from stems containing
between $5 \times 10^7$ and $10^9$ CFU/g stem, as determined by dilution plating a ground stem section (Supplementary Fig. 2B). Each of the three biological replicates contained pooled RNA from 4 or 5 plants.
**Fig. 2.** Nitric oxide causes broad changes in *R. solanacearum* gene expression. 

A, Design of RNA-seq experiment. We extracted RNA from *R. solanacearum* grown with altered NO levels from stems of infected tomato plants (*in planta*) and from denitrifying VDM medium (in culture) and NO levels were increased chemically by adding the NO donor CysNO to wild type (WT) and increased genetically by using a NO-accumulating ΔnorB mutant. The ΔnarG mutant has reduced levels of NO. H$_2$O$_2$-treated WT was included as a control for non-nitrosative general stress responses. 

B, Whole-transcriptome datasets were used for principal component analysis; plot created in R with the package Vegan (Oksanen et al. 2019). Each data point represents one biological replicate, with three replicates per condition. Ellipses represent a 95% confidence interval for each condition (some ellipses are so small that they appear as lines).

Around 97% of RNA-seq reads from *in vitro* samples mapped to the *R. solanacearum* genome (Supplementary Fig. 3A). Because tomato stem slices also included host RNA, only 9.2% of the *in planta* RNA reads mapped to the *R. solanacearum* genome. However, the absolute number of bacterial transcripts from the *in planta* samples still exceeded 10$^6$ reads per replicate. Principal component analysis clustered the *in vitro* samples together based on treatment, and the *in planta* samples were even more tightly clustered, reflecting less variation in gene expression than the culture samples (Fig. 2B). Genes were considered differentially expressed if they had
an adjusted P-value < 0.05. A full list of gene expression values can be found in the supplementary materials.

We validated our transcriptomic results by using qRT-PCR to measure the expression of two regulators of T3SS, *hrpB* and *hrpG*, and one well-expressed T3E, *ripX*. Expression levels of each of these genes in the ΔnarG and ΔnorB mutants generally matched those obtained from RNA-seq analysis (Fig. 3). As with *hrpB* expression, *hrpG* and *ripX* were both activated by NO, whether it was produced endogenously or exogenously.

**Fig. 3.** Expression analysis of selected T3SS-related genes by qRT-PCR validated RNA-seq results. RNA was extracted from *R. solanacearum* cultured in VDM media at 0.1% oxygen for 16 hours for both qRT-PCR and RNA-seq analysis. *R. solanacearum*
samples were: wild type strain GMI1000 (WT, untreated control reference), non-denitrifying low-NO mutant (ΔnarG) and NO-accumulating mutant (ΔnorB), and WT treated for 4h with exogenous NO (CysNO). Gene expression levels in all samples were normalized using serC (Jacobs et al. 2012). Error bars indicate standard error of the mean. Black bars, log₂ fold-change expression of T3SS regulatory gene hrpB; green bars, log₂ fold-change expression of T3SS regulatory gene hrpG; blue bars, log₂ fold-change expression of Type 3-secreted effector gene ripX. * P<0.05, for qRT, student’s t-test compared to gene expression level in untreated WT bacteria; for RNA-seq, FDR using Benjamini-Hochberg.

Denitrifying conditions in culture do not replicate in planta conditions

Because xylem fluid contains relatively small amounts of nutrients, minimal media has often been considered to mimic the xylem environment (Arlat et al. 1992; Lowe-Power et al. 2018a). Because VDM is a minimal medium and R. solanacearum experiences low-oxygen conditions and uses denitrifying respiration during tomato infection, we asked if anaerobic growth in VDM would better mimic the tomato xylem environment than rich media (Dalsing et al. 2015). However, when we examined expression of R. solanacearum genes in tomato stems compared to in VDM, we found a total of 1038 genes were differentially regulated in these conditions (Supplementary Table 2). Most genes involved in denitrification were expressed at a higher level in VDM than in planta. Other broad categories that were altered include sugar metabolism, flagellar motility, amino acid transporters, iron uptake genes, and ribosomal proteins. In
line with previous results, T3SS-related genes were among the most highly upregulated genes *in planta* (Jacobs et al. 2012). Overall, growth in VDM did not closely replicate the *in planta* environment and the bacterium seemed more reliant on denitrifying metabolism in VDM.

### Altering denitrification and NO levels changes expression of denitrification and virulence-related genes *in planta*

To determine if the effects of NO on the T3SS were specific or a part of a non-specific general response, we examined genes differentially regulated the non-denitrifying $\Delta narG$ and NO-accumulating $\Delta norB$ *in planta*. In the stem, 187 genes were significantly altered by mutating *narG* and thus blocking denitrification and NO production, while only 49 genes were altered in the NO accumulating $\Delta norB$ mutant. In $\Delta narG$, nine of the most significantly upregulated genes were directly involved in nitrate sensing and uptake or were components of the nitrate reductase protein. In both $\Delta narG$ and $\Delta norB$, genes involved in motility and sulfur metabolism were decreased compared to WT (Supplementary Table 2). Overall, this muted response highlights the robust regulatory state of *R. solanacearum* cells growing *in planta* and shows that they can tolerate even changes in fundamental aspects of its biology (Peyraud et al. 2018).

Consistent with the observation that the entire transcriptome of WT *R. solanacearum* was very different *in planta* than in culture (Fig. 2aA) the transcriptomic profiles of the denitrification mutants grown in culture were much more divergent from that of WT, with 3,145 significantly altered genes in $\Delta narG$ and 3,976 altered in $\Delta norB$. 
In the non-denitrifying ΔnarG, the bacterium showed decreased expression in many KEGG pathways involved in growth and metabolic activity such as purine and pyrimidine metabolism, oxidative phosphorylation, and tRNA synthetases (Supplementary Table 3).

In culture, genes up-regulated in the NO-accumulating ΔnorB mutant were enriched in the KEGG pathways of flagellar assembly, sulfur relay system and fatty acid degradation (Supplementary Table 3). Downregulated genes were enriched in central metabolic KEGG pathways like oxidative phosphorylation, pyrimidine metabolism, biosynthesis of amino acids, and fatty acid biosynthesis. In contrast to the activation of the T3SS, CysNO-treated R. solanacearum cells and ΔnorB saw reduction in expression of Types I, II, and VI secretion systems. Overall, the transcriptomic responses of Rs cells to either increased or decreased NO did not align well with their responses in planta. This is consistent with previous studies showing that cultured Rs cells do not reflect biology of the pathogen in its natural habitat (Jacobs et al. 2012; Perrier et al. 2018).

R. solanacearum encounters more nitrosative stress in VDM than in planta

To estimate the degree of nitrosative stress induced by our treatments, we looked at the expression of norB and hmpX, which directly detoxify NO in R. solanacearum (Farr and Kogoma 1991; Dalsing et al. 2015). Compared with R. solanacearum growing in denitrifying culture conditions, norB and hmpX expression decreased 5-fold and 20-fold in planta, suggesting that in this environment the
bacterium experiences lower nitrosative stress levels (Supplementary Table 2). In striking contrast, expression of the oxidative stress-response catalase gene katE was increased 35-fold in planta. In planta, neither the non-denitrifying ΔnarG strain nor the NO overproducing ΔnorB strain showed significant changes norB or hmpX expression. Conversely, the ΔnorB mutant grown in culture had an 11-fold up-regulation of the NO-detoxifying hmpX. In culture, our oxidative stress control treatment with H$_2$O$_2$ increased expression of katE, as expected, but also had increased expression of NO detoxifying genes hmpX and norB. Overall, these results showed some overlap in the response to ROS and RNS stress in culture but suggested that R. solanacearum cells experienced less nitrosative stress in tomato xylem than when denitrifying in VDM culture.

**Nitric oxide induced expression of T3SS-related genes both in vitro and in planta.**

To determine if in addition to hrpB, other T3SS-associated genes are induced by NO in culture, we measured relative expression of all 107 known R. solanacearum T3SS genes under each tested condition. These genes encode T3SS structural elements, regulators, secreted effectors, and chaperones (Fig. 4) (Cunnac et al. 2004; Peeters et al. 2013; Van Gijsegem et al. 1995; Plener et al. 2010; Lonjon et al. 2017; Zhang et al. 2015b, 2018, 2011; Brito et al. 1999; Marc Marenda et al. 1998; Lonjon et al. 2015; Génin et al. 2005). Cells of the NO-accumulating ΔnorB mutant grown in culture had broadly increased T3SS gene expression relative to wild type, with nine of sixteen structural elements significantly increased. Only one structural element, hrpF, trended non-significantly towards reduced expression (Fig. 4A). Five out of thirteen
known regulators of T3SS were significantly upregulated in ΔnorB, although expression
of the prhA, prhG, prhO, and the prhKLM operon were all reduced. Two out of the four
T3E chaperones had significantly elevated expression in ΔnorB, as did most T3Es. The
differential expression of effectors did not seem to correlate with their known
interactions with chaperones (Supplementary Table 4). The expression of T3SS genes
in CysNO-treated cells largely mirrored that of norB, with 80% of tested genes having a
similar trend. However, the magnitude of the effect was lower. T3SS gene expression in
ΔnarG was more varied, with fewer significantly differentially regulated genes than
ΔnorB. In contrast to ΔnorB, ΔnarG, and CysNO-treated cultures, H2O2-treated cultures
had only eight significantly changed T3SS genes (Fig. 4A). Overall, we saw a greater
induction of T3SS genes in culture by our high-NO conditions than low-NO conditions.
ΔnarG ΔnorB CysNO H₂O₂

A vs WT in culture

Structural proteins
Regulators
Chaperones
Secreted proteins

Log₂(Fold Change)

B vs WT in planta

Structural proteins
Regulators
Chaperones
Secreted proteins

Log₂(Fold Change)

C WT in culture vs WT in planta

Structural proteins
Regulators
Chaperones
Secreted proteins

Log₂(Fold Change)
Fig. 4. Nitric oxide alters expression of T3SS-related genes both in vitro and in planta.

A, Heatmap showing relative expression levels of 107 T3SS-associated genes in *R. solanacearum* cells growing in rich medium with varying NO levels. All fold-change numbers are compared to levels in untreated wild-type (WT) cells. Gene annotations, fold change, and significance levels can be found in the supplementary materials. NO levels were modified genetically using mutants lacking *narG* (deleting this nitrate reductase reduces endogenous NO) and *norB* (deleting this nitric oxide reductase increases endogenous NO). NO levels were increased chemically by adding 1 mM CysNO. Cells treated with 100 µM hydrogen peroxide (*H₂O₂*) were a non-NO control to show general stress responses. B, Relative expression levels of 107 T3SS-associated genes in *R. solanacearum* cells growing in planta. About 2000 CFU of WT *R. solanacearum, ΔnarG*, or *ΔnorB* were introduced into 17-day old Bonny Best tomato plants through a cut petiole and RNA was harvested from a stem slice 3 days after inoculation. C, Expression of most T3SS-associated genes in *R. solanacearum* strain GMI1000 was enhanced when the bacterium grew in tomato plants relative to when *R. solanacearum* cells multiplied in vitro under denitrifying conditions (VDM medium, 0.1% *O₂*).

To determine if altering NO levels affects *R. solanacearum* T3SS gene expression in a biologically relevant environment with all natural T3SS regulating inputs, we examined changes in T3SS gene expression in *ΔnarG* and *ΔnorB in planta*. Compared to *R. solanacearum* grown in culture, wild type *R. solanacearum in planta*
had much higher expression of almost every T3SS-associated gene (Fig. 3C). When growing in tomato stems, ΔnorB grown in planta had no significantly differentially regulated T3SS genes, possibly because relatively high levels of NO are already present in the plant environment (Fig. 3B). In contrast, the non-denitrifying ΔnarG mutant showed an overall trend of lower T3SS gene expression, with expression of nine genes significantly reduced (Fig. 3B). These included two components of the T3SS structure, hrpF and hrcN, the regulators hrpB and prhG, the effector chaperone hpaG, and four T3SS effectors. Even in the highly T3SS-inducing in planta environment, reducing R. solanacearum-produced NO was sufficient to reduce T3SS gene expression in tomato xylem.

Discussion

The T3SS is essential for R. solanacearum virulence, as it is for many Gram-negative pathogenic bacteria. Involving at least 16 structural proteins, this secretion system is metabolically expensive to synthesize and operate, so the bacterium is under strong selection pressure to express it only when it increases fitness (Genin et al. 1992; Boucher et al. 1985). Many factors contribute to the regulation of T3SS in R. solanacearum, including the presence of undefined host cell wall materials, unknown soluble factors, unknown metabolic cues, and (in culture) quorum sensing (Zuluaga et al. 2013; Brito et al. 1999; Aldon et al. 2000; Genin and Denny 2012; Zhang et al. 2018, 2011; Senuma et al. 2020). While many T3SS regulators have been described, open questions remain, such as why T3SS is repressed in rich media and whether specific
host cell wall components activate PrhA. In addition, many genes that affect T3SS gene
expression do so via unknown mechanisms (Zhang et al. 2011, 2015b, 2018). The
results presented here suggest that NO participates in this complex regulatory network.

Despite its toxicity at high concentrations, NO is widely used as a signaling
molecule in all domains of life (Asgher et al. 2017; Astuti et al. 2018; Simmonds et al.
2014). Many effects of NO are mediated by NO-dependent protein post-translational
modifications, including binding transition metals, S-nitrosylation of cysteine, and
tyrosine nitration (Simmonds et al. 2014; Cooper 1999; Leitner et al. 2009; Wünsche et
al. 2011; Kolbert et al. 2017; Zhang et al. 2015a; Russwurm and Koesling 2004). While
most well-characterized mechanisms of NO signaling have been described in
eukaryotes, examples of NO signaling in bacteria have also been identified. Species in
Legionella, Shewanella, Vibrio, and Silicibacter have been shown to use H-NOX
proteins to sense NO and regulate biofilm formation (Rao et al. 2015; Henares et al.
2012; Liu et al. 2012; Network et al. 2012; Carlson et al. 2010). NO also contributes to
biofilm regulation in Pseudomonas aeruginosa via the protein NosP (Hossain and Boon
2017). Both H-NOX proteins and NosP bind NO using iron centers, but no orthologues
of these genes are apparent in the R. solanacearum genome. The first example of S-
nitrosylation in bacteria was the oxidative stress response protein OxyR in E. coli (Seth
et al. 2012). OxyR regulates a distinct set of genes when a key cysteine is nitrosylated
rather than oxidized. In addition to this, nitrosative stress damage triggers S-
nitrosylation of the Salmonella enterica redox sensor SsrB and affects the S-nitroso
proteome of Mycobacterium tuberculosis (Rhee et al. 2005; Husain et al. 2010).
Although we show NO activates a key virulence factor in \textit{R. solanacearum}, its specific mechanism of action is not yet known. Using \textit{in silico} predictions of NO-dependent post-translational modifications, we could identify and selectively modify potentially important residues in T3SS regulatory proteins to determine the mechanism of NO-dependent T3SS activation (Xue et al. 2010; Liu et al. 2011).

We found that expression of T3SS-associated genes was enhanced by the presence of a plant host and by exogenous NO released by the NO donor CysNO, and the plant-mediated induction was suppressed by the NO scavenger cPTIO (Fig. 1C). T3SS gene expression also increased in response to nitric oxide produced by \textit{R. solanacearum} itself, both at natural levels generated by denitrifying respiration and at enhanced levels caused by deleting \textit{norB}. As previously reported (Monteiro et al. 2012a; Jacobs et al. 2012; Khokhani et al. 2017), \textit{R. solanacearum} cells infecting tomato plants highly expressed most T3SS genes, but reducing \textit{R. solanacearum}-produced NO by preventing denitrifying respiration with a \textit{ΔnarG} mutation significantly decreased expression of some T3SS-related genes \textit{in planta}. These included genes encoding structural components, HrpF and HrcN, key regulators HrpB and PrhG, the chaperone HpaG, and four T3-secreted effectors (Fig. 4B). This was especially striking because overall gene expression in \textit{ΔnarG} was otherwise very similar to that of wild type. These results raise the possibility that \textit{R. solanacearum} uses the presence of NO as an indirect indicator of the host xylem environment. There was no increase in T3SS gene expression in the NO-overproducing \textit{ΔnorB} mutant, which may indicate that T3SS
genes are already so highly expressed in planta that the addition of more NO does not further increase this elevated level of expression.

Besides its effects on the T3SS, altering NO concentrations in culture elicited broad changes in gene expression. We reduced NO levels using the non-denitrifying mutant ΔnarG. However, preventing the cells from using the denitrification pathway has significant effects on the biology and metabolic state of the cells, making the interpretation of these results challenging. For example, the ΔnarG cells grown in culture showed evidence of decreases in growth rate and metabolic activity, indicated by decreased expression in the biosynthetic pathways of nucleotides, reduced expression of the oxidative phosphorylation system, and decreased production of many tRNAs. Treating the cells with NO, either with exogenously applied CysNO or with the NO-overproducing ΔnorB, caused similar changes in many metabolic pathways in culture. However, a primary force in culture was the stress caused by increased NO concentrations. This manifested in decreased expression of denitrification and nitrate uptake functions and an increased expression of the NO-detoxifying enzyme hmpX. This stress was associated with decreased expression of most R. solanacearum secretion systems. However, expression of the T3SS showed the opposite trend, indicating the specificity of NO-dependent T3SS regulation.

When denitrification mutants grew in planta they had far fewer alterations in gene expression relative to wild type than when the mutants grew in culture. This suggests that inputs from the plant host help R. solanacearum control its metabolism and behaviors. Overall, the cells grown in planta seem to encounter less nitrosative stress
than cells grown in denitrifying conditions in culture. This could mean that NO stress is not as important in planta than in culture, but it does not take into account differences in the microenvironments of the xylem environment. Bacteria grown in culture experience a uniform environment with consistent NO stress. In contrast, *R. solanacearum* cells colonizing plant stems occupy multiple different microenvironments, which likely contain varying NO levels.

A previous study compared gene expression between *R. solanacearum* grown in rich media and *R. solanacearum* living in tomato xylem (Jacobs et al. 2012). Although there were methodological differences between that experiment and this one, we saw many similarities in the DEGs present between in culture and in planta *R. solanacearum*. In both experiments, many of the most highly upregulated genes are involved in the T3SS. Of the genes that were upregulated in planta in both experiments, 31 out of 141 (~22%) encoded structural components, regulators, or effectors of the T3SS. Three exceptions to this trend were *prhKLM*. Mutating any of these genes leads to a loss of T3SS gene expression, yet all three were highly downregulated in planta compared to either CPG or VDM (Supplementary Table 4) (Zhang et al. 2011; Jacobs et al. 2012). Several other genes that were highly upregulated in planta in both experiments encode metabolism of sucrose or myo-inositol, two carbon sources important for *R. solanacearum* during infection (Lowe-Power et al. 2018b; Hamilton et al. 2020; Xian et al. 2020). Additionally, genes involved in flagellar motility were upregulated in both experiments. A similar number of genes (137) were downregulated in planta in both experiments. These included several genes predicted to be involved in
amino acid transport and metabolism and the superoxide dismutase genes sodB and sodC.

Components of the denitrification pathway were among the most highly upregulated *R. solanacearum* genes *in planta* compared to in rich medium. Consistent with this observation, *R. solanacearum* mutants unable to denitrify were reduced in growth *in planta* and in bacterial wilt virulence (Dalsing et al. 2015). However, the same genes were around 20-fold more highly expressed in denitrifying culture than *in planta*. Further, the RNS-responsive NO-detoxifying genes norB and hmpX were upregulated *in planta* compared to rich media but were expressed much less *in planta* than in denitrifying conditions in culture. This indicates that *Rs* experiences more nitrosative stress when growing in denitrifying culture conditions than when growing in the plant host, which in turn is more nitrosatively stressful than aerobic growth in rich media. These data add to the strong evidence that *R. solanacearum* gene expression and metabolism are very different in the biologically realistic host environment than in culture. This may be due to the complex structure of the xylem environment, where host inputs modulate *R. solanacearum* gene expression, the ability to form defensive biofilms and multiple microenvironments with different nutrient or oxygen concentrations, or because the xylem is a flowing environment, constantly bringing new nutrients into the bacterial habitat. The bacterium’s behavior in culture should be regarded as artifactual unless proven otherwise.

We attempted to directly measure the concentrations of NO in healthy and infected tomato xylem collected from fluid pooling on a cut stem using the NO-reactive
fluorescent dye DAF-FM, electro paramagnetic resonance (EPR), and an NO-sensitive microsensor (Namin et al. 2013; Kleschyov et al. 2007; Yoshioka et al. 1996; Schreiber et al. 2008). While a fluorescent signal was observed when DAF-FM was added to xylem sap, no signal was detected through EPR using the NO spin trap cPTIO or with the microsensor (Unisense, Aarhus, Denmark), suggesting that the DAF-FM fluorescence was not specific to NO. These results highlight the need to measure NO using multiple methods in biological systems (Gupta and Igamberdiev 2013). This failure to detect NO in tomato xylem may not reflect true levels of NO in an intact plant.

We collected xylem sap by de-topping tomato plants just above the cotyledons and allowing root pressure to force xylem fluid to pool on the cut stem. Any material exuded in the first two minutes after cutting was removed in an attempt to limit the amount of cellular debris and phloem in the collected material. This method, while useful for obtaining sap to analyze for stable metabolites may not work with a transient metabolite like NO (Lowe-Power et al. 2018a). Cutting the stems stimulates production of reactive oxygen species, which quickly react with NO to form other reactive nitrogen species, potentially diluting any NO signal (Milling et al. 2011; Beckman and Koppenol 1996; Van Faassen and Vanin 2007). Furthermore, this approach can only measure NO levels in bulk xylem sap. The xylem is a dynamic and physically heterogenous environment.

Xylem flow, xylem structure, and *R. solanacearum*-produced biofilms may all create gradients or microenvironments with altered NO concentrations in the xylem of intact plants (Zimmermann, 1983; Kim *et al.*, 2016; Tran *et al.*, 2016; Lowe-Power, Khokhani and Allen, 2018). These factors complicate direct measurement of xylem NO. Confocal
microscopy of intact infected stems using fluorescent markers to visualize *R. solanacearum*, biofilms, and NO may eventually give a more complete understanding of this challenging landscape.

Overall, these results reveal a strong connection between NO levels and expression of the *R. solanacearum* T3SS. They suggest that *R. solanacearum* regulates virulence in part by using denitrifying metabolism and the resulting NO as a signal of the hypoxic high-nitrogen host xylem environment. Ongoing work is exploring how *R. solanacearum* manages nitrosative stress and identify additional effects of NO on biology of both host plant and bacterial pathogen.

**Materials and Methods**

**Bacterial strains and culture conditions.**

All strains, plasmids, and primers used in this study are listed in Supplementary Table 1. *R. solanacearum* was cultured in either CPG or a modified VDM medium at 28°C (Hendrick and Sequeira 1984; Dalsing et al. 2015). *E. coli* was grown in Luria-Bertani (LB) media at 37°C. As needed, cultures were supplemented with antibiotics: 25 µg/mL kanamycin or 15 µg/mL gentamicin.

**Strain construction.**
An *R. solanacearum* GMI1000 mutant lacking *hrpB* (RSp0873) was created using a modified *sacB*-dependent positive selection vector, pUFR80 (Castañeda et al. 2005). The regions directly upstream and downstream of *hrpB* were amplified using the Δ*hrpBup/dwn* primer pairs. These fragments were then inserted into pUFR80 digested with *Hind*III using Gibson assembly (Sievers et al. 2011). The resulting plasmid, pUFR80-Δ*hrpB* was transformed into *R. solanacearum* GMI1000 and successful plasmid integrations were selected for kanamycin resistance. The resulting colonies were then counter selected on CPG + 5% w/v sucrose. Successful deletions were confirmed using PCR, sequencing, and a functional screen for the ability to induce HR on the incompatible host *N. tabacum* (Poueymiro et al. 2009).

The Δ*hrpB* mutation was complemented by inserting a DNA fragment encoding *hrpB* and *hrcC* with the ~600 bp upstream containing their native promoter into the selectively neutral *att* site in the *R. solanacearum* chromosome using pRCK (Monteiro et al. 2012b). The fragment was amplified using hrpBcomp_F/R and inserted into *AvRlII/XbaI*-digested pRCK using Gibson assembly. This vector was then transformed into Δ*hrpB* and selected on CPG+kan.

**Plant growth conditions.**

Wilt-susceptible tomato cv. Bonny Best were germinated and grown in BM2 all-purpose germination and propagation mix (Berger, Saint-Modeste, QC). Plants were grown at 28°C with a 12 hr photoperiod. Seedlings were transplanted after 14 days. After transplanting, plants were watered on alternate days with 1/2 strength Hoagland
solution. Tomato seeds for axenic seedling production were sterilized by washing with 10% bleach for ten minutes, followed by two five-minute washes in 70% ethanol, then rinsed five times with water and germinated on water agar plates.

**Lux reporter assays.**

To test the effect of denitrification on T3SS gene expression, *R. solanacearum* strain GMI1000 carrying a *hrpB::lux* reporter gene fusion was grown in denitrifying conditions as follows (Monteiro et al. 2012a). Overnight cultures grown in CPG were resuspended in VDM with or without 30 mM potassium nitrate to an initial OD$_{600}$ of 0.001. These cultures were grown in 200 µL volumes in clear-bottomed white-walled 96 well plates at 28°C in 0.1% O$_2$ with shaking. Every half hour, the Abs$_{600}$ and luminescence was measured using a BioTek SynergyHT microplate reader (BioTek, Winooski, VT, USA). To determine the effects of the NO scavenger cPTIO on T3SS gene expression, *hrpB::lux* cultures were resuspended in 24-well plates in 1 mL of water, either with or without two five day-old sterile tomato seedlings. As appropriate, cPTIO was added to a final concentration of 1 mM. The plates were incubated at 28°C with shaking for ~24 hours. The pigmented liquid was removed from the seedlings and transferred to 1.5 mL Eppendorf tubes, after which the bacteria were pelleted by centrifugation. The pigmented supernatant was removed, and the pellets resuspended in 200 µL of water. These suspensions were transferred to a new plate and the Abs$_{600}$ and luminescence was measured as detailed above.
RNA extraction for sequencing.

To profile gene expression of *R. solanacearum* at various levels of NO stress, we extracted RNA from *R. solanacearum* grown in culture and *in planta*. For each condition, we used *R. solanacearum* from three separate overnight cultures as biological replicates. For the *in vitro* samples, overnight cultures grown in CPG were resuspended to a final OD$_{600}$ of 0.01 in 50 mL of VDM + 30 mM potassium nitrate in conical tubes. These cultures were incubated without shaking for 12 hours at 28˚C in 0.1% O$_2$. Four hours before harvesting, CysNO or H$_2$O$_2$ were added to the relevant cultures to a final concentration of 1 mM and 100 µM, respectively. CysNO was synthesized by adding HCl to a solution containing 200 mM L-cysteine and 200 mM sodium nitrite. The solution was incubated in the dark for ten minutes before it was neutralized by adding sodium hydroxide to a final concentration of 200 mM. The molarity of the solution was determined using the absorbance at 334 nm and the extinction coefficient 90/M*cm. After 16h total incubation, sub-samples were collected for dilution plating to determine CFU/ml and the tubes were capped and centrifuged at room temperature for 5 min at 8000 rpm. The supernatant was removed and pellets were frozen in liquid nitrogen. RNA extractions were carried out using a modified version of the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA, USA), as follows. Pellets were resuspended in 400 µL of ice cold TE pH 8 with 1 mg/mL lysozyme, 0.25 µL Superase Inhibitor (Ambion, Austin, TX, USA), and 80 µL of 10% SDS, vortexed for 10s, transferred to a new 2 mL tube, and shaken at ~300 rpm for 2 min. 800 µL of RNA-Lysis lysis buffer was added, the tubes were vortexed again for 10 s, cleaned according
to the kit manufacturer’s instructions, and eluted in 100 µL of water. Nucleic acid concentrations were estimated using a nanodrop, normalized to 200 ng/µL, and cleaned using the DNA-free DNAse kit (Invitrogen, Carlsbad, CA, USA). Samples were incubated at 37°C for 1 hour, with 2 µL more of DNAse added at 30 min. After DNAse inactivation, samples were further cleaned by chloroform extraction, then precipitated overnight at -20°C with 100 µM Sodium Acetate pH 5.5 and 66 % ethanol. Samples were checked for concentration on a Nanodrop, for DNA contamination by PCR using the qRT-PCR primers serC_F/R, and for RNA integrity (RIN) using an Agilent Bioanalyzer 21000 (Agilent, Santa Clara, CA, USA). All samples had RIN values above 7.3.

The in planta samples were harvested after 21 day old Bonny Best tomatoes were inoculated with ~2000 CFU of each bacteria strain through the cut petiole of the first true leaf. At 3 dpi, approximately 0.1 g of stem tissue was collected from the site of inoculation, immediately frozen in liquid nitrogen, and stored at -80°C. Another ~0.1 g of tissue was collected from directly below the inoculation site and was ground in bead beater tubes using a PowerLyzer (Qiagen, Hilden, Germany) for two cycles of 2200 rpm for 90 s in each with a 4 min rest between cycles. This material was then dilution plated to measure bacterial colonization. From each biological replicate, we chose six plants with the most similar level of colonization. RNA was extracted from these using a modified hot phenol-chloroform method (Jacobs et al. 2012). Between 4 and 5 individual plants were pooled per biological replicate. Nucleic acid sample quality was checked
using a nanodrop, Agilent bioanalyzer, and qRT-PCR primers Actin_F/R. All samples had RIN values of above 7.2.

**RNA sequencing and data analysis.**

All RNA was sent to Novogene (Beijing, China) for library preparation, sequencing, and analysis. rRNA depletion, fragmentation, and library construction were done using NEBNext® μLtra™ Directional RNA Library Prep Kit for Illumina® (NEB, Ipswitch, MA, USA) following the manufacturer’s recommendation and starting with 3 µg of RNA per sample. After the libraries were constructed, their quality was assessed using an Agilent Bioanalyzer 21000 system and sequenced using an Illumina platform to generate paired-end reads. Between 19,000,000 and 40,000,000 reads were produced per sample. Raw reads were converted to FASTQ files using Illumina CASAVA v1.8, and then filtered for quality, removing reads with adaptor contamination, greater than 10% uncertain nucleotides, or more than 50% of nucleotides with a \( Q_{\text{pred}} \) less than or equal to 5. Over 95% of reads were of good quality, and were mapped to the *R. solanacearum* GMI1000 genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_000009125.1) using Bowtie2 -2.2.3 (Langmead et al. 2009). From this, gene expression was calculated using HTseq v0.6.1 and differential gene expression was calculated using DESeq 1.18.0. P-values calculated by DESeq were adjusted to control for the false discovery rate (FDR) using the Benjamini-Hochberg approach. Gene Ontology enrichment was analyzed using
GOseq Release2.12 and KEGG enrichment was calculated using KOBAS v2.0 software.

Measuring T3SS expression with qRT-PCR.

Expression of T3SS genes was measured in denitrification mutants grown in 40 mL of VDM + 30 mM nitrate grown in GasPak™ EZ microaerobic pouches (BD, Franklin Lakes, NJ, USA) at 28°C with shaking for 18 hours starting from an OD$_{600}$ of 0.001. RNA extractions were carried out using a hot phenol-chloroform method (Jacobs et al. 2012). Extractions were carried out using the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA, USA). cDNA synthesis was done using the Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). qPCR reactions were carried out in 10 µL volumes using 5 ng of total template using PowerUp Syber Green Master) in a QuantStudio 5 Real-Time PCR System Mix (Applied Biosystems, Foster City, CA, USA).

Data Availability

The data supporting this research are openly available in the Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE160024, GEO accession: GSE160024.

Acknowledgments
The authors would like to thank Jon Jacobs for discussion and advice throughout the project and Max Miao for his expertise and discussion about statistics and R. CGH and BLD were supported by NSF predoctoral fellowships. This material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program under Grant No. DGE-1747503. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. The authors declare no competing financial interests.

References

Aldon, D., Brito, B., Boucher, C., and Genin, S. 2000. A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. Embo J. 19:2304–2314

Arlat, M., Gough, C. L., Zischek, C., Barberis, P. a, Trigalet, A., and Boucher, C. a. 1992. Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. Mol. Plant. Microbe. Interact. 5:187–193

Asgher, M., Per, T. S., Masood, A., Fatma, M., Freschi, L., Corpas, F. J., and Khan, N. A. 2017. Nitric oxide signaling and its crosstalk with other plant growth regulators in plant responses to abiotic stress. Environ. Sci. Pollut. Res. 24:2273–2285

Astuti, R. I., Nasuno, R., and Takagi, H. 2018. Nitric Oxide Signalling in Yeast. Adv. Microb. Physiol. 72:29–63

Beckman, J. S., and Koppenol, W. H. 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am. J. Physiol. 271:C1424-37

Boucher, C. A., Barberis, P. A., Trigalet, A. P., and Demery, D. A. 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn5-induced avirulent mutants. J. Gen. Microbiol. 131:2449–2457

Brito, B., Marenda, M., and Barberis, P. 1999. *prhJ* and *hrpG*, two new components of
the plant signal-dependent regulatory cascade controlled by PrhA in *Ralstonia solanacearum*. Mol. Microbiol. 31:237–251

Carlson, H. K., Vance, R. E., and Marletta, M. A. 2010. H-NOX Regulation of c-di-GMP Metabolism and Biofilm Formation in *Legionella pneumophila*. Mol. Microbiol. 77:930–942

Castañeda, A., Reddy, J., El-Yacoubi, B., and Gabriel, D. 2005. Mutagenesis of all eight *avr* genes in *Xanthomonas campestris pv. campestris* had no detected effect on pathogenicity, but one *avr* gene affected race specificity. Mol. Plant-Microbe Interact. 18:1306–1317

Cooper, C. E. 1999. Nitric oxide and iron proteins. Biochim. Biophys. Acta - Bioenerg. 1411:290–309

Cunnac, S., Occhialini, A., Barberis, P., Boucher, C., and Genin, S. 2004. Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*: identification of novel effector proteins translocated to plant host cells through the type III secretion system. Mol. Microbiol. 53:115–128

D'Alessandro, S., Posocco, B., Costa, A., Zahariou, G., Schiavo, F. Lo, Carbonera, D., and Zottini, M. 2013. Limits in the use of cPTIO as nitric oxide scavenger and EPR probe in plant cells and seedlings. Front. Plant Sci. 4:340

Dalsing, B. L., Truchon, A. N., Gonzalez-Orta, E. T., Milling, A. S., and Allen, C. 2015. *Ralstonia solanacearum* uses inorganic nitrogen metabolism for virulence, ATP production, and detoxification in the oxygen-limited host xylem environment. MBio. 6:1–13

Delledonne, M., Xia, Y., Dixon, R. a, and Lamb, C. 1998. Nitric oxide functions as a signal in plant disease resistance. Nature. 394:585–588

Elphinstone, J. 2005. The current bacterial wilt situation: a global overview. Pages 9–28 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex., C. Allen, P. Prior, and A.C. Hayward, eds. Society, American Phytopathological, St Paul, MN.

Van Faassen, E., and Vanin, A. F. 2007. *Nitric oxide radicals and their reactions*. Elsevier B.V.

Farr, S. B., and Kogoma, T. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. 55:561–585

Ge, S. X., Son, E. W., and Yao, R. 2018. iDEP: An integrated web application for differential expression and pathway analysis of RNA-Seq data. BMC Bioinformatics. 19:1–24
Génin, S., Brito, B., Denny, T. P., and Boucher, C. 2005. Control of the Ralstonia solanacearum Type III secretion system (Hrp) genes by the global virulence regulator PhcA. FEBS Lett. 579:2077–2081

Genin, S., and Denny, T. P. 2012. Pathogenomics of the Ralstonia solanacearum species complex. Annu. Rev. Phytopathol. 50:67–89

Genin, S., Gough, C. L., Zischek, C., and Boucher, C. A. 1992. Evidence that the hrpB gene encodes a positive regulator of pathogenicity genes from Pseudomonas solanacearum. Mol. Microbiol. 6:3065–3076

Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. 1995. The hrp gene locus of Pseudomonas solanacearum, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol. Microbiol. 15:1095–1114

Gupta, K. J., and Igamberdiev, A. U. 2013. Recommendations of using at least two different methods for measuring NO. Front. Plant Sci. 4:2–5

Hamilton, C. D., Steidl, O. R., Macintyre, A. M., Hendrich, C. G., and Allen, C. 2020. Ralstonia solanacearum depends on catabolism of myo-inositol, sucrose, and trehalose for virulence in an infection stage-dependent manner. MPMI.

Hanahan, D. 1983. Studies on Transformation of Escherichia coli with Plasmids. J. Mol. Biol. 166:557–580

Henares, B. M., Higgins, K. E., and Boon, E. M. 2012. Discovery of a Nitric Oxide Responsive Quorum Sensing Circuit in Vibrio harveyi. ACS Chem. Biol. 7:1331–1336

Hendrick, C. A., and Sequeira, L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen Pseudomonas solanacearum. Appl. Environ. Microbiol. 48:94–101

Hossain, S., and Boon, E. M. 2017. Discovery of a Novel Nitric Oxide Binding Protein and Nitric-Oxide-Responsive Signaling Pathway in Pseudomonas aeruginosa. ACS Infect. Dis. 3:454–461

Husain, M., Jones-carson, J., Song, M., Mccollister, B. D., and Bourret, T. J. 2010. Redox sensor SsrB Cys 203 enhances Salmonella fitness against nitric oxide generated in the host immune response to oral infection. Proc Natl Acad Sci U S A. 107:4–13

Jacobs, J. M., Babujee, L., Meng, F., Milling, A. S., and Allen, C. 2012. The in planta transcriptome of Ralstonia solanacearum: conserved physiological and virulence strategies during bacterial wilt of tomato. MBio. 3:e00114-12
Khokhani, D., Lowe-Power, T. M., Tran, T. M., and Allen, C. 2017. A Single Regulator Mediates Strategic Switching between Attachment / Spread and Growth / Virulence in the Plant Pathogen *Ralstonia solanacearum*. MBio. 8:1–20

Kim, M. K., Ingremeau, F., Zhao, A., Bassler, B. L., and Stone, H. A. 2016. Local and global consequences of flow on bacterial quorum sensing. Nat. Microbiol. 1:15005

Kleschyov, A. L., Wenzel, P., and Munzel, T. 2007. Electron paramagnetic resonance (EPR) spin trapping of biological nitric oxide. J. Chromatogr. B Anal. Technol. Biomed. Life Sci.

Kolbert, Z., Feigl, G., Bordé, Á., Molnár, Á., and Erdei, L. 2017. Protein tyrosine nitration in plants: Present knowledge, computational prediction and future perspectives. Plant Physiol. Biochem. 113:56–63

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10

Leitner, M., Vandelle, E., Gaupels, F., Bellin, D., and Delledonne, M. 2009. NO signals in the haze. Nitric oxide signalling in plant defence. Curr. Opin. Plant Biol. 12:451–458

Liu, N., Xu, Y., Hossain, S., Huang, N., Coursolle, D., and Gralnick, J. A. 2012. Nitric Oxide Regulation of Cyclic di-GMP Synthesis and Hydrolysis in *Shewanella woodyi*. Biochemistry. 51:2087–2099

Liu, Z., Cao, J., Ma, Q., Gao, X., Ren, J., and Xue, Y. 2011. GPS-YNO2: computational prediction of tyrosine nitration sites in proteins. Mol. Biosyst. 7:1197

Lonjon, F., Lohou, D., Cazalé, A. C., Büttner, D., Ribeiro, B. G., Péanne, C., Genin, S., and Vailleau, F. 2017. HpaB-Dependent Secretion of Type III Effectors in the Plant Pathogens *Ralstonia solanacearum* and *Xanthomonas campestris pv. vesicatoria*. Sci. Rep. 7:1–14

Lonjon, F., Turner, M., Henry, C., Rengel, D., Lohou, D., van de Kerkhove, Q., Cazale, A.-C., Peeters, N., Genin, S., and Vailleau, F. 2015. Comparative Secretome Analysis of *Ralstonia solanacearum* Type 3 Secretion-Associated Mutants Reveals a Fine Control of Effector Delivery, Essential for Bacterial Pathogenicity. Mol. Cell. Proteomics.

Lowe-Power, T. M., Hendrich, C. G., von Roepenack-Lahaye, E., Li, B., Wu, D., Mitra, R., Dalsing, B. L., Ricca, P., Naidoo, J., Cook, D., Jancewicz, A., Masson, P., Thomma, B., Lahaye, T., Michael, A. J., and Allen, C. 2018a. Metabolomics of tomato xylem sap during bacterial wilt reveals *Ralstonia solanacearum* produces abundant putrescine, a metabolite that accelerates wilt disease. Environ. Microbiol.
Lowe-Power, T. M., Khokhani, D., and Allen, C. 2018b. How *Ralstonia solanacearum* Exploits and Thrives in the Flowing Plant Xylem Environment. Trends Microbiol. 26:929–942

Marc Marenda, Callard, B. B. D., Genin, S., Barberis, P., Boucher, C., and Arlat, M. 1998. PrhA controls a novel regulatory pathway required for the specific induction of *Ralstonia solanacearum* hrp genes in the presence of plant cells. Mol. Microbiol. 27:437–453

Milling, A. S., Babujee, L., and Allen, C. 2011. *Ralstonia solanacearum* extracellular polysaccharide is a specific elicitor of defense responses in wilt-resistant tomato plants. PLoS One. 6

Monteiro, F., Genin, S., van Dijk, I., and Valls, M. 2012a. A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection. Microbiol. (United Kingdom). 158:2107–2116

Monteiro, F., Solé, M., van Dijk, I., and Valls, M. 2012b. A chromosomal insertion toolbox for promoter probing, mutant complementation, and pathogenicity studies in *Ralstonia solanacearum*. Mol. Plant. Microbe. Interact. 25:557–68

Namin, S. M., Nofallah, S., Joshi, M. S., Kavallieratos, K., and Tsoukias, N. M. 2013. Kinetic analysis of DAF-FM activation by NO: Toward calibration of a NO-sensitive fluorescent dye. Nitric Oxide - Biol. Chem. 28:39–46

Network, C. S., Plate, L., and Marletta, M. A. 2012. Article Nitric Oxide Modulates Bacterial Biofilm Formation through a Multicomponent. Mol. Cell. 46:449–460

Oksanen, J., Blanchet, G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O’Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., and Wagner, H. 2019. vegan: Community Ecology Package. R package version 2.5-6. Available at: https://cran.r-project.org/package=vegan.

Peeters, N., Carrère, S., Anisimova, M., Plener, L., Cazalé, A.-C., and Genin, S. 2013. Repertoire, unified nomenclature and evolution of the Type III effector gene set in the *Ralstonia solanacearum* species complex. BMC Genomics. 14:859

Perrier, A., Barlet, X., Peyraud, R., Rengel, D., Guidot, A., and Genin, S. 2018. Comparative transcriptomic studies identify specific expression patterns of virulence factors under the control of the master regulator PhcA in the *Ralstonia solanacearum* species complex. Microb. Pathog. 116:273–278

Peyraud, R., Cottret, L., Marmiesse, L., and Genin, S. 2018. Control of primary metabolism by a virulence regulatory network promotes robustness in a plant
pathogen. Nat. Commun. 9:418

Pfeiffer, S., Leopold, E., Hemmens, B., Schmidt, K., Werner, E. R., and Mayer, B. 1997. Interference of carboxy-PTIO with nitric oxide-and peroxynitrite-mediated reactions. Free Radic. Biol. Med. 22:787–794

Plener, L., Manfredi, P., Valls, M., and Genin, S. 2010. PrhG, a transcriptional regulator responding to growth conditions, is involved in the control of the type III secretion system regulon in Ralstonia solanacearum. J. Bacteriol. 192:1011–1019

Poueymiro, M., Cunnac, S., Barberis, P., Deslandes, L., Peeters, N., Cazale-Noel, A.-C., Boucher, C., and Genin, S. 2009. Two Type III Secretion System Effectors from Ralstonia solanacearum GMI1000 Determine Host-Range Specificity on Tobacco. Mol. Plant-Microbe Interact. 22:538–550

Poueymiro, M., and Genin, S. 2009. Secreted proteins from Ralstonia solanacearum: a hundred tricks to kill a plant. Curr. Opin. Microbiol. 12:44–52

Rao, M., Smith, B. C., and Marletta, M. A. 2015. Nitric Oxide mediates biofilm formation and symbiosis in Silicibacter sp. strain TrichCH4B. MBio. 6:1–10

Rhee, K. Y., Erdjument-Bromage, H., Tempst, P., and Nathan, C. F. 2005. S-nitroso proteme of Mycobacterium tuberculosis: Enzymes of intermediary metabolism and antioxidant defense. Proc. Natl. Acad. Sci. U. S. A. 102:467–72

Russwurm, M., and Koesling, D. 2004. NO activation of guanylyl cyclase. EMBO J. 23:4443–4450

Scheler, C., Durner, J., and Astier, J. 2013. Nitric oxide and reactive oxygen species in plant biotic interactions. Curr. Opin. Plant Biol. 16:534–539

Schreiber, F., Polerecky, L., and De Beer, D. 2008. Nitric oxide microsensor for high spatial resolution measurements in biofilms and sediments. Anal. Chem. 80:1152–1158

Senuma, W., Takemura, C., Hayashi, K., Ishikawa, S., Kiba, A., Ohnishi, K., Kai, K., and Hikich, Y. 2020. The putative sensor histidine kinase PhcK is required for the full expression of phcA encoding the global transcriptional regulator to drive the quorum-sensing circuit of Ralstonia solanacearum strain OE1-1. Mol. Plant Pathol. :1–15

Seth, D., Hausladen, A., Wang, Y.-J., and Stamler, J. S. 2012. Endogenous Protein S-Nitrosylation in E. coli: Regulation by OxyR. Science (80-. ). 336:470–3

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D., and Higgins, D. G. 2011.
Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7:539–545

Simmonds, M. J., Detterich, J. A., and Connes, P. 2014. Nitric oxide, vasodilation and the red blood cell. Biorheology. 51:121–134

Simontacchi, M., Galatro, A., Ramos-Artuso, F., and Santa-María, G. E. 2015. Plant Survival in a Changing Environment: The Role of Nitric Oxide in Plant Responses to Abiotic Stress. Front. Plant Sci. 6:977

Swanson, J. K., Montes, L., Mejia, L., and Allen, C. 2007. Detection of latent infections of Ralstonia solanacearum race 3 biovar 2 in geranium. Plant Dis. 91:828–834

Tran, T., MacIntyre, A., Khokhani, D., Hawes, M. C., and Allen, C. 2016. Extracellular DNases of Ralstonia solanacearum modulate biofilms and facilitate bacterial wilt virulence. Environ. Microbiol. Environ. Microbiol. Reports. 00:1–15

Vasse, J., Frey, P., and Trigalet, A. 1994. Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by Pseudomonas solanacearum. MPMI. 8:241–251

Wilson, I. D., Neill, S. J., and Hancock, J. T. 2008. Nitric oxide synthesis and signalling in plants. Plant, Cell Environ. 31:622–631

Wünsche, H., Baldwin, I. T., and Wu, J. 2011. S-Nitrosoglutathione reductase (GSNOR) mediates the biosynthesis of jasmonic acid and ethylene induced by feeding of the insect herbivore Manduca sexta and is important for jasmonate-elicited responses in Nicotiana attenuata. J. Exp. Bot. 62:4605–4616

Xian, L., Yu, G., Wei, Y., Rufian, J. S., Li, Y., Zhuang, H., Xue, H., Morcillo, R. J. L., and Macho, A. P. 2020. A Bacterial Effector Protein Hijacks Plant Metabolism to Support Pathogen Nutrition. Cell Host Microbe. :1–10

Xue, Y., Liu, Z., Gao, X., Jin, C., Wen, L., Yao, X., and Ren, J. 2010. GPS-SNO: Computational prediction of protein s-nitrosylation sites with a modified GPS algorithm. PLoS One. 5:1–7

Yoshioka, T., Iwamoto, N., and Ito, K. 1996. An application of electron paramagnetic resonance to evaluate nitric oxide and its quenchers. J. Am. Soc. Nephrol. 7:961–965

Yuliar, Nion, Y. A., and Toyota, K. 2015. Recent trends in control methods for bacterial wilt diseases caused by Ralstonia solanacearum. Microbes Environ. 30:1–11

Zhang, R., Hess, D. T., Qian, Z., Hausladen, A., Fonseca, F., Chaube, R., Reynolds, J. D., and Stamler, J. S. 2015a. Hemoglobin βCys93 is essential for cardiovascular
function and integrated response to hypoxia. Proc. Natl. Acad. Sci. U. S. A. 112:6425–6430

Zhang, Y., Kiba, A., Hikichi, Y., and Ohnishi, K. 2011. prhKLM genes of Ralstonia solanacearum encode novel activators of hrp regulon and are required for pathogenesis in tomato. FEMS Microbiol. Lett. 317:75–82

Zhang, Y., Li, J., Zhang, W., Shi, H., Luo, F., Hikichi, Y., Shi, X., and Ohnishi, K. 2018. A putative LysR-type transcriptional regulator PrhO positively regulates the type III secretion system and contributes to the virulence of Ralstonia solanacearum. Mol. Plant Pathol. :1–12

Zhang, Y., Luo, F., Wu, D., Hikichi, Y., Kiba, A., Igarashi, Y., Ding, W., and Ohnishi, K. 2015b. PrhN, a putative marR family transcriptional regulator, is involved in positive regulation of type III secretion system and full virulence of Ralstonia solanacearum. Front. Microbiol. 6:1–12

Zimmermann, M. H. 1983. Xylem Structure and the Ascent of Sap. Springer-Verlag, Berlin.

Zuluaga, A. P., Puigvert, M., and Valls, M. 2013. Novel plant inputs influencing Ralstonia solanacearum during infection. Front. Microbiol. 4:1–7
### Supplementary Table 1. Bacterial strains, plasmids, and primers

| Strains          | Relevant characteristics                                                                 | Reference                |
|------------------|-----------------------------------------------------------------------------------------|--------------------------|
| *R. solanacearum*|                                          |                          |
| GMI1000          | Phylotype I seq 18 strain isolated from tomato in French Guyana                          | (Boucher et al. 1985)    |
| *hrpB::lux*      | GMI1000 expressing the bacterial *lux* reporter controlled by the *hrpB* promoter, Kan^R| (Monteiro et al. 2012 a) |
| *ΔnarG*          | GMI1000 lacking *narG*, a nitrate reductase. Cannot denitrify and does not produce NO, Gm^R| (Dalsing et al. 2015)    |
| *ΔnorB*          | GMI1000 lacking *norB*, a nitric oxide reductase. Can denitrify, but accumulates NO, Gm^R| (Dalsing et al. 2015)    |
| *ΔhrpB*          | GMI1000 lacking *hrpB*. Avirulent and does not express a T3SS                            | This study               |
| *hrpB comp*      | *ΔhrpB* complemented., Kan^R                                                             | This study               |
| *E. coli*        |                                          |                          |
| DH5α             | dlacZ, Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1 | (Hanahan 1983)           |
| **Plasmid**      | **Description**                                                                          | **Reference**            |
| pUFR80           | Positive sucrose selection vector, Suc^S^, Kan^R                                        | (Castañeda et al. 2005)  |
| pRCK-GWY         | Gateway destination vector that integrates into the GMI1000 genome at the neutral *att* site; Kan^R | (Monteiro et al. 2012 b) |
| pUFR80-ΔhrpB     | Vector used to make unmarked *R. solanacearum ΔripB*, Suc^S^, Kan^R                     | This study               |
| pRCK-hrpBco      | Complementation vector containing WT *hrpB* and *hrcC*, Kan^R                           | This study               |
| Primer         | Sequence                                                                 | Reference        |
|---------------|--------------------------------------------------------------------------|------------------|
| ΔhrpBup_F     | 5’ – gcagggccagt.gcaGTTCGATACCAACCAGAGAAAGC                              | This study       |
| ΔhrpBup_R     | 5’ – cggaggGcGCCATGCTCTGAAGCG target locus: RSp0873                       | This study       |
| ΔhrpBdwn_F    | 5’ – gcatgcGCCCCTCggGACGGCC target locus: RSp0873                        | This study       |
| ΔhrpBdwn_R    | 5’ – acctccaggcatgcaGCTGGTGCCGACGTATTGACC GGTGC target locus: RSp0873    | This study       |
| hrpBcomp_F    | 5’ – tcgcgacggaggattgcGACAACGTCTCCCGGTTCC target locus: RSp0873-RSp0874 | This study       |
| hrpBcomp_R    | 5’ – cgcacctagttaagatcttCTTCGGCATCGCAATG target locus: RSp0873-RSp0874  | This study       |
| hrpB_F        | 5’ – CCTGCCGAGATACGCAAATG target locus: RSp0873                          | This study       |
| hrpB_R        | 5’ – CGAATGGCGGATCAGGCGCT target locus: RSp0873                          | This study       |
| hrpG_F        | 5’ – GCAGAACGTGGAATGTGTCC target locus: R. solanacearump0852            | This study       |
| hrpG_R        | 5’ – AGACTGAATGCGCTCGGTACG target locus: RSp0852                         | This study       |
| ripAA_F       | 5’ – GGAATACAGCAACTCGTGCGTCG target locus: RSc0608                       | This study       |
| ripAA_R       | 5’ – TTGTAGTTGCGGACCCCTCAG targeting locus: RSc0608                      | This study       |
| serC_F        | 5’ – CGGCGAAATACGGTGAGTGACGATTG target locus: R. solanacearumc0903      | This study       |
| serC_R        | 5’ – GTGCACAGATGCACGTAAGC target locus: RSc0903                          | This study       |
| Actin_F       | 5’ – TCGAAGCTGGGATGATATG target locus: BT013524                          | (Milling et al. 2011) |
Actin_R

| 5’ – TTAGGGTTGAGAGGTGCTTC target locus: BT013524 (Milling et al. 2011) |

*a* Capitalized bp anneal to the target locus, non-capitalized bp anneal to other fragments in a Gibson assembly reaction

**Supplementary Fig. 1.** Growth and NO levels of *R. solanacearum* strains under denitrifying conditions in culture. Cultures were started at a concentration of $10^6$ CFU/mL in VDM containing the NO-detecting fluorescent dye DAF-FM DA at 10 µM. Cultures were incubated in clear-bottomed, white-walled 96-well plates at 30°C for 24 h at $[O_2]=0.1\%$. Culture growth (dotted lines) is shown as absorbance at 600 nm. NO
concentration (solid lines) was measured as DAF-FM fluorescence at 495/515 nm. The $\Delta norB$ mutant strain accumulates NO, which significantly impairs its growth.
**Supplementary Fig. 2.** In vitro cell densities and tomato stem colonization of samples used in RNA-seq. **A,** Cell densities of culture samples as measured by dilution plating. One H$_2$O$_2$-treated plate was uncountable due to plate contamination. OD$_{600}$ values of each culture were also collected and had comparable values. **B,** Colonization of tomato plants used for transcriptome sequencing. Roughly 0.1 g of stem tissue was collected from directly below the sample used for sequencing. The tissue was ground and dilution plated to enumerate CFU/g stem.
Supplementary Fig. 3. General RNA-seq quality A, Total and mapped reads per RNA-seq sample. The *in planta* samples had lower percentage of mapped reads due to the presence of host RNA. B, Hierarchical clustering and expression heat map of the 1000 most variable genes in the dataset. Blue indicates higher expression and brown indicates lower expression. All genes were normalized to the mean gene expression across all conditions. Samples collected from *R. solanacearum* grown *in planta* are denoted with the prefix “p”. Heatmap created using iDEP.90 (Ge et al. 2018).

Supplementary Table 2 Complete list of gene expression and log$_2$ fold change values for all conditions included in this study. All fold-change numbers are compared to levels in untreated wild-type (WT) cells. NO levels were modified genetically using mutants lacking *narG* (deleting this nitrate reductase reduces endogenous NO) and *norB* (deleting this nitric oxide reductase increases endogenous NO). NO levels were increased chemically by adding 1 mM CysNO. Cells treated with 100 µM hydrogen peroxide (H$_2$O$_2$) were a non-NO control to identify general stress responses. *P*-values were calculated by DESeq were adjusted to control for the false discovery rate (FDR) using the Benjamini-Hochberg approach.

Supplementary Table 3 KEGG enrichment of genes altered in each condition. enrichment was calculated using KOBAS v2.0 software. *P* values were calculated using a hypergeometric test and adjusted using the Benjamini and Hochberg FDR correction method.
Supplementary Table 4 Annotations, Log$_2$ fold change relative to wild-type and adjusted p-values for *R. solanacearum* T3SS related genes in response to changes in NO concentration both *in planta* and in culture (see Figure 3 for heatmap of these data). All fold-change numbers are compared to levels in untreated wild-type (WT) cells. *In planta* samples are denoted by a ‘p’ in front of the treatment (e.g. *pnorB*). Genes organized by their category, including structural genes, regulatory genes, chaperones, and effectors. Effectors are subgrouped based on their known association with chaperones (Lonjon et al. 2015).