Received: 28 June 2020 | Revised: 27 January 2021 | Accepted: 31 January 2021

DOI: 10.1111/pce.14019

ORIGINAL ARTICLE

Plant, Cell & Environment WILEY

Nitrogen forms and metabolism affect plant defence to foliar and root pathogens in tomato

Shuting Ding[1] | Xiangqi Shao[1] | Jianxin Li[1] | Golam Jalal Ahammed[2] | Yanlai Yao[3] | Jian Ding[4] | Zhangjian Hu[1] | Jingquan Yu[1,5] | Kai Shi[1,5]

[1]Department of Horticulture, Zhejiang University, Hangzhou, China

[2]College of Horticulture and Plant Protection, Henan University of Science and Technology, Luoyang, China

[3]Institute of Environment, Resource, Soil and Fertilizer, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

[4]Zhejiang Agricultural Technical Extension Center, Hangzhou, China

[5]Key Laboratory of Horticultural Plants Growth, Development and Quality Improvement, Agricultural Ministry of China, Hangzhou, China

Correspondence
Kai Shi, Department of Horticulture, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, China.
Email: kaishi@zju.edu.cn

Funding information
Key Research and Development Program of Zhejiang Province, Grant/Award Number: 2021C02040; Cooperative Extension Plan of Major Agricultural Technologies of Zhejiang Province, Grant/Award Number: 2019XTTGSC04; Natural Science Foundation of Zhejiang Province, Grant/Award Number: LR19C150001; Fundamental Research Funds for the Central Universities; National Natural Science Foundation of China, Grant/Award Numbers: 31772355, 31822046

Abstract

Nitrogen (N) influences a myriad of physiological processes while its effects on plant defences and the underlying mechanisms are largely unknown. Here, the interaction between tomato and pathogens was examined under four N regimes (sole NO_3^- or mixed NO_3^-/NH_4^+ of total 1 and 7 mM N, denoting low and high N regimes, respectively) followed by inoculation with two bacterial pathogens, *Pseudomonas syringae* and *Ralstonia solanacearum*. Tomato immunity against both pathogens was generally higher under low N as well as NO_3^- as the sole N source. The disease susceptibility was reduced by silencing N metabolism genes such as *NR*, *NiR* and *Fd-GOGAT*, while increased in *NiR1*-overexpressed plants. Further studies demonstrated that the N-modulated defence was dependent on the salicylic acid (SA) defence pathway. Low N as well as the silencing of N metabolism genes increased the SA levels and transcripts of its maker genes, and low N-enhanced defence was blocked in NahG transgenic tomato plants that do not accumulate SA, while exogenous SA application attenuated the susceptibility of OE-*NiR1*. The study provides insights into the mechanisms of how nitrogen fertilization and metabolism affect plant immunity in tomato, which might be useful for designing effective agronomic strategies for the management of N supply.

KEYWORDS
nitrate reductase, nitrite reductase, plant–microbe interaction, *Pseudomonas syringae*, *Ralstonia solanacearum*, salicylic acid, *Solanum lycopersicum*

1 | INTRODUCTION

Nitrogen (N) is an essential macronutrient required by plants throughout their life cycle. It is an indispensable component of proteins, nucleic acids, cell wall and chlorophylls (Bloom, 2015). Despite significant contributions of N fertilizers to doubling food production, excessive N inputs have resulted in severe negative consequences during recent decades. These include N-caused environmental pollution, such as water eutrophication, soil acidification and greenhouse gas (N_2O) emission (Wang, Cheng, Chen, & Tsay, 2018; Xu, Fan, & Miller, 2012). In most cases, N use efficiency ranges from 30% to 50%, while 50% to 70% of the applied N fertilizers are lost from soil to crop systems, which not only affect agricultural systems but also threaten human health (Li, Wang, Hu, Gao, & Stewart, 2009; Wang et al., 2018; Zhang et al., 2015). Therefore, it is indispensable to understand the response of plants to N fertilizers for developing rational N input strategies.

Beside soil nutrient status, disease-causing plant pathogens are considered as major yield-limiting factors, which cause billions of dollars of crop yield losses every year. Three main components, including (a) host, (b) pathogen and (c) environmental conditions and their interaction, largely influence disease incidence. Therefore, it is highly likely that altered environmental conditions associated with N fertilization

would potentially affect the abundance of plant pathogens. The effect of N fertilization to various host plants on disease development appears to be variable. Some studies show that the same pathogen can be affected in an opposite manner by increased N supply to different host species. For instance, high N fertilization rates increase disease severity caused by *Botrytis cinerea* in lettuce (Lecompte, Abro, & Nicot, 2013), while elevated N concentrations result in reduced susceptibility to this fungus in tomato (Hoffland, van Beusichem, & Jeger, 1999; Vega et al., 2015). Furthermore, contrasting responses of different *B. cinerea* isolates were observed in tomato plants grown under different NO_3^- regimes (Lecompte, Abro, & Nicot, 2010). Nitrogen modulation of *Medicago truncatula* resistance to root pathogen *Aphanomyces euteiches* varied among plant genotypes (Thalineau et al., 2018). These contradictions may be, in part, explained by the impact of N supply on the nutrient availability to pathogens, the production of defence metabolites in plants and the pathogen virulence (Fagard et al., 2014; Lecompte et al., 2010). Apart from the N doses, N forms in fertilizers also affect plant resistance to pathogens. A number of studies have reported that plants grown with NH_4^+ display attenuated resistance to pathogen attack with respect to plants grown with NO_3^- fertilizer, for example, in tobacco exposed to *Pseudomonas syringae* pv. *phaseolicola* (Gupta et al., 2013), cucumber infected with *Fusarium oxysporum* (Wang et al., 2016) or rice attacked by *Rhizoctonia solani* (Chi et al., 2019). Conversely, several works have reported increased resistance in sole NH_4^+-fed plants such as tomato exposed to *P. syringae* (Fernández-Crespo, Scalschi, Llorens, García-Agustín, & Camañes, 2015; González-Hernández et al., 2019). In this case, the positive effect of NH_4^+ on tomato resistance might be a consequence of NH_4^+ toxicity-provoked priming that eventually increased the resistance against pathogen infection (Fernández-Crespo et al., 2015; González-Hernández et al., 2019). Most plants prefer nitrate nutrition and grow well under sole NO_3^-, or low NH_4^+ supply combined with a high proportion of NO_3^-, while the plants may exhibit ammonium toxicity and yield decrease when supplied with NH_4^+ as the sole N source (Ben-Oliel et al., 2004; Borgognone et al., 2013). Many studies have investigated the effects of different ratios of NO_3^- and NH_4^+ under specific total N concentrations on tomato growth, development and responses to abiotic stresses. Nonetheless, few studies investigated the effects of combined N forms on plant susceptibility and the mechanism involved.

In plants, upon absorption of N fertilizers by nitrate transporters, nitrate is first reduced to nitrite by nitrate reductase (NR) in the cell cytosol. Then, nitrite is reduced to ammonium by nitrite reductase (NiR) in plastids or chloroplasts, and ammonium is then assimilated into amino acids through glutamine synthetase/glutamine-2-oxoglutarate aminotransferase (GS/GOGAT) cycle (Xu et al., 2012). NR is a rate-limiting enzyme in N assimilation and the double mutants of *Arabidopsis thaliana*, *nia1nia2*, show reduced amino acid contents with increased susceptibility to *P. syringae* pv. *maculicola* (Oliveira, Justino, Sodek, & Salgado, 2009). However, alterations in the Arabidopsis *Nitrate Trasnporter2.1* (*NRT2.1*) gene modify basal susceptibility, and the *nrt2.1* mutant is less susceptible to *P. syringae* (Camañes et al., 2012). Similarly, rice mutants lacking *Fd-GOGAT* show enhanced resistance to seven *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains (Chen et al., 2016). It seems that N-related genes are affected by pathogen attack, and the gene modification can alter N metabolism, leading to remarkable effects on disease development. However, the relationships between N metabolism and plant–pathogen interaction are not simply trophic rather more complex, which can be affected by the forms of N fertilizers, host plant species and the types of pathogens (e.g. [hemi]biotrophic and necrotrophic). Thus, how the N regimes and the N metabolism are associated with plant defence and the underlying mechanisms need to be tested in a biological context using the same system, which might account for the highly specific nature of host–pathogen interactions in response to N conditions.

Plants have evolved complex signalling networks to respond to pathogen attacks. During both compatible and incompatible plant–pathogen interactions, salicylic acid (SA) is a major plant defence hormone whose levels rise, especially in response to biotrophic and hemibiotrophic pathogens (Vlot, Dempsey, & Klessig, 2009). Endogenous SA manipulation and exogenous SA application can induce systemic acquired resistance (SAR) and the expression of defence-related genes, such as *pathogenesis-related protein 1* (*PR1*) (Klessig, Choi, & Dempsey, 2018). SA accumulation and *PR1a* transcripts decreased in NH_4^+-fed tobacco plants compared with NO_3^--fed plants, which was suggested to mediate the influence of N forms on tobacco immunity against *P. syringae* pv. *phaseolicola* (Gupta et al., 2013). Disturbance in N uptake due to a deletion in the nitrate high-affinity transporter *NRT2.1* results in faster priming of SA defence pathway and reduced susceptibility to *P. syringae* in *Arabidopsis* (Camañes et al., 2012), suggesting that impairment in N metabolism potentially favours SA pathway in *Arabidopsis*. However, in tomato, SA generation and its signalling pathways have been demonstrated to not be required for the NH_4^+-mediated defence response (Fernández-Crespo et al., 2015). Thus, the role of SA pathway in the N-modulated plant defence still remains elusive.

Tomato is one of the most economically important vegetable crops, cultivated on large-scale throughout the world. More than 200 pests and diseases affect tomato production, causing significant economic losses (Bai & Lindhout, 2007). In particular, *P. syringae*, a hemibiotrophic bacterial pathogen that causes speck and wilt diseases in aboveground parts of plants, resulting a huge yield penalty of vegetable crops. It is also used as a model pathogen to investigate the mechanism of plant–pathogen interaction. Furthermore, *Ralstonia solanacearum*, a soilborne biotrophic bacterial pathogen, is one of the most aggressive and destructive pathogens in the world because of the high mortality rates of diseased plants and the lack of effective control measures (French, Kim, Rivera-Zuluaga, & Iyer-Pascuzzi, 2018). In the present study, we used *Pst* DC3000 and *R. solanacearum* as bacterial agents and explored the tomato–bacteria interaction under different N regimes, considering the vital roles of N in plant growth and defence response. Furthermore, target gene overexpression (OE) and virus-induced gene silencing (VIGS) tools were used to better understand the relationship between N metabolism and plant defences and the mechanism involved. Our data indicate that low N, as well as NO_3^-, favours plant basal defence against both pathogens,

Pst DC3000 and *R. solanacearum*, and SA pathway is involved in N-modulated defence. This information not only provides new insights into the modulation of N metabolism in plant immune responses but also paves new ways for the strategical development of plant disease management.

2 | MATERIALS AND METHODS

2.1 | Plant materials and treatments

Tomato (*Solanum lycopersicum* L.) genotype 'Condine Red' was used in most experiments. NahG transgenic line (in which overexpression of salicylate hydroxylase abolishes SA accumulation) and its wild-type control line Moneymaker (MM) were obtained from the laboratory of J.D.G. Jones (Sainsbury Laboratory, Norwich, UK). Seeds were germinated in trays of growth medium containing a mixture of vermiculite and perlite (1/1, v/v) in a greenhouse. After emergence, groups of four seedlings were transferred to 1.5-L tanks filled with half-strength Enshi nutrient solution (Yu & Matsui, 1997). The solution was continuously aerated with an air pump, and the growth conditions were as follows: 500 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), 12 hr daily light period, 22/20°C (day/night) air temperature and 80% relative humidity.

About 4 weeks after sowing, tomato seedlings at 4- to 5-leaf stages were subjected to an N-free hydroponic solution for 2 days and then supplied with different N regimes. Half of the plants were fed with a dose of N supply previously shown to be non-limiting for tomato seedling growth (Claussen, 2002; Royer, Larbat, Le Bot, Adamowicz, & Robin, 2013) referred to as 'high (H) N', with N regulated at 7 mM. The low N dose, sub-optimal for tomato seedling growth regulated at 1 mM, is referred to as 'low (L) N'. N was provided either as pure NO_3^- or as NO_3^-/NH_4^+ mixture (70/30 mol/mol). The hydroponic culture solutions were prepared with water and pure salt. Nitrate was brought by KNO_3 and $Ca(NO_3)_2$. H-NO_3^- nutrient solution consists of the following macronutrients (in mM): KNO_3 (2), $Ca(NO_3)_2$ (2.5), $MgSO_4$ (1), KH_2PO_4 (1) and K_2SO_4 (1.15). The L-NO_3^- solution at 1 mM was composed of $Ca(NO_3)_2$ (0.5), $MgSO_4$ (1), KH_2PO_4 (1), K_2SO_4 (1.15), $CaCl_2$ (2) and KCl (2). Ammonium was brought by $(NH4)_2SO_4$. In H-NO_3^-/NH_4^+nutrient solution, they were: KNO_3 (0.9), $Ca(NO_3)_2$ (2), $(NH_4)_2SO_4$ (1.05), $MgSO_4$ (1), KH_2PO_4 (1), K_2SO_4 (0.1), $CaCl_2$ (0.5) and KCl (3.2).The L-NO_3^-/NH_4^+ nutrient solution contained $Ca(NO_3)_2$ (0.35), $(NH_4)_2SO_4$ (0.15), $MgSO_4$ (1), KH_2PO_4 (1), K_2SO_4 (1), $CaCl_2$ (2.15) and KCl (2.3). In all solutions, the trace elements were provided as previously described (Royer et al., 2013). The pH of the nutrient solution was adjusted to 5.5 using 1 M KOH and was continuously renewed every 2 days to maintain a stable nutrient composition. Tomato plants were grown in different nutrient solutions for 7 days before inoculation with *Pst* DC3000 or *R. solanacearum*.

For experiments without different N regime treatments, tomato seedlings were grown in plastic pots (diameter, 10.5 cm; depth, 10.5 cm; one plant per pot) filled with a mixture of peat and vermiculite (2:1, v/v) and were fertilized with complete nutrient, with intermediate nitrogen concentration at 5 mM NO_3^- (Claussen, 2002; Debouba, Maâroufi-Dghimi, Suzuki, Ghorbel, & Gouia, 2007). In the experiments employing a combined treatment of pathogen inoculation and SA, the chemical pre-treatments were applied to the tomato 2 hr prior to pathogen inoculation.

2.2 | Generation of virus-induced gene silencing and gene overexpression plants

VIGS was performed by infiltrating the fully expanded cotyledons of 10-day-old tomato seedlings with tobacco rattle virus (TRV) vectors using a mixture of pTRV1 and pTRV2, as previously described (Liu, Schiff, & Dinesh-Kumar, 2002). Fragments from tomato *NR*, *NiR1*, *NiR2* and *Fd-GOGAT* cDNAs were PCR-amplified using the primers shown in Table S1 and cloned into pTRV2. The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. An empty pTRV2 vector was used as a native control. pTRV:*NiR1/2* was an equal mixture of pTRV:*NiR1* and pTRV:*NiR2*. The silencing efficiency was evaluated by quantitative real-time-PCR using specific primers shown in Table S2. Only plants showing a significant silencing efficiency were used for experiments.

For generating the *NiR1* overexpression plants, the 1,761 bp full-length coding DNA sequence (CDS) of *NiR1* was cloned by PCR using specific primers (Table S1). The PCR product was digested with AscI and KpnI and inserted behind the CaMV 35S promoter in the plant transformation vector pFGC1008-HA. The recombinant vector was transferred into *A. tumefaciens* strain GV3101. Transgenic plants overexpressing the *NiR1* were identified by western blotting using an anti-HA (Pierce) monoclonal antibody. Two independent homozygous lines (OE-*NiR1*#4, #5) of the F_2 progeny were used in this study.

2.3 | Pathogen inoculation and pathogenicity assays

The bacterial strain *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) was cultured in King's B solid medium containing rifampicin (25 mg ml^{-1}) at 28°C overnight. The bacteria were diluted with 10 mM $MgCl_2$. The whole plant was sprayed with bacteria suspension at a concentration of 10^7 colony-forming units per millilitre (CFU ml^{-1}) using 0.02% Silwet L-77, according to a previously described method (Katagiri, Thilmony, & He, 2002). Negative control was carried out with 10 mM $MgCl_2$. *In planta*, bacterial population was assessed at 2 days post inoculation (dpi) as described previously (Wolfe, Hutcheon, Higgins, & Cameron, 2000). Trypan blue staining was performed 2 dpi with *Pst* DC3000 to visualize tissue damage according to a previously described method (Bai et al., 2012). Based on the ratio of dying area, trypan blue staining was quantified by Image J software.

The bacteria *R. solanacearum* was grown in Casamino Peptone Agar (CPG) containing 1% triphenyltetrazolium chloride (TZC) at 28°C for 2 days. Bacteria were harvested with sterile water, and bacterial suspensions were adjusted to a density of 1.0 at 600 nm wavelength

corresponding to approximately 10^9 CFU ml^{-1}. Approximately, 5-week-old plants were inoculated by soil drenching with 40 ml of the bacterial suspension per pot, whereas soil drenched with sterile water was the negative control. For hydroponic plants, 50 ml bacterial suspension was added into each pot containing 500 ml nutrient solution. *In planta*, *R. solanacearum* colonization in the stem was measured as described previously (Kiirika, Stahl, & Wydra, 2013). The disease index was measured based on the percentage of wilted leaves in each individual plant, according to the following disease rating scores: 0 = 0%, 1 = 1% to 25%, 2 = 26% to 50%, 3 = 51% to 75% and 4 = 76% to 100% (Denancé et al., 2013).

Unless otherwise stated, for *Pst* DC3000 experiments, samples analysed were collected from leaves, while for *R. solanacearum* experiments, most samples were collected from roots, except for the *R. solanacearum* colonization samples that were collected from midstems of inoculated plants.

2.4 | RNA isolation and transcript analysis

Total RNA was isolated using RNA extraction kits (Easy-Do Biotech) and reverse transcribed using a ReverTra Ace qPCR RT kit (Toyobo) following the manufacturer's instructions. qRT-PCR was performed using the Light Cycler 480 real-time PCR system (Roche Diagnostics). Each reaction (20 μl) consisted of 10 μl of SYBR Green PCR Master Mix (Takara), 7.2 μl of water, 2 μl of cDNA and 0.4 μl each of forward and reserve primers. The PCR was run at 94°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min. The housekeeping genes *Actin* and *UBI3* were used as internal control, and *Actin* was chosen for calculating the relative expression of target genes. Gene-specific primer pairs as well as the amplification efficiency are listed in Table S2.

2.5 | Analysis of endogenous SA, JA, NO_3^- and NH_4^+ content

The concentrations of SA and JA were measured using an HPLC-MS/MS-based method as described previously (Zhang et al., 2018). Nitrate in plant tissues was assayed using the salicylic acid–sulphuric acid method (Cataldo, Haroon, Schrader, & Youngs, 1975). The absorbance was recorded at 410 nm. The nitrate content was calculated using a standard curve of KNO_3. Ammonium contents in leaf and root samples were assayed using 40% $KNaC_4H_4O_6$ and the Nessler reagent (Molins-Legua, Meseguer-Lloret, Moliner-Martinez, & Campíns-Falcó, 2006). The absorbance was measured at 430 nm, and ammonium content was calculated using a standard curve of NH_4Cl.

2.6 | Statistical analysis

At least three independent biological replicates from different plants were conducted for each determination. The experiments were independently performed three times. The data were subjected to analysis of variance using SAS software, version 8 (SAS Institute), and means were compared using Tukey's test at the 5% level.

3 | RESULTS

3.1 | Effects of nitrogen forms and concentrations on bacterial pathogen incidence

To study the role of N in plant defence against bacterial pathogens, hydroponic cultures were conducted for 7 days before pathogen inoculation, using sole NO_3^- and a mixture of NO_3^- and NH_4^+ (70/30 mol/mol ratio) as N source at different concentrations. Plant susceptibility was firstly compared between the sole NO_3^- or mixed NO_3^-/NH_4^+ of total 1 mM [low (L)] and 7 mM [high (H)] N conditions (Figure 1). At 5 days post *Pst* DC3000 inoculation (dpi), the surface of the infected leaves showed visible disease lesions (Figure 1a). Notably, the leaves supplied with low N levels, regardless of its forms, showed fewer disease lesions compared with high N supply. Moreover, under the same N concentrations, the numbers of disease lesions on tomato leaves seemed reduced in plants grown under NO_3^- as compared with NO_3^-/NH_4^+. As a reliable marker of pathogen-caused symptoms, trypan blue staining was used to visualize the cell death in tomato leaves (Figure 1b), and the dying area was quantified (Figure 1c). The extent of cell death on the leaves was not evident in low N supply as that in high N supply, and NO_3^- form reduced the area of dead cells compared with NO_3^-/NH_4^+ under the same N concentration. In addition, growth analysis of *Pst* DC3000 in inoculated leaves at 2 dpi showed that plants supplied with H-NO_3^-/NH_4^+ had the highest bacterial colony counts, followed by H-NO_3^-. However, the infected leaves supplied with L-NO_3^- showed the least bacterial colony counts (Figure 1d). The effects of N forms and concentrations on plant resistance against *R. solanacearum* were similar to that of *Pst* DC3000 (Figure 1e–g). *R. solanacearum*-inoculated plants that received low N dose–exhibited delayed symptom development (leaf wilting) compared with high N-treated plants. Moreover, under the same N concentration, tomato plants that received NO_3^-/NH_4^+ wilted more severely than those received only NO_3^- (Figure 1e,f). Bacterial colony counts were consistent with the phenotype of plants (Figure 1g).

3.2 | Genes involved in N metabolism are responsive to bacterial invasion

In plants, absorbed nitrate is reduced to nitrite by NR, followed by further reduction of nitrite to ammonium, which is then directly assimilated into amino acids. In this study, the NO_3^--N concentrations in leaves and roots generally increased with increasing NO_3^- and decreased when the mixture of NO_3^-/NH_4^+ was supplied to the nutrient solution. The endogenous NH_4^+-N content also increased at high

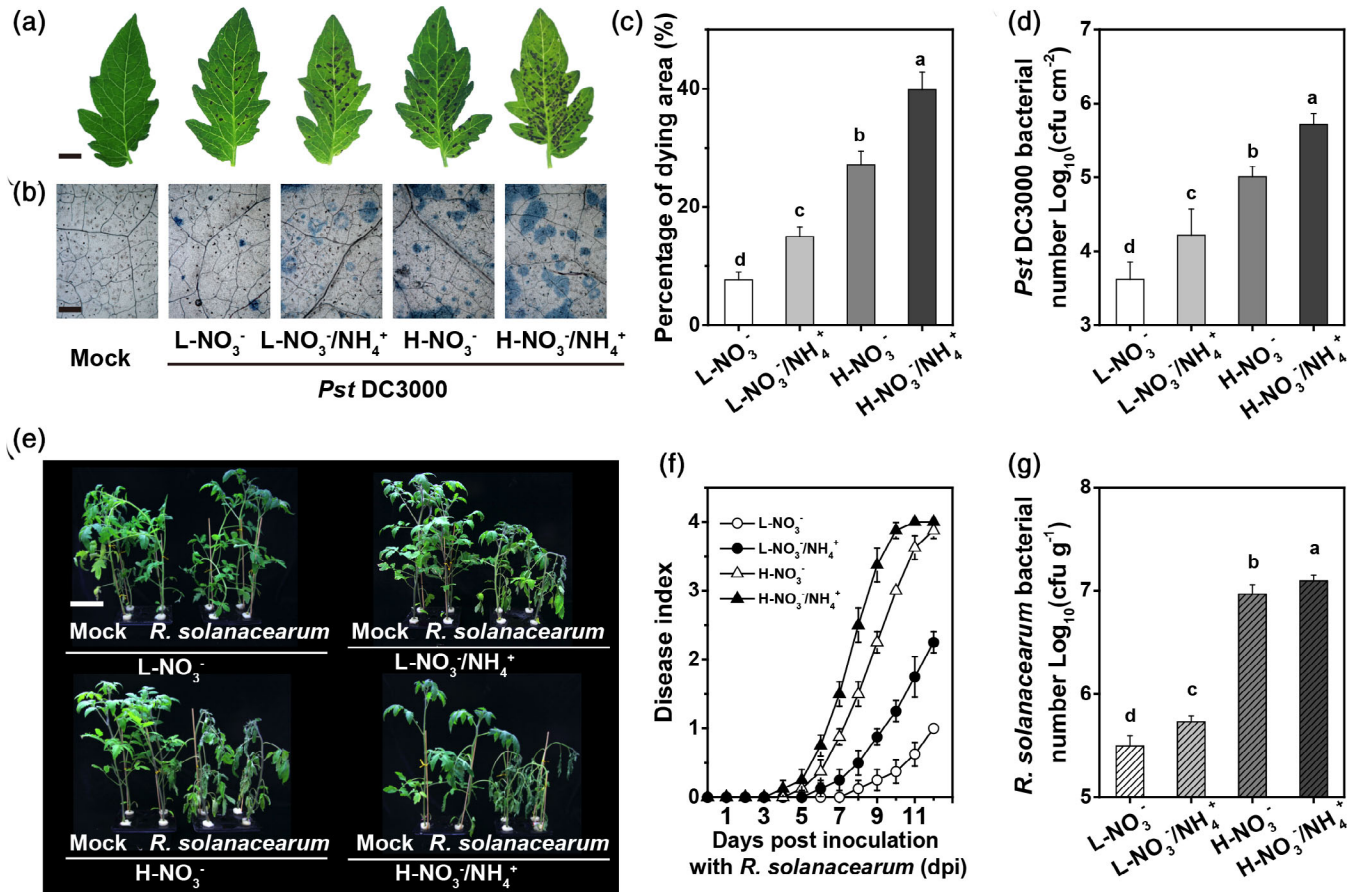

FIGURE 1 Effects of nitrogen (N) forms and concentrations on the susceptibility of tomato plants to *Pst* DC3000 and *R. solanacearum*. Plants were grown hydroponically and treated as described in Section 2. (a) Disease symptoms photographed at 5 days post inoculation (dpi) with *Pst* DC3000. Bar = 1 cm. (b) Trypan blue staining for visualizing cell death was carried out at 2 dpi with *Pst* DC3000. Bar = 500 μm. (c) Quantitative data of trypan blue staining of *Pst*-inoculated leaves at 2 dpi. (d) *Pst* DC3000 bacterial population at 2 dpi. (e) The phenotypes of plants inoculated with *R. solanacearum* at 12 dpi. Bar = 10 cm. (f) Mean disease index ± SE following *R. solanacearum* inoculation under four different nitrogen treatments; $n = 8$. (g) *R. solanacearum* bacterial population at 12 dpi. The results in (d) and (g) are presented as mean values ± SD, $n = 4$. Different letters depict significant differences between the treatments ($p < .05$). The above experiments were repeated three times with similar results

N treatment, but the increase was most evident in the mixture of NO_3^-/NH_4^+ treatment. Both endogenous NO_3^--N and NH_4^+-N contents generally decreased in the pathogen-inoculated condition compared to their mock-inoculated counterparts (Figure S1).

To examine the effect of bacterial inoculation on N assimilation, the transcript levels of four key genes, including *NR*, *NiR1*, *NiR2* and *Fd-GOGAT* involved in N assimilation, were investigated at four different N regimes after inoculation with two pathogens (Figure 2). Under mock-inculated condition, the transcripts of these four genes generally increased with higher total N concentration and decreased when the mixture of NO_3^-/NH_4^+ was supplied. Among them, the effect of N forms on *Fd-GOGAT* expression was less evident. On the other hand, inoculation of both pathogens significantly decreased the transcript levels of these four genes, and the changes of these genes expression were generally more evident under high N condition compared with that under low N. Notably, the expression of *NR* fluctuated in leaves after *Pst* DC3000 inoculation, while the expression of *NiR1* gradually decreased over time in response to both *Pst* DC3000 and *R. solanacearum* inoculation. These results indicate that pathogen infections potentially alter N metabolism in plants.

3.3 | Genetic manipulation of N metabolism genes alters disease resistance in tomato

To investigate the relationship between N metabolism and plant defence, genes involved in N metabolism (*NR*, *NiR1/2* and *Fd-GOGAT*) were silenced using VIGS in tomato plants. This method reduced the transcript abundance of target genes to about 42% compared with the TRV:0 plants (Figure S2). The leaves of TRV:*Fd-GOGAT* plants showed variegated chlorosis, while the TRV:*NiR1/2* plants showed normal phenotype as of the TRV:0 plants (Figure S3). We found that silencing *NiR1/2* and *Fd-GOGAT*

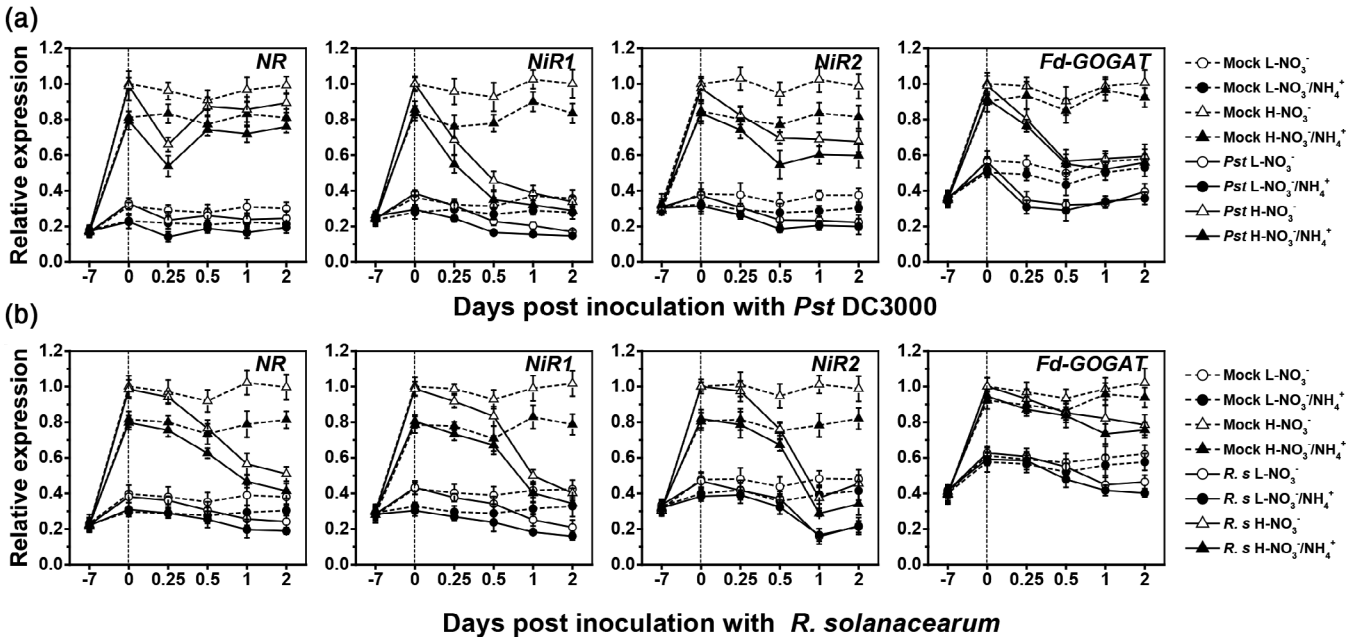

FIGURE 2 Effects of *Pst* DC3000 and *R. solanacearum* inoculation on the transcript abundance of key genes involved in nitrogen metabolism. (a) Time-course changes in expression of nitrogen metabolism–related genes in tomato leaves in response to *Pst* DC3000- or mock-inoculated condition. (b) Time-course of changes in expression of target genes in tomato roots in response to *R. solanacearum*- or mock-inoculated condition. Leaf or root tissues were sampled at 7 days before inoculation (−7 dpi, i.e., the 0 day of different nitrogen treatments), 0 dpi, 0.25 dpi, 0.5 dpi, 1 dpi, and 2 dpi. The expression of each gene under H-NO_3^- with mock inoculation at 0 dpi was defined as 1. The results are presented as mean values ± SD, $n = 4$. The results are presented as mean values ± SD, $n = 4$. The above experiments were repeated three times with similar results

significantly enhanced the resistance to *Pst* DC3000, as reflected by reduced cell death and bacterial population in tomato leaves compared with TRV:0 plants. This effect was more profound in TRV:*Fd-GOGAT* plants than TRV:*NiR1/2* plants (Figure 3a–c). Similar to the response of plants to *Pst* DC3000, *NR*-, *NiR1/2*- and *Fd-GOGAT*-silenced plants showed enhanced resistance to *R. solanacearum* inoculation, as revealed by plant phenotype and bacterial colony counts (Figure 3d–f). At 12 dpi, the TRV:0 plants showed severe wilting phenotype, while the silenced plants exhibited minor symptoms (Figure 3d). These results suggest that suppression of N metabolism-related genes could enhance plant resistance against bacterial pathogens in tomato.

To further clarify the role of N assimilation in plant defence, we generated *NiR1* overexpression plants (OE-*NiR1* #4 and OE-*NiR1*#5). Phenotypically, there were no differences between the wild-type (WT) and OE-*NiR1* plants (Figure S4); however, OE-*NiR1* plants were found to be more susceptible to both *Pst* DC3000 and *R. solanacearum* (Figure 4). Leaf phenotype observation and trypan blue staining revealed an increased cell death in OE-*NiR1* leaves compared with WT in response to both pathogens (Figure 4a,b,e). Moreover, bacterial colony counts showed that overexpression of *NiR1* significantly increased the bacterial population in tomato (Figure 4d,g). These results indicated that enzymes involved in N assimilation could negatively regulate plant immunity against the bacterial disease, which further confirmed that low N dose would enhance plant resistance against bacterial pathogens.

3.4 | N-modulated defence is associated with the SA pathway

Phytohormone SA mediates plant defence response to bacteria. To clarify whether SA defence pathway is involved in N-modulated defence, SA accumulation was measured in tomato leaves with or without bacterial inoculation (Figures 5 and 6). The basal levels of SA showed no significant differences among different N treatments in leaves of mock plants. However, at 24-hr post infection (hpi) with *Pst* DC3000, the SA content in leaves significantly increased in all N treatments, and its levels were significantly higher in low N-treated plants than that treated with high N supply. More precisely, the content of SA under L-NO_3^- was almost twice than that under H-NO_3^-/NH_4^+ after *Pst* DC3000 infection. In addition, tomato plants fed with NO_3^- showed a higher SA content than those treated with NO_3^-/NH_4^+ under the same N concentrations (Figure 5a). In plants, there are two distinct pathways in SA biosynthesis, the isochorismate (IC) pathway and the phenylalanine ammonia lyase (PAL) pathway (Dempsey, Vlot, Wildermuth, & Klessig, 2011). We found that *PAL1* and *PAL2* were induced by inoculation with *Pst* DC3000, and the induction was consistent with SA accumulation, while the expression of *ICS* decreased significantly after *Pst* DC3000 inoculation (Figure 5b and Figure S5a). The expression of *PRs* is considered as a reliable marker of SA-mediated plant defence against pathogenic microbes (Uknes et al., 1993; Uppalapati et al., 2008). In our experiment, the relative expression of *PR1*, *PR2*, *PR4* and *PR5* showed similar trends when

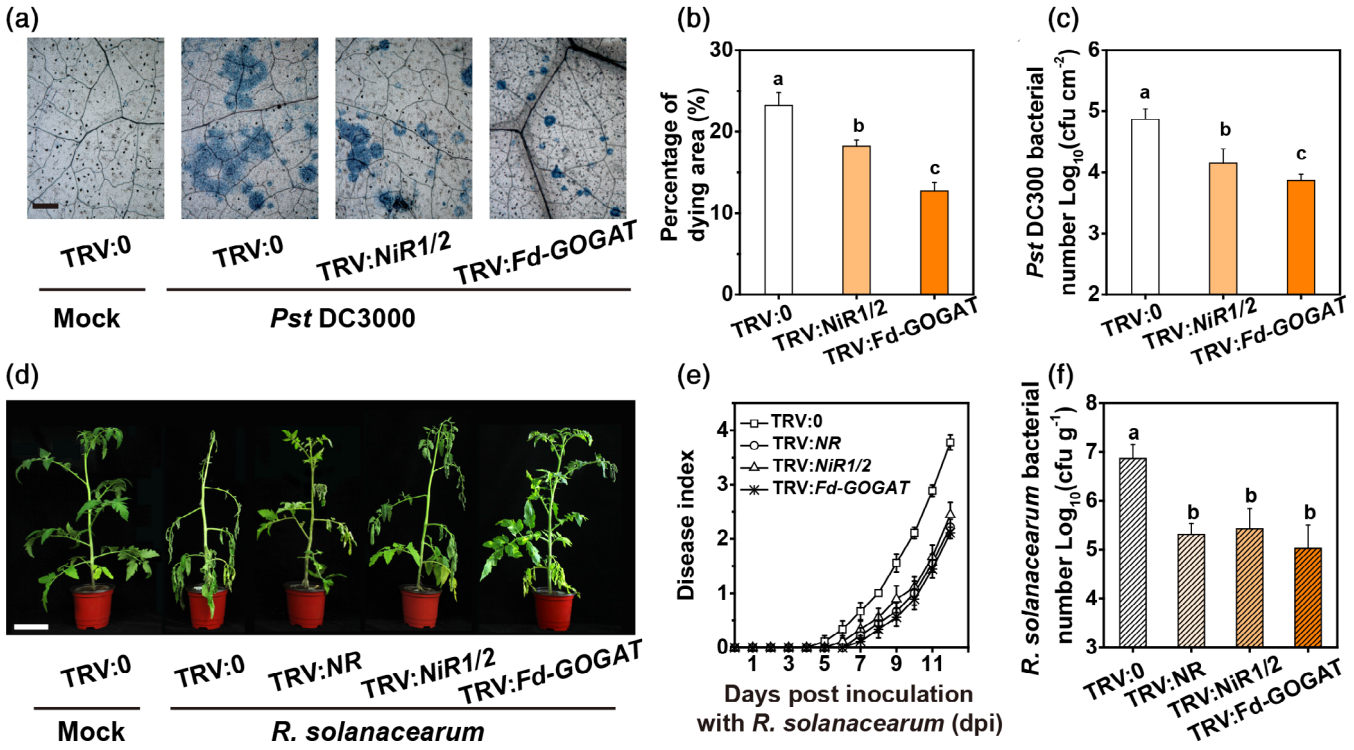

FIGURE 3 Effects of silencing nitrogen metabolism–related key genes on the susceptibility of tomato plants to *Pst* DC3000 and *R. solanacearum* pathogens. (a) Trypan blue staining for cell death in tomato leaves sampled at 2 dpi with *Pst* DC3000. Bar = 500 μm. (b) Quantitative data of trypan blue staining of *Pst*-inoculated leaves at 2 dpi. (c) *Pst* DC3000 bacterial population at 2 dpi. (d) The phenotypes of plants at 12 dpi with *R. solanacearum*. Bar = 10 cm. (e) Mean disease index ± SE following *R. solanacearum* inoculation of gene-silenced plants; $n = 9$. (f) *R. solanacearum* bacterial population at 12 dpi. The results in (c) and (f) are presented as mean values ± SD, $n = 4$. Different letters indicate significant differences between treatments ($p < .05$). The above experiments were repeated three times with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

inoculated with *Pst* DC3000 (Figure 5b and Figure S5a). The transcript levels of *PR2* and *PR4* under L-NO_3^- were about fourfold higher than H-NO_3^-/NH_4^+ at 12 hpi. We then analysed SA content and transcript abundances of *PALs* and *PRs* in *NiR1/2*- and *Fd-GOGAT*-silenced plants with or without *Pst* DC3000 infection (Figure 5e,f and Figure S5b). Although SA content was not significantly different between pTRV:0 and TRV:*NiR1/2* plants in mock treatment, the SA content in TRV:*Fd-GOGAT* was 17-fold higher than pTRV:0 without inoculation. At 24 hpi, SA accumulation in leaves significantly increased, and it was much higher in silenced plants than pTRV:0 (Figure 5e). Under the mock condition, the transcript abundance of *PR2* in TRV:*Fd-GOGAT* was 23-fold of pTRV:0, and the transcript abundance of *PR4* in TRV:*Fd-GOGAT* was 84-fold (Figure 5f).

The SA content and the relative expression of *PALs* and *PRs* showed similar trends in roots after inoculation with *R. solanacearum*, although their levels were lower than that in leaves with *Pst* DC3000 infection (Figure 6 and Figure S6). Unlike leaf SA content, the SA content in the roots of mock plants was significantly higher in low N supply than that in high N supply (Figure 6a). Furthermore, at 24 hpi, the transcript levels of *PALs* and *PRs* significantly increased in all N treatments with the highest transcript abundance under L-NO_3^- treatment (Figure 6b and Figure S6a). Analysis of SA content in roots revealed that silencing *Fd-GOGAT* significantly increased SA content in roots under mock conditions. Moreover, infection with *R. solanacearum* significantly increased the SA content in all genotypes (Figure 6e). Upon inoculation, the transcript levels of *PAL1*, *PR2* and *PR4* significantly increased, and silenced plants showed a greater expression level than TRV:0 plants at 24-hpi (Figure 6f). All these results indicated that low N status in plants could trigger SA defence pathway more intensely towards enhanced resistance to bacterial pathogens. Several lines of evidence indicate that crosstalk between SA and JA plays an essential role in plant response to pathogens (Glazebrook, 2005; Liu et al., 2016; Vega, O'Brien, & Gutiérrez, 2019). Therefore, we analysed endogenous JA levels, and the transcript levels of genes involved in JA biosynthesis and signalling (Figures 5 and 6). The concentrations of JA increased in leaves under all N conditions after *Pst* DC3000 inoculation; however, the extent of increase was far lower than SA in leaves. Meanwhile, JA levels were significantly higher in H-NO_3^-/NH_4^+-treated plants than that treated with L-NO_3^- (Figure 5c). The transcript levels of *allene oxide cyclase* (*AOC*) and *proteinase inhibitors* (*PIs*) also increased in a similar trend after inoculation with *Pst* DC3000 (Figure 5d). In addition, JA content and the transcript levels of *AOC* and *PIs* were analysed in silenced plants. Upon inoculation with *Pst* DC3000, JA content increased in all plants; however, the JA levels and the transcript abundance of *AOC*, *PI-I* and *PI-II* in TRV:*Fd-GOGAT* plants were significantly lower than that of TRV:0 plants

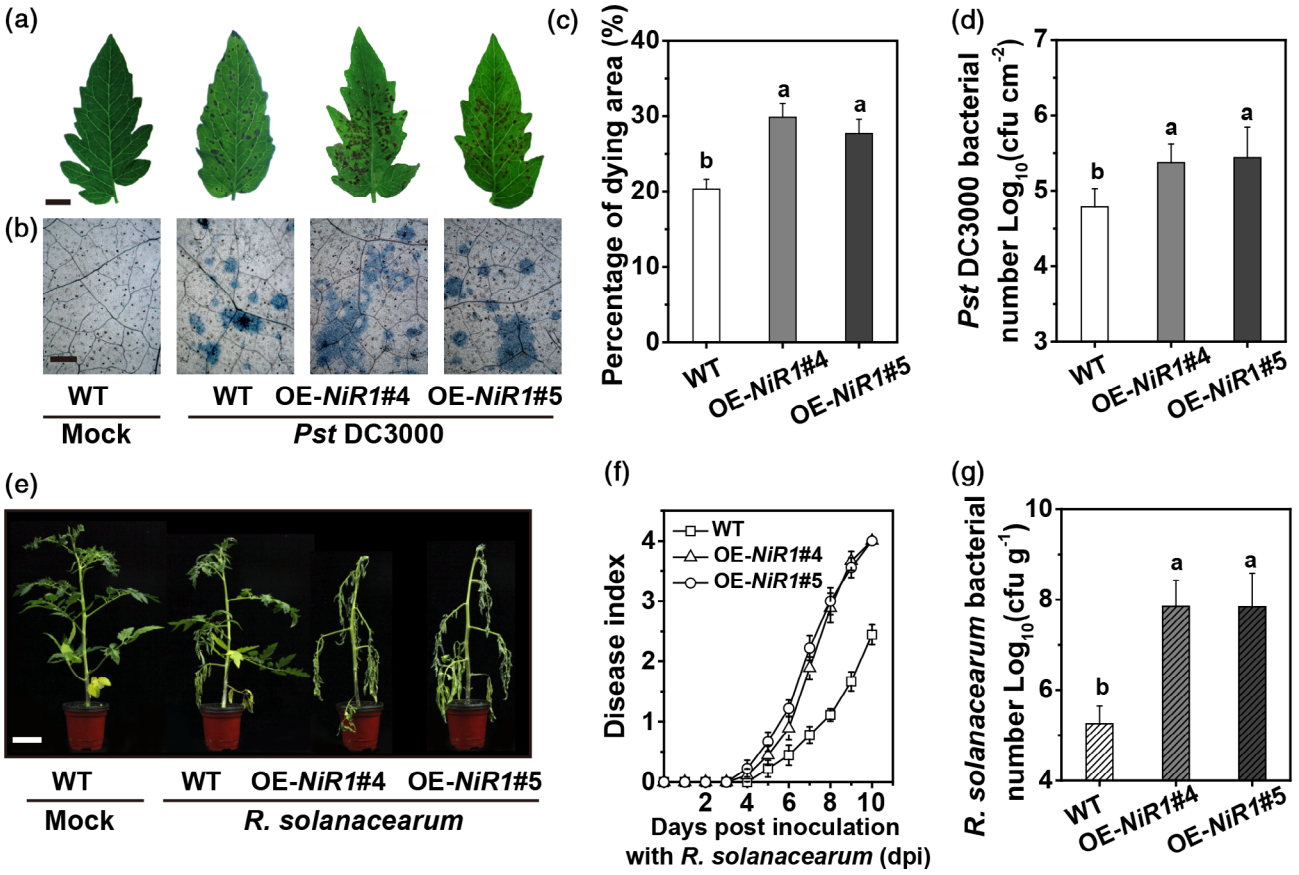

FIGURE 4 Effects of *NiR1* overexpression on the susceptibility of tomato plants to *Pst* DC3000 and *R. solanacearum* pathogens. (a) Disease symptoms photographed at 5 dpi with *Pst* DC3000. Bar = 1 cm. (b) Trypan blue staining for cell death in tomato leaves performed at 2 dpi with *Pst* DC3000. Bar = 500 μm. (c) Quantitative data of trypan blue staining of *Pst*-inoculated leaves at 2 dpi. (d) *Pst* DC3000 bacterial population at 2 dpi. (e) The phenotypes of plants at 10 dpi with *R. solanacearum*. Bar = 10 cm. (f) Mean disease index ± SE following *R. solanacearum* inoculation of *NiR1*-overexpressed plants; $n = 9$. (g) *R. solanacearum* bacterial population at 10 dpi. The results in (d) and (g) are presented as mean values ± SD, $n = 4$. Different letters indicate significant differences between treatments ($p < .05$). The above experiments were repeated three times with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 5g,h). When the tomato plants were inoculated with *R. solanacearum*, the JA levels and the transcript abundance of *AOC*, *PI-I* and *PI-II* increased in a similar trend in the roots (Figure 6).

To further determine the role of SA in N-modulated defence, tomato NahG plants that do not accumulate SA, as well as the wild-type control Moneymaker (MM), were treated with N regimes under both mock- and pathogen-inoculated conditions. The NahG plants exhibited increased susceptibility to both pathogens as compared with MM (Figure 7). Strikingly, N regimes did not affect the susceptibility of the NahG plants in response to *Pst* DC3000 (Figure 7a,b). Similarly, the N-mediated defence response was also compromised when inoculated with *R. solanacearum* (Figure 7c–e), which was distinct from that of MM plants (Figure 7). Furthermore, exogenous SA application on OE-*NiR1* plants compromised the susceptibility of OE-*NiR1* plants to *Pst* DC3000 and *R. solanacearum*, which was evident from the phenotype and bacterial population counts (Figure 8). SA application decreased the growth of *Pst* DC3000 and *R. solanacearum* to WT level (Figure 8b,e). These results indicate that the N-modulated plant defence against bacteria is mainly dependent on SA content.

4 | DISCUSSION

Nitrogen status in plants greatly influences plant growth, productivity and adaptation to biotic and abiotic stresses. Understanding the mechanisms of N-modulated plant response to pathogens is indispensable for efficient disease management. However, previous studies dealing with the assessment of plant disease under different N conditions showed that N-modulated defence widely varied (Fagard et al., 2014; Gupta et al., 2013; Hoffland et al., 1999), and only a few studies investigated the effect of mixed N nutrition on plant susceptibility to diseases. In this study, by applying low and high N concentrations with different NO_3^-/NH_4^+ ratios as well as silencing and overexpression of N metabolism genes, we revealed how N forms and metabolism affect plant immunity to the foliar and root bacterial pathogens and demonstrated that the N-modulated defence variation is associated with SA defence pathway.

Both N concentrations and its forms are critical for evaluating the effect of N regimes on plant defence. Few studies have investigated the N-modulated defence against above- and below-ground plant

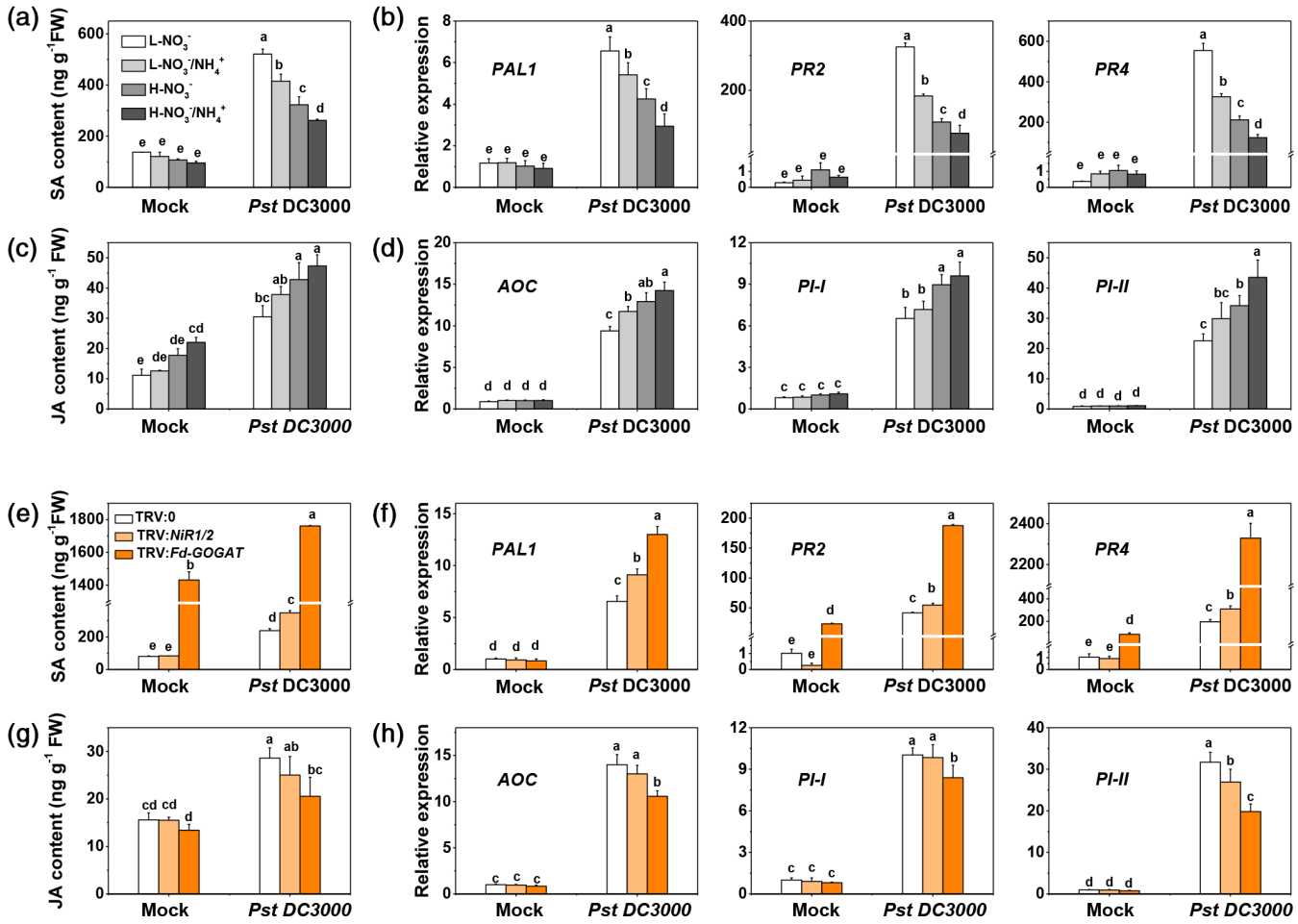

FIGURE 5 Endogenous salicylic acid (SA) and jasmonic acid (JA) concentrations and relative expression of genes involved in SA and JA biosynthesis and signalling pathways in tomato leaves under mock- and *Pst* DC3000-inoculated conditions. Plants were grown and treated as described in Section 2. (a) SA content in tomato leaves under four different nitrogen treatments. (b) *Phenylalanine ammonialyase 1* (*PAL1*) and *pathogenesis-related proteins* (*PRs*) expression in tomato leaves under four different nitrogen treatments. (c) JA content in tomato leaves under four different nitrogen treatments. (d) *Allene oxide cyclase* (*AOC*) and *proteinase inhibitors* (*PIs*) expression in tomato leaves under different nitrogen treatments. The expression of each gene under H-NO_3^- and mock-inoculated condition was defined as 1. (e) SA content in target gene-silenced plants. (f) *PAL1*, *PR2* and *PR4* expression in target gene-silenced plants. (g) JA content in target gene-silenced plants. (h) *AOC*, *PI-I* and *PI-II* expression in target gene-silenced plants. The expression of each gene under mock-inoculated condition of TRV:0 plants was defined as 1. Leaf samples were collected at 1 dpi for hormone contents detection, whereas leaves for gene expression assay were sampled at 0.5 dpi. The results are expressed as the mean values ± SD, $n = 4$. Different letters indicate significant differences ($p < .05$). The above experiments were repeated three times with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

pathogens in the same system, despite the crucial importance of above- and below-ground defence allocation (van Dam, 2009). Despite diverse pathobiology of *Pst* DC3000 and *R. solanacearum*, we found that low N conditions enhanced tomato resistance to both bacterial pathogens, and the resistance to bacteria was attenuated when the plants were fed with NO_3^-/NH_4^+ compared with the plants that were solely fed with NO_3^- under the same N concentrations (Figure 1), indicating that N application–caused changes in N metabolism have the similar effect on defence allocation between above- and below-ground parts. Thus, low N supply may provide a broad-spectrum resistance to bacterial pathogens that are capable of causing diseases in either above- or below-ground parts of plants. The high N-induced susceptibility in the current study is in accordance with previous studies that high N fertilizers generally increase the susceptibility of plants to biotrophs, whereas high N generally decreases the susceptibility of plants to necrotrophs (Fagard et al., 2014; Snoeijers, Perez-Garcia, Joosten, & De Wit, 2000).

With respect to the N forms, many plant species, including tomato, develop symptoms of toxicity when NH_4^+ is the only, or predominant N source, which ranges widely, and generally appears with external NH_4^+ concentrations above 0.1–0.5 mM (Britto & Kronzucker, 2002). But as NH_4^+ assimilation requires less energy than that of NO_3^-, many studies have investigated the effects of different ratio of NO_3^- and NH_4^+ under specific total N concentrations on tomato growth, development and responses to abiotic stresses and found that combinations of NO_3^- and NH_4^+ at an appropriate ratio

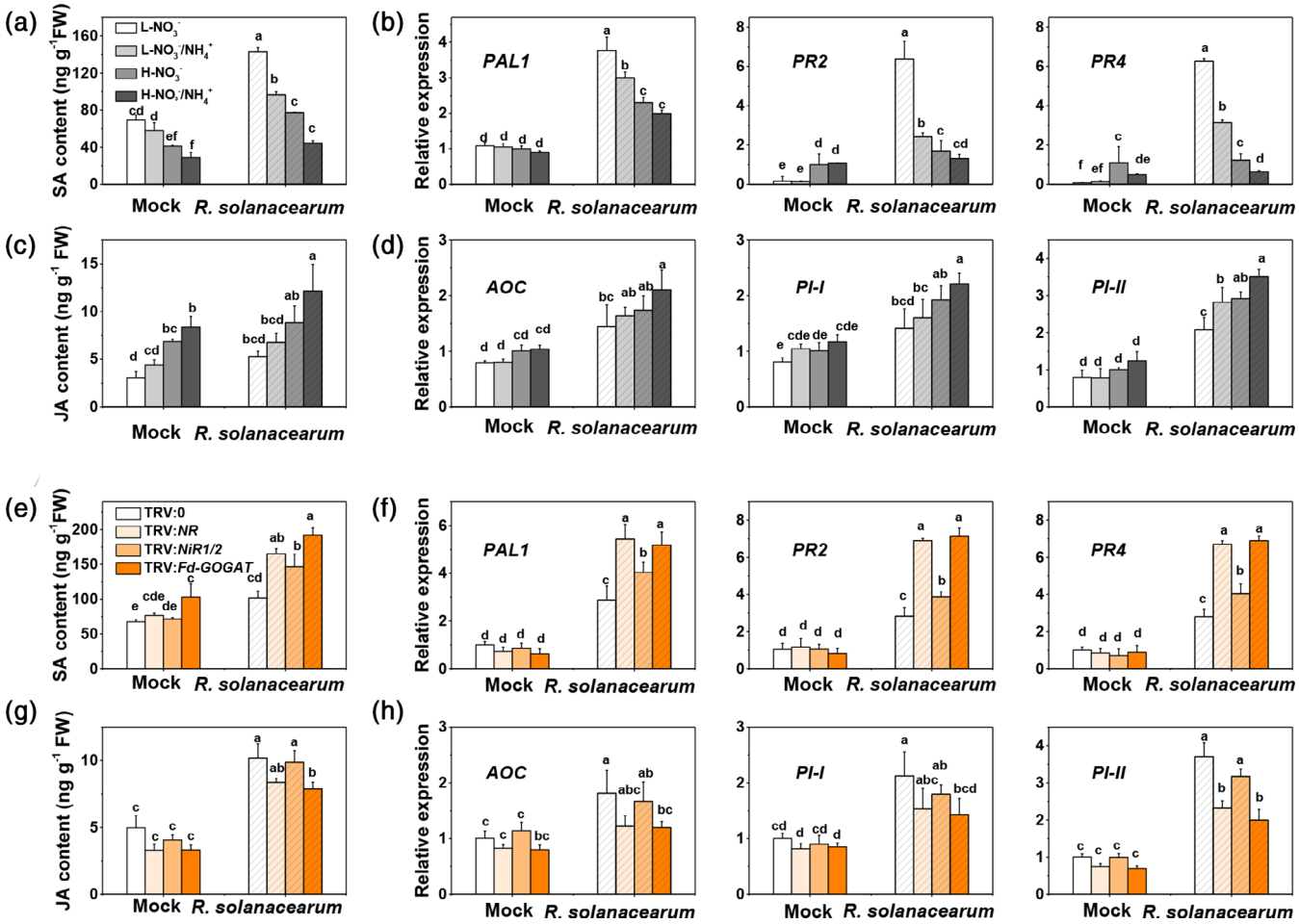

FIGURE 6 Endogenous salicylic acid (SA) and jasmonic acid (JA) concentrations and relative expression of genes involved in SA and JA biosynthesis and signalling pathways in tomato roots under mock- and *R. solanacearum*-inoculated conditions. Plants were grown and treated as described in Section 2. (a) SA content in tomato roots under four different nitrogen treatments. (b) *Phenylalanine ammonialyase 1* (*PAL1*) and *pathogenesis-related proteins* (*PRs*) expression in tomato roots under four different nitrogen treatments. (c) JA content in tomato roots under four different nitrogen treatments. (d) *Allene oxide cyclase* (*AOC*) and *proteinase inhibitors* (*PIs*) expression in tomato roots under four different nitrogen treatments. The expression of each gene under H-NO_3^- and mock-inoculated condition was defined as 1. (e) SA content in target gene-silenced plants. (f) *PAL1*, *PR2* and *PR4* expression in target gene-silenced plants. (g) JA content in target gene-silenced plants. (h) *AOC*, *PI-I* and *PI-II* expression in target gene-silenced plants. The expression of each gene under mock-inoculated condition of TRV:0 plants was defined as 1. Root samples were collected at 2 dpi for hormone levels detection, whereas roots for gene expression assay were sampled at 1 dpi. The results are expressed as the mean values ± SD, $n = 4$. Different letters indicate significant differences ($p < .05$). The above experiments were repeated three times with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

did not affect or even increased the plant growth and yield in tomato (Ben-Oliel et al., 2004; Borgognone et al., 2013; Claussen, 2002). Nonetheless, few studies investigated the effects of combined N forms on plant susceptibility. In the current study, the plants cultivated at a higher concentration of NO_3^-/NH_4^+ showed higher susceptibility against both pathogens than that of plants at the same concentrations of sole NO_3^- resources (Figure 1), which is similar to the observation in cucumber plants challenged with *F. oxysporum* (Wang et al., 2016). Similarly, knock-out mutant *amt1.1* (high-affinity ammonium transporter) shows significant metabolic changes and displays enhanced resistance against *Plectosphaerella cucumerina* and reduced susceptibility to *P. syringae* in *Arabidopsis* (Pastor et al., 2014).

Nitrogen metabolism is a complex biochemical process, which includes N uptake, assimilation and remobilization. NR, NiR and Fd-GOGAT are key enzymes in N metabolism, mainly participating in N assimilation. In the present study, the expression of N metabolism genes significantly decreased under pathogen-inoculated condition (Figure 2), revealing that plant primary metabolism is affected by the bacterial pathogen infection. It seems an interconnection between metabolic and stress signalling pathways is required for proper and efficient resource allocation. It is known that photosynthesis is decreased, and the related genes are also down-regulated upon pathogens challenge (Bilgin et al., 2010). Thus, it was speculated that silencing or overexpressing the important genes involved in N assimilation would disturb N metabolism in plants. In rice, the deletion of

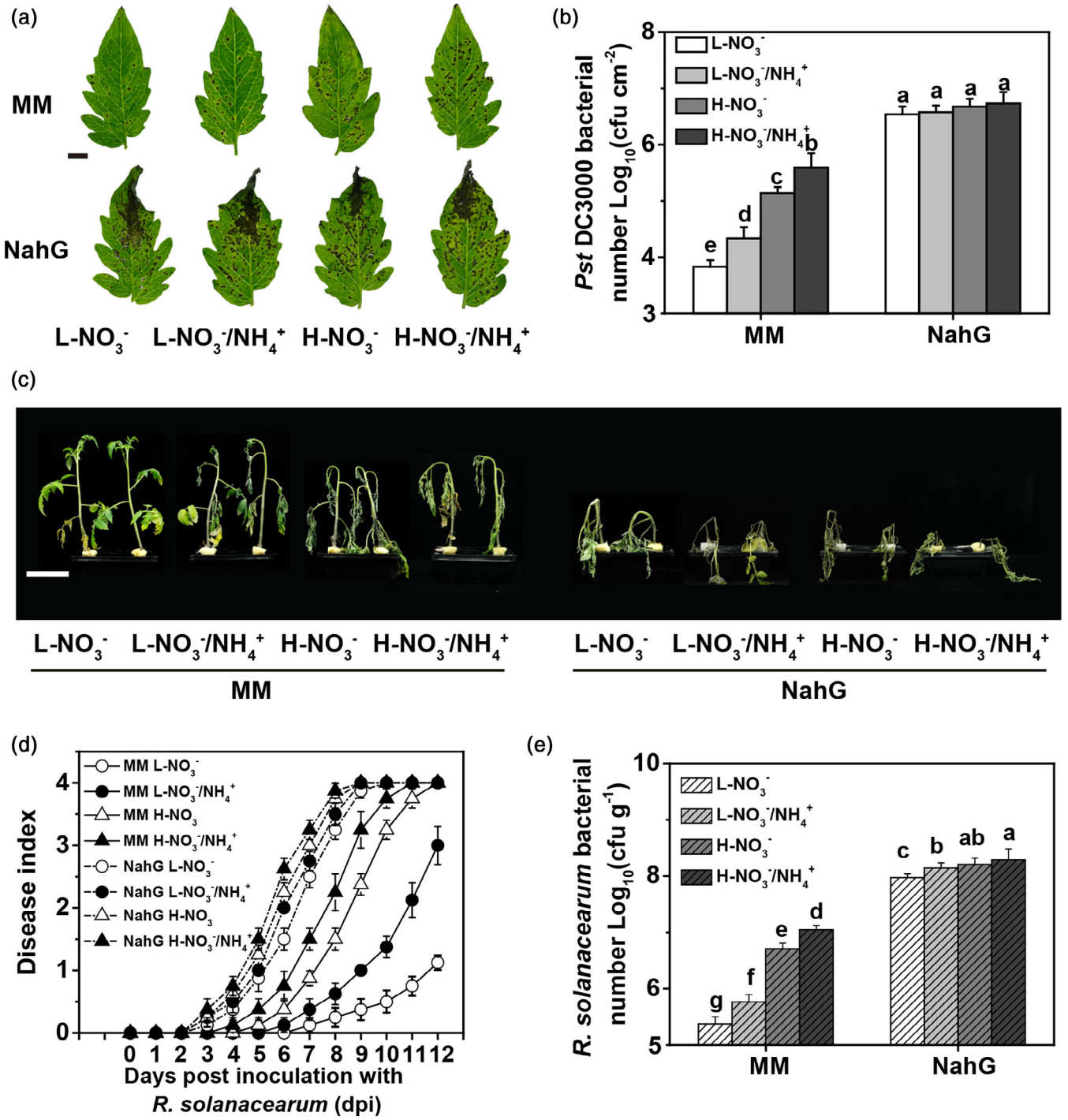

FIGURE 7 Effects of nitrogen (N) forms and concentrations on the susceptibility of tomato plants in SA accumulation defective transgenic NahG and its wild-type line cv. MM under *Pst* DC3000- and *R. solanacearum*-inoculated conditions. Plants were grown and treated as described in Section 2. (a) Disease symptoms photographed at 5 dpi with *Pst* DC3000. Bar = 1 cm. (b) *Pst* DC3000 bacterial population at 2 dpi. (c) The phenotypes of plants inoculated with *R. solanacearum* at 12 dpi. Bar = 10 cm. (d) Mean disease index ± SE following *R. solanacearum* inoculation; $n = 8$. (e) *R. solanacearum* bacterial population at 12 dpi. The results in (b) and (e) are presented as mean values ± SD, $n = 4$. Different letters depict significant differences between the treatments ($p < .05$). The above experiments were repeated three times with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

Fd-GOGAT reduces the nitrate, free amino acid, chlorophyll and sugar content and also increases plant resistance against *Xoo* (Chen et al., 2016). Moreover, amino acid metabolism changes due to a deletion of *LYSINE HISTIDINE TRANSPORTER1* (*LHT1*), leading to an increased plant immunity (Liu et al., 2010). The expression of *NR*, *NiR1/2* and *Fd-GOGAT* decreased in Rio Grande (RG)-PtoR resistant

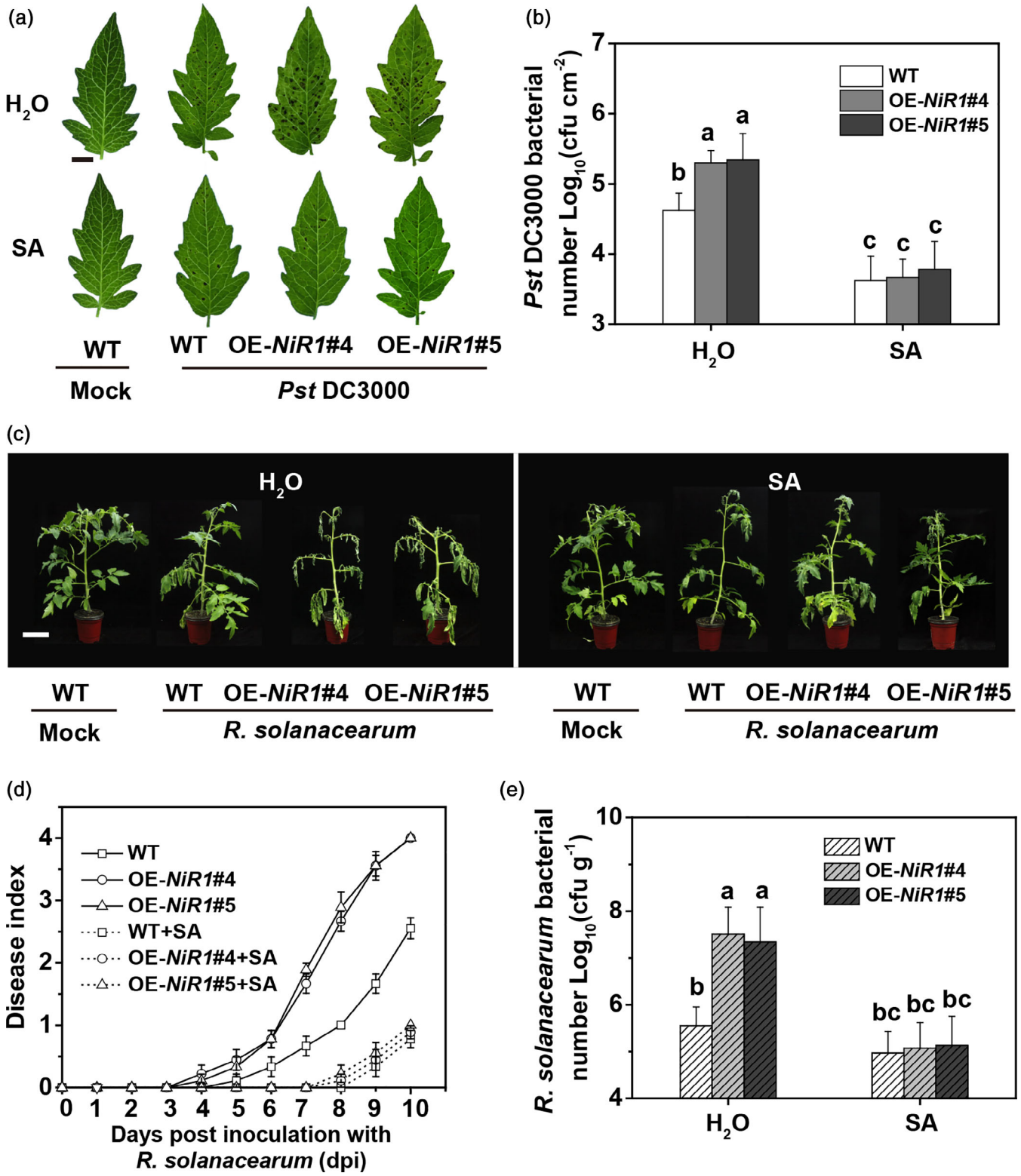

FIGURE 8 Effects of exogenous salicylic acid (SA) application on the susceptibility of tomato plants to *Pst* DC3000 and *R. solanacearum*. Tomato leaves were pre-treated with 2 mM SA at 2 hr prior to *Pst* DC3000 inoculation, and roots were pre-treated with 1 mM SA at 2 hr prior to *R. solanacearum* inoculation, respectively. (a) Disease symptoms photographed at 5 dpi with *Pst* DC3000. Bar = 1 cm. (b) *Pst* DC3000 bacterial population at 2 dpi. (c) The phenotypes of plants at 10 dpi with *R. solanacearum*. Bar = 10 cm. (d) Mean disease index ± SE following *R. solanacearum* inoculation; $n = 9$. (e) *R. solanacearum* bacterial population at 10 dpi. The results in (b) and (e) are presented as mean values ± SD, $n = 4$. Different letters indicate significant differences between treatments ($p < .05$). The above experiments were repeated three times with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

plants compared with that in susceptible plants after *Pst* DC3000 inoculation (Pombo et al., 2014). In the present study, silencing the N metabolism genes enhanced resistance (Figure 3), while *NiR1* overexpression increased plant susceptibility to both pathogens (Figure 4). Therefore, changes in N metabolism could influence the defence, and lower N or down-regulation of the N metabolism genes improves plant immunity, which seems to be the consequence of the trade-off between plant growth and defence responses (Fagard et al., 2014).

The remaining outstanding question to be addressed is what is the underlying mechanism for N-modulated plant defence. At the metabolic level, plant growth results from the primary metabolism that supplies resources to new tissues, and the defence relies on the secondary metabolism via defence-related molecules (Croteau, Kutchan, & Lewis, 2000). In a previous study on tomato plants with varying concentrations of N and CO_2, C:N ratio is suggested to be an indicator of the C-based compound allocation between primary and defence-related secondary metabolism (Royer et al., 2013). This implies that the N-modulated defence variations could be associated with the alteration in defence-related molecules. In particular, SA is an important C-based secondary metabolite involved in plant immunity, especially in plant response to (hemi)biotrophic pathogens (Vlot et al., 2009). A previous study showed that mutations in the *NRT2.1* gene in *Arabidopsis* resulted in an increase in SA content and priming of SA signalling (Camañes et al., 2012). However, SA generation and its signalling are not correlated with NH_4^+-mediated defence in tomato (Fernández-Crespo et al., 2015). In this study, based on the analysis of gene silencing/overexpression lines and the SA-deficient NahG tomato plants, we proposed that SA defence pathway participates in N-modulated defence, leading to immunity against bacterial pathogens in tomato. This conclusion is based on several lines of evidence: (1) low N conditions primed the constitutive levels of SA in roots, although the increase in leaves was not significantly different from that in high N supply in the absence of bacteria (Mock) (Figures 5a and 6a). (2) After inoculation with bacteria, low N, as well as the use of NO_3^- as the sole N source, showed lower levels of susceptibility, which was accompanied by higher levels of SA content and transcripts of SA defence pathway genes (Figures 5 and 6). (3) Low N-induced defence was comprised in NahG-transgenic plants (Figure 7). (4) In N metabolism gene–silenced plants, SA content and transcripts of SA response genes were significantly higher than pTRV:0 after infection with bacteria (Figures 5 and 6). (5) Exogenous SA application enhanced resistance in OE-*NiR1* plants, suggesting that SA functions downstream of N metabolism to mediate N-modulated defence in response to bacterial pathogens (Figure 8). SA and JA signals antagonize each other in plants (Glazebrook, 2005), and the JA signalling pathway is activated against necrotrophic pathogens (Tsuda & Katagiri, 2010). In this study, N regime treatments with higher SA content generally had a lower JA level under both mock- and pathogen-inoculated conditions (Figures 5 and 6). Tomato plants showed higher susceptibility against necrotrophic fungus *B. cinerea* in nitrate-limiting conditions (Hoffland et al., 1999; Lecompte et al., 2010; Vega et al., 2015), may be due to the repressed JA signalling by higher SA compounds under low N. In agreement with our study, N limitation induced the accumulation of SA without pathogen inoculation in *Arabidopsis* (Yaeno & Iba, 2008), and knockout of an amino acid transporter, LHT1, confers broad-spectrum disease resistance in *Arabidopsis* in an SA-dependent manner (Liu et al., 2010). However, it is still not clear how N regulates SA accumulation at the molecular level. NR can also produce nitric oxide (NO) through a reductive pathway in an NADPH-dependent process (Modolo, Augusto, Almeida, Magalhaes, & Salgado, 2005). It has been suggested that NO initiates the biosynthesis of SA or participates in localized plant defence and SAR (Espunya, De Michele, Gomez-Cadenas, & Martinez, 2012), whereas different N forms-affected defence is linked to NO production in NO_3^--fed plants as compared with NH_4^+ plants (Gupta et al., 2013). Besides, NO_3^- could also trigger Ca^{2+} signalling and subgroup III Ca^{2+}-sensor protein kinases (CPKs) in *Arabidopsis*, which might directly or indirectly modulate SA biosynthesis and thus orchestrating primary nitrate responses as master regulators (Liu et al., 2017). Further detailed studies on the molecular event may shed light on the in-depth mechanism of the relationship between the N-modulated defence and the SA defence pathway.

In conclusion, our data indicate that low N supply reduced plant susceptibility to both foliar and root bacterial pathogens in tomato, and NO_3^- as a sole N source had a better effect than the combined supply of NO_3^- and NH_4^+ under the same N concentrations. Disease susceptibility was reduced by down-regulation of N metabolism genes while aggravated by overexpression. The N-modulated defence is mediated by SA defence pathway, and SA functions downstream of N metabolism in plant defence response to bacteria. These findings provide important insights into the mechanism behind N-modulated tomato–bacteria interaction and can be useful for designing effective agronomic strategies of N fertilization. In particular, sole NO_3^- can be preferred for field application rather than a combination of NO_3^- and NH_4^+ (Ben-Oliel et al., 2004; Borgognone et al., 2013; Claussen, 2002). Moreover, low N impacts tomato growth for a prolonged period, and thus an appropriate N dose may not only enhance plant growth but also improve resistance to bacterial pathogens. However, further studies are required to ascertain the large-scale implication of this proposition.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31822046, 31772355), the Natural Science Foundation of Zhejiang Province for Distinguished Young Scholar (LR19C150001), the Key Research and Development Program of Zhejiang Province (2021C02040) and the Cooperative Extension Plan of Major Agricultural Technologies of Zhejiang Province (2019XTTGSC04).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Kai Shi conceived the research; Shuting Ding and Kai Shi designed the experiments; Shuting Ding, Xiangqi Shao, Jianxin Li, Yanlai Yao, Jian Ding and Zhangjian Hu performed the research and analysed the data;

Jingquan Yu provided technical/intellectual support for the research; Shuting Ding, Golam Jalal Ahammed and Kai Shi wrote the article with contributions from other authors.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Golam Jalal Ahammed https://orcid.org/0000-0001-9621-8431
Jingquan Yu https://orcid.org/0000-0002-7626-1165
Kai Shi https://orcid.org/0000-0001-5351-1910

REFERENCES

Bai, S., Liu, J., Chang, C., Zhang, L., Maekawa, T., Wang, Q., … Shen, Q. H. (2012). Structure-function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance. *PLoS Pathogens*, *8*, e1002752.

Bai, Y., & Lindhout, P. (2007). Domestication and breeding of tomatoes: What have we gained and what can we gain in the future? *Annals of Botany*, *100*, 1085–1094.

Ben-Oliel, G., Kant, S., Naim, M., Rabinowitch, H. D., Takeoka, G. R., Buttery, R. G., & Kafkafi, U. (2004). Effects of ammonium to nitrate ratio and salinity on yield and fruit quality of large and small tomato fruit hybrids. *Journal of Plant Nutrition*, *27*, 1795–1812.

Bilgin, D. D., Zavala, J. A., Zhu, J., Clough, S. J., Ort, D. R., & DeLucia, E. H. (2010). Biotic stress globally downregulates photosynthesis genes. *Plant, Cell & Environment*, *33*, 1597–1613.

Bloom, A. J. (2015). The increasing importance of distinguishing among plant nitrogen sources. *Current Opinion in Plant Biology*, *25*, 10–16.

Borgognone, D., Colla, G., Rouphael, Y., Cardarelli, M., Rea, E., & Schwarz, D. (2013). Effect of nitrogen form and nutrient solution pH on growth and mineral composition of self-grafted and grafted tomatoes. *Scientia Horticulturae*, *149*, 61–69.

Britto, D. T., & Kronzucker, H. J. (2002). NH_4^+ toxicity in higher plants: A critical review. *Journal of Plant Physiology*, *159*, 567–584.

Camañes, G., Pastor, V., Cerezo, M., García-Andrade, J., Vicedo, B., García-Agustín, P., & Flors, V. (2012). A deletion in *NRT2.1* attenuates *Pseudomonas syringae*-induced hormonal perturbation, resulting in primed plant defenses. *Plant Physiology*, *158*, 1054–1066.

Cataldo, D. A., Haroon, M., Schrader, L. E., & Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Communications in Soil Science and Plant Analysis*, *6*, 71–80.

Chen, H. L., Li, C. R., Liu, L. P., Zhao, J. Y., Cheng, X. Z., Jiang, G. H., & Zhai, W. X. (2016). The Fd-GOGAT1 mutant gene *lc7* confers resistance to *Xanthomonas oryzae* pv. *Oryzae* in rice. *Scientific Reports*, *6*, 26411.

Chi, W. J., Wang, Z. Y., Liu, J. M., Zhang, C., Wu, Y. H., & Bai, Y. J. (2019). Ammonium uptake and assimilation are required for rice defense against sheath blight disease. *Cereal Research Communications*, *47*, 98–110.

Claussen, W. (2002). Growth, water use efficiency, and proline content of hydroponically grown tomato plants as affected by nitrogen source and nutrient concentration. *Plant and Soil*, *247*, 199–209.

Croteau, R., Kutchan, T. M., & Lewis, N. G. (2000). Natural products (secondary metabolites). In B. Buchanan, W. Gruissem, & R. Jones (Eds.), *Biochemistry and molecular biology of plants* (pp. 1250–1318). Rockville, MD: American Society of Plant Nutrition.

Debouba, M., Maâroufi-Dghimi, H., Suzuki, A., Ghorbel, M. H., & Gouia, H. (2007). Changes in growth and activity of enzymes involved in nitrate reduction and ammonium assimilation in tomato seedlings in response to NaCl stress. *Annals of Botany*, *99*, 1143–1151.

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., & Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *The Arabidopsis Book*, *9*, e0156.

Denancé, N., Ranocha, P., Oria, N., Barlet, X., Rivière, M. P., Yadeta, K. A., … Marco, Y. (2013). Arabidopsis *wat1* (*walls are thin1*)-mediated resistance to the bacterial vascular pathogen, *Ralstonia solanacearum*, is accompanied by cross-regulation of salicylic acid and tryptophan metabolism. *The Plant Journal*, *73*, 225–239.

Espunya, M. C., De Michele, R., Gomez-Cadenas, A., & Martinez, M. C. (2012). *S*-Nitrosoglutathione is a component of wound- and salicylic acid-induced systemic responses in *Arabidopsis thaliana*. *Journal of Experimental Botany*, *63*, 3219–3227.

Fagard, M., Launay, A., Clément, G., Courtial, J., Dellagi, A., Farjad, M., … Masclaux-Daubresse, C. (2014). Nitrogen metabolism meets phytopathology. *Journal of Experimental Botany*, *65*, 5643–5656.

Fernández-Crespo, E., Scalschi, L., Llorens, E., García-Agustín, P., & Camañes, G. (2015). NH_4^+ protects tomato plants against *Pseudomonas syringae* by activation of systemic acquired acclimation. *Journal of Experimental Botany*, *66*, 6777–6790.

French, E., Kim, B. S., Rivera-Zuluaga, K., & Iyer-Pascuzzi, A. S. (2018). Whole root transcriptomic analysis suggests a role for auxin pathways in resistance to *Ralstonia solanacearum* in tomato. *Molecular Plant-Microbe Interactions*, *31*, 432–444.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*, *43*, 205–227.

González-Hernández, A. I., Fernández-Crespo, E., Scalschi, L., Hajirezaei, M. R., von Wirén, N., García-Agustín, P., & Camañes, G. (2019). Ammonium mediated changes in carbon and nitrogen metabolisms induce resistance against *Pseudomonas syringae* in tomato plants. *Journal of Plant Physiology*, *239*, 28–37.

Gupta, K. J., Brotman, Y., Segu, S., Zeier, T., Zeier, J., Persijn, S. T., … Mur, L. A. J. (2013). The form of nitrogen nutrition affects resistance against *Pseudomonas syringae* pv. *phaseolicola* in tobacco. *Journal of Experimental Botany*, *64*, 553–568.

Hoffland, E., van Beusichem, M. L., & Jeger, M. J. (1999). Nitrogen availability and susceptibility of tomato leaves to *Botrytis cinerea*. *Plant and Soil*, *210*, 263–272.

Katagiri, F., Thilmony, R., & He, S. Y. (2002). The *Arabidopsis thaliana-Pseudomonas springae* interaction. In *The Arabidopsis Book* (eds C. R. Somerville & E. M. Meyerowitz), pp. 1–35.American Society of Plant Biologists, Rockville, MD.

Kiirika, L. M., Stahl, F., & Wydra, K. (2013). Phenotypic and molecular characterization of resistance induction by single and combined application of chitosan and silicon in tomato against *Ralstonia solanacearum*. *Physiological and Molecular Plant Pathology*, *81*, 1–12.

Klessig, D. F., Choi, H. W., & Dempsey, D. A. (2018). Systemic acquired resistance and salicylic acid: Past, present, and future. *Molecular Plant-Microbe Interactions*, *31*, 871–888.

Lecompte, F., Abro, M. A., & Nicot, P. C. (2010). Contrasted responses of *Botrytis cinerea* isolates developing on tomato plants grown under different nitrogen nutrition regimes. *Plant Pathology*, *59*, 891–899.

Lecompte, F., Abro, M. A., & Nicot, P. C. (2013). Can plant sugars mediate the effect of nitrogen fertilization on lettuce susceptibility to two necrotrophic pathogens: *Botrytis cinerea* and *Sclerotinia sclerotiorum*? *Plant and Soil*, *369*, 387–401.

Li, S. X., Wang, Z. H., Hu, T. T., Gao, Y. J., & Stewart, B. A. (2009). Nitrogen in dryland soils of China and its management. *Advances in Agronomy*, *101*, 123–181.

Liu, G., Ji, Y., Bhuiyan, N. H., Pilot, G., Selvaraj, G., Zou, J., & Wei, Y. D. (2010). Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in *Arabidopsis*. *The Plant Cell*, *22*, 3845–3863.

Liu, K. H., Niu, Y., Konishi, M., Wu, Y., Du, H., … Sheen, J. (2017). Discovery of nitrate-CPK-NLP signaling in central nutrient-growth networks. *Nature*, *545*, 311–316.

Liu, L., Sonbol, F., Huot, B., Gu, Y., Withers, J., Mwimba, M., … Dong, X. (2016). Salicylic acid receptors activate jasmonic acid signalling through a non-canonical pathway to promote effector-triggered immunity. *Nature Communications*, *7*, 13099.

Liu, Y., Schiff, M., & Dinesh-Kumar, S. P. (2002). Virus-induced gene silencing in tomato. *The Plant Journal*, *31*, 777–786.

Modolo, L. V., Augusto, O., Almeida, I. M. G., Magalhaes, J. R., & Salgado, I. (2005). Nitrite as the major source of nitric oxide production by *Arabidopsis thaliana* in response to *Pseudomonas syringae*. *FEBS Letters*, *579*, 3814–3820.

Molins-Legua, C., Meseguer-Lloret, S., Moliner-Martinez, Y., & Campíns-Falcó, P. (2006). A guide for selecting the most appropriate method for ammonium determination in water analysis. *Trends in Analytical Chemistry*, *25*, 282–290.

Oliveira, H. C., Justino, G. C., Sodek, L., & Salgado, I. (2009). Amino acid recovery does not prevent susceptibility to *Pseudomonas syringae* in nitrate reductase double-deficient *Arabidopsis thaliana* plants. *Plant Science*, *176*, 105–111.

Pastor, V., Gamir, J., Camañes, G., Cerezo, M., Sánchez-Bel, P., & Flors, V. (2014). Disruption of the ammonium transporter *AMT1.1* alters basal defenses generating resistance against *Pseudomonas syringae* and *Plectosphaerella cucumerina*. *Frontiers in Plant Science*, *5*, 231.

Pombo, M. A., Zheng, Y., Pozo, N. F., Dunham, D. M., Fei, Z. J., & Martin, G. B. (2014). Transcriptomic analysis reveals tomato genes whose expression is induced specifically during effector-triggered immunity and identifies the Epk1 protein kinase which is required for the host response to three bacterial effector proteins. *Genome Biology*, *15*, 492–508.

Royer, M., Larbat, R., Le Bot, J., Adamowicz, S., & Robin, C. (2013). Is the C:N ratio a reliable indicator of C allocation to primary and defense-related metabolisms in tomato? *Phytochemistry*, *88*, 25–33.

Snoeijers, S. S., Perez-Garcia, A., Joosten, M. H. A. J., & De Wit, P. J. G. M. (2000). The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. *European Journal of Plant Pathology*, *106*, 493–506.

Thalineau, E., Fournier, C., Gravot, A., Wendehenne, D., Jeandroz, S., & Truong, H. N. (2018). Nitrogen modulation of *Medicago truncatula* resistance to *Aphanomyces euteiches* depends on plant genotype. *Molecular Plant Pathology*, *19*, 664–676.

Tsuda, K., & Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Current Opinion in Plant Biology*, *13*, 459–465.

Uknes, S., Dincher, S., Friedrich, L., Negrotto, D., Williams, S., Thompsontaylor, H., … Ryals, J. (1993). Regulation of pathogenesis-related protein-1a gene expression in tobacco. *The Plant Cell*, *5*, 159–169.

Uppalapati, S. R., Ishiga, Y., Wangdi, T., Urbanczyk-Wochniak, E., Ishiga, T., Mysore, K. S., & Bender, C. L. (2008). Pathogenicity of *Pseudomonas syringae* pv. *tomato* on tomato seedlings: Phenotypic and gene expression analyses of the virulence function of coronatine. *Molecular Plant-Microbe Interactions*, *21*, 383–395.

van Dam, N. M. (2009). Belowground herbivory and plant defenses. *Annual Review of Ecology, Evolution, and Systematics*, *40*, 373–391.

Vega, A., Canessa, P., Hoppe, G., Retamal, I., Moyano, T. C., Canales, J., … Rubilar, J. (2015). Transcriptome analysis reveals regulatory networks underlying differential susceptibility to *Botrytis cinerea* in response to nitrogen availability in *Solanum lycopersicum*. *Frontiers in Plant Science*, *6*, 911.

Vega, A., O'Brien, J. A., & Gutiérrez, R. A. (2019). Nitrate and hormonal signaling crosstalk for plant growth and development. *Current Opinion in Plant Biology*, *52*, 155–163.

Vlot, A. C., Dempsey, D. A., & Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology*, *47*, 177–206.

Wang, M., Sun, Y., Gu, Z., Wang, R., Sun, G., Zhu, C., … Shen, Q. (2016). Nitrate protects cucumber plants against *Fusarium oxysporum* by regulating citrate exudation. *Plant and Cell Physiolology*, *57*, 2001–2012.

Wang, X. Z., Zou, C. Q., Gao, X. P., Guan, X. X., Zhang, Y. Q., Shi, X. J., & Chen, X. P. (2018). Nitrate leaching from open-field and greenhouse vegetable systems in China: A meta-analysis. *Environmental Science and Pollution Research*, *25*, 31007–31016.

Wang, Y. Y., Cheng, Y. H., Chen, K. E., & Tsay, Y. F. (2018). Nitrate transport, signaling, and use efficiency. *Annual Review of Plant Biology*, *69*, 85–122.

Wolfe, J., Hutcheon, C. J., Higgins, V. J., & Cameron, R. K. (2000). A functional gene-for-gene interaction is required for the production of an oxidative burst in response to infection with avirulent *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana*. *Physiological and Molecular Plant Pathology*, *56*, 253–261.

Xu, G. H., Fan, X. R., & Miller, A. J. (2012). Plant nitrogen assimilation and use efficiency. *Annual Review of Plant Biology*, *63*, 153–182.

Yaeno, T., & Iba, K. (2008). BAH1/NLA, a RING-type ubiquitin E3 ligase, regulates the accumulation of salicylic acid and immune responses to *Pseudomonas syringae* DC3000. *Plant Physiology*, *148*, 1032–1041.

Yu, J. Q., & Matsui, Y. (1997). Effects of root exudates of cucumber (*Cucumis sativus*) and allelochemicals on ion uptake by cucumber seedlings. *Journal of Chemical Ecology*, *23*, 817–827.

Zhang, H., Hu, Z. J., Lei, C., Zheng, C. F., Wang, J., Shao, S. J., … Shi, K. (2018). A plant phytosulfokine peptide initiates auxin-dependent immunity through cytosolic Ca^{2+} signaling in tomato. *The Plant Cell*, *30*, 652–667.

Zhang, X., Davidson, E. A., Mauzerall, D. L., Searchinger, T. D., Dumas, P., & Shen, Y. (2015). Managing nitrogen for sustainable development. *Nature*, *528*, 51–59.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Ding S, Shao X, Li J, et al. Nitrogen forms and metabolism affect plant defence to foliar and root pathogens in tomato. *Plant Cell Environ*. 2021;44:1596–1610. https://doi.org/10.1111/pce.14019