Transcriptome analysis reveals regulatory networks underlying differential susceptibility to *Botrytis cinerea* in response to nitrogen availability in *Solanum lycopersicum*

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Nitrogen (N) is one of the main limiting nutrients for plant growth and crop yield. It is well documented that changes in nitrate availability, the main N source found in agricultural soils, influences a myriad of developmental programs and processes including the plant defense response. Indeed, many agronomical reports indicate that the plant N nutritional status influences their ability to respond effectively when challenged by different pathogens. However, the molecular mechanisms involved in N-modulation of plant susceptibility to pathogens are poorly characterized. In this work, we show that *Solanum lycopersicum* defense response to the necrotrophic fungus *Botrytis cinerea* is affected by plant N availability, with higher susceptibility in nitrate-limiting conditions. Global gene expression responses of tomato against *B. cinerea* under contrasting nitrate conditions reveals that plant primary metabolism is affected by the fungal infection regardless of N regimes. This result suggests that differential susceptibility to pathogen attack under contrasting N conditions is not only explained by a metabolic alteration. We used a systems biology approach to identify the transcriptional regulatory network implicated in plant response to the fungus infection under contrasting nitrate conditions. Interestingly, hub genes in this network are known key transcription factors involved in ethylene and jasmonic acid signaling. This result positions these hormones as key integrators of nitrate and defense against *B. cinerea* in tomato plants. Our results provide insights into potential crosstalk mechanisms between necrotrophic defense response and N status in plants.

Keywords: nitrate nutrition, tomato, defense mechanisms, jasmonic acid, gene network analysis, ethylene signaling, microarray analysis
INTRODUCTION

Nitrogen (N) is an essential macronutrient whose availability significantly impacts plant growth and development. Since natural environments and agricultural fields have limited amounts of N, the production of high-yielding crops relies on the application of large quantities of nitrogenous fertilizers, which come at considerable economic (Good et al., 2004) and environmental costs (Hirel et al., 2011).

The relevance of N for plants is clearly exemplified by its effects on leaf growth (von Wirén et al., 2000), senescence (Vanacker et al., 2006), root system architecture (Zhang et al., 2007; Vidal et al., 2010) and flowering time (Castro Marín et al., 2010). Besides growth and developmental effects, it is also clear that N nutrition can impact the plant's ability to cope with environmental challenges such as plant pathogen attacks (Snoeijers et al., 2000; Walters and Bingham, 2007; Dordas, 2008; Fagard et al., 2014). Different studies have shown that N availability impacts the outcome of plant-pathogen interactions, although the mechanisms underlying this connection are poorly understood, and the effect of N on this process is highly dependent on the crop being studied and on the particular life style of the pathogen involved (Snoeijers et al., 2000; Walters and Bingham, 2007; Dordas, 2008; Fagard et al., 2014). Consequently, it is difficult to derive general rules for the role of N in this process.

The plant defense response is a complex biological process involving numerous changes at the biochemical, physiological, and molecular (transcriptional) level, all governed by an intricate grid of hierarchical and regulatory interactions (Windram et al., 2014). These defense mechanisms are triggered partly by the defense hormones salicylic acid (SA) and jasmonic acid (JA) (Thomma et al., 2001; Glazebrook, 2005; Pieterse et al., 2009; Caarls et al., 2015). In general terms, SA is involved in resistance against biotrophic pathogens (Vlott et al., 2009), while JA participates in the regulation of defense response against necrotrophic pathogens and insects (Farmer et al., 2003).

Since plant defense is an active and energetically costly response mechanism, it is expected that the metabolic state of the plant plays a fundamental role in the outcome of the plant-pathogen interaction. A few agronomic reports indicate that high N availability increases the incidence of crop diseases (Hoffland et al., 2000; Olesen et al., 2003; Ballini et al., 2013). On the other hand, some studies reported that a reduction in N fertilization increases disease severity (Hoffland et al., 1999; Long et al., 2007; Lingquist et al., 2008). In the model plant Arabidopsis thaliana, proteins involved in plant resistance to infections are up-regulated in response to changes in N levels (Lau and Hamer, 1996; Dietrich et al., 2004). In the case of Botrytis cinerea, one of the most important fungal plant pathogens with regard to both its scientific and agronomic importance (Dean et al., 2012), plant N status can either promote or impede infection, depending on the plant species. For instance, while high N fertilization rates increase disease severity in legumes (Davidson et al., 2007) and lettuce (Lecompte et al., 2013), elevated N concentrations result in reduced susceptibility to this fungus in tomato (Hoffland et al., 1999; Lecompte et al., 2010). As there appears to be a trade-off between plant growth and defense responses (Walters and Heil, 2007), an intricate interconnection between metabolic and stress signaling pathways is required for proper and efficient resource allocation (Hey et al., 2010). The relationship between N metabolism and plant defense responses however, has not been analyzed in detail, although it has recently been recognized that this interconnection may shed new light on the complexity of plant defense strategies (Fagard et al., 2014).

In this study, we analyzed changes in global gene expression patterns during B. cinerea infections in tomato plants grown under contrasting nitrate regimes, with the goal of characterizing the interaction between N supply and defense responses at molecular level. High nitrate availability reduced plant susceptibility to this fungus when measured in leaves and tomato fruits. Global gene expression patterns confirmed that the tomato plant defense response is affected by nitrate availability. To get a broader overview of the interconnection between N metabolism and the plant defense response, a gene network analysis was performed. This strategy identified a transcriptional regulatory network controlling plant susceptibility to this fungus depending on nitrate condition. After validating a network-derived prediction by RT-qPCR in leaves and fruits infected by the fungus, we conclude that the expression of key transcription factors (TFs) involved in ethylene (ET) and JA signaling is modulated by the plant N status when tomato is infected by B. cinerea, suggesting that these hormones play a role in the nitrate-defense response interaction in tomato. Our study identified crosstalk points between N-nutrition, defense response and the ET/JA pathways in plants.

RESULTS

Plant Nitrate Regimes Alter the Progression of B. cinerea Infection in Tomato

As a first step to evaluate a connection between plant nitrate availability and plant response to pathogen infection, we evaluated the growth of S. lycopersicum cv. MicroTom under N (nitrate) conditions that ranged from limiting to sufficient. Tomato plants were grown in pots with vermiculite, an inert growing substrate without N sources, and irrigated with a complete mineral nutrient solution without N supplemented with 2, 4, 6, or 12 mM nitrate (final concentration). As expected, nitrate concentration had a significant impact on tomato plant growth. Maximal growth was attained with 6 mM (N-sufficient condition) under our experimental conditions, and 2 mM and 4 mM nitrate produced severe and mild growth phenotypes, respectively (N-limiting conditions). To evaluate higher N input, tomato plants were grown using 12 mM nitrate. The amount of shoot biomass was reduced upon lowering the amount of nitrate to that normally present in the N-sufficient condition (6 mM), while higher concentrations did not lead to significant changes (Figure S1).

To evaluate whether contrasting nitrate concentrations impact the susceptibility of tomato plants to fungal infection, plants were grown under 2, 4, 6, or 12 mM nitrate as the only N...
source and challenged with *B. cinerea*. Leaves in planta were inoculated with an aqueous suspension of $5 \times 10^3$ conidia. Typical *B. cinerea* symptoms, such as necrotic lesions, were observed in leaves under all N regimes. The first visual symptoms of the infections were detected 2 days post inoculation (dpi), at which time darkening of the leaf surface under the inoculum was observed (data not shown). As shown in Figure 1, disease symptoms developed faster in plants grown under N-limiting than in N-sufficient conditions. At 3 dpi, larger lesions were observed under N-limiting conditions, with evident tissue maceration surrounding primary infection sites in leaves (Figure 1A). Conversely, only discrete necrotic lesions were observed under N-sufficient conditions (Figure 1A). Even though, disease symptoms (expanding necrosis, chlorosis, and tissue maceration) were observed in leaves from plants grown under all N regimes at 5 dpi (data not shown), the size of the lesions and the percentage of the leaf exhibiting symptoms were always larger in leaves from plants grown under N-limiting conditions (Figures 1B,C).

To quantitatively assess fungal growth on plant tissue, we employed a quantitative PCR (qPCR) assay based on the relative quantification of fungal and plant DNA in infected plants, as described (Gachon and Saindrenan, 2004). As shown in Figure S2A, and consistent with the results shown in Figure 1, an increase in fungal growth was observed in all N-conditions as disease progressed, with larger quantities in infected plants grown under N-limiting conditions.

Previous studies have shown that nitrate responses are organ and developmental stage-dependent (Wang et al., 2003, 2004; Vidal et al., 2014). To evaluate whether there was a difference in the aforementioned results when fruits instead of vegetative plant tissue was used, and considering that ripening promotes fruit susceptibility to pathogens (Alba et al., 2005; Giovannoni, 2007; Cantu et al., 2009), we evaluated the response of unripe green and ripe red tomato fruits to *B. cinerea* infection. Figure 2 shows disease symptoms and lesion progression in two developmental stages known as “mature green” (MG) and “red ripe” (RR) fruits obtained from plants grown under the same N conditions described above. Under all nitrate conditions evaluated, MG tomatoes were significantly less susceptible to *B. cinerea* compared to RR fruits. When MG tomato fruits were inoculated, a small necrotic lesion was observed at the site of infection 3 dpi (Figure 2A). On RR tomato fruits, on the other hand, tissue rotting, and fungal growth were already
FIGURE 2 | Tomato fruits susceptibility to B. cinerea obtained from plants grown under different nitrate concentrations. (A) Representative inoculated fruits (3 dpi) for each nitrate concentration and ripening stage (dpa, days post-anthesis). (B,C) Lesion size (diameter) for inoculated MG and RR fruits, respectively. For MG fruits, white arrows indicate the site of infection with minimal necrotic lesion. Different letters indicate significant differences among treatments at a given time point (p \leq 0.05; error bars indicate SEM; n = 4).

evident 3 dpi and extended into the pericarp tissue (Figure 2A). The severity of the disease symptoms (Figures 2B,C) and the accumulation of fungal biomass (Figures S2B,C) in infected MG and RR fruits from plants grown under N-limiting conditions were larger than those obtained from a N-sufficient regime. These results indicate that plants grown under N-limiting conditions exhibit enhanced susceptibility to B. cinerea infection.

Global Gene Expression Analysis Supports a Connection between Plant N Status and Susceptibility to Fungal Infection in Tomato

To better understand the molecular changes underlying the impact of nitrate availability on plant susceptibility to B. cinerea, we performed transcriptome-profiling assays on mock-treated and infected plants grown under severe limiting (2 mM) and sufficient (6 mM) N conditions, using GeneChip Tomato Genome Arrays (Affymetrix). We also included 12 mM nitrate in our analysis, as a higher N input. These experiments were performed using shoots since most plant defense responses have been described in this particular vegetative tissue (Glazebrook, 2005). As shown in Figure S3, our analysis suggests a significant interaction between the plant N-status and B. cinerea infection (Two-way ANOVA, p < 0.01), where 2110 genes showed significant Nitrogen/B. cinerea (N/B) interaction, representing approximately 4.7% of the analyzed tomato transcriptome (Table S1). Since this result suggests a nitrate and defense signaling crosstalk in response to B. cinerea infection, this set of genes was selected for further analysis.

Principal component analysis (PCA) (Figure 3A) showed that the first principal component (PC1) accounted for 45% of the total variation and segregated mock (M) from infected (B) samples, regardless of N-conditions. In addition, PC1 also allows the differentiation between N-status in B. cinerea-infected samples. The second principal component (PC2) accounted for 13% of the variation, and differentiated limiting vs. sufficient plant N-status in mock-treated samples. Indeed, mock-treated samples from plants grown under N-limiting conditions (2 mM nitrate) cluster more closely with B. cinerea-infected samples, rather than with non-infected samples, under both N-sufficient concentrations (6 mM and 12 mM nitrate). This result suggests that the transcriptome state of infected plants may be similar to N-limiting conditions. Moreover, the total number of differentially expressed genes (DEG) in response to pathogen infection was comparable among all N conditions evaluated (Figure 3B). For DEG analysis, genes with significant N/B model were used for a pairwise comparison between mock and infected samples in each N-condition, using Rank-products (p < 0.05). However, only 18% of up-regulated (22/123) genes were common to all N concentrations analyzed (Figure 3C). Although, the susceptibility phenotypes of plant grown under 2 mM and 6 mM nitrate were different in our experimental conditions, these N-conditions present more common up-regulated and down-regulated genes when compared with plants grown in 12 mM nitrate. These results suggest that higher N-input also affect
plant defense response, indicating a complex link between N-metabolism and *B. cinerea* infections.

**Differentially Expressed Genes under N-sufficient Condition supports better Defense Response in Tomato**

We performed a functional classification of the DEG in each N-condition (*Tables S2–S4*) using the Generic Gene Ontology (GO) Term Finder tool (Boyle et al., 2004). This analysis showed a wide range of biological processes that were affected due to fungal infection under different N-conditions (**Figure 4**). Processes associated with metabolism, both primary and secondary, were significantly affected by the pathogen under all N conditions analyzed (*p* < 0.01). This observation is consistent with previous reports that show that pathogens can reprogram host metabolism, strongly affecting primary, and secondary metabolism in plants (Kliebenstein et al., 2005; Rojas et al., 2014). **Figure 4B** shows a general overview of metabolic pathways affected by *B. cinerea* infections in each N-condition, using MapMan software (Thimm et al., 2004). Cell wall metabolisms were strongly affected by the fungus infections in plants grown in N-limiting conditions. In contrast, secondary metabolisms were most affected by the fungus infections in N-sufficient conditions. This finding is consistent with the fact that the accumulation of secondary metabolites is a plant defense mechanism triggered by several fungus pathogens (Kliebenstein et al., 2005; Ward et al., 2010; Smith et al., 2014; Pusztahelyi et al., 2015). Interestingly, we found that genes associated with N transporters (NRT2), N and amino acid metabolism were induced in response to *B. cinerea* infection depending on the plant’s N-status, with it occurring most in N-sufficient conditions (**Figure 4B**). In agreement with this observation, it has been shown in a recent report that the expression of several genes involved in N metabolism and its transport are strongly affected in response to infections with *B. cinerea*, *Phytophthora infestans*, *Phytophthora parasitica*, and *Pseudomonas syringae* (Fagard et al., 2014). Other biological functions modified by the *B. cinerea* infection, and specifically over-represented under N-sufficient conditions, were transport and oxidation-reduction processes (**Figure 4, p* < 0.01). Redox status significantly impacts both plant defenses and *B. cinerea* infection (Lamb and Dixon, 1997; Torres et al., 2006).

Our enrichment analysis also identified genes that encode proteins previously implicated in defense responses (AbuQamar et al., 2006; Cantu et al., 2009; Windram et al., 2012; Blanco-Ulate et al., 2013). This group of genes was closely examined...
to evaluate possible correlations between reduced expression of defense-related genes and susceptibility to <i>B. cinerea</i> infection. Table 1 shows a subset of these genes induced in plants grown under N-sufficient conditions or repressed under N-limiting conditions. These genes include anthranilate synthase 1 (ASA1), 2-oxophytodienoate reductase 3 (OPR3), and pathogenesis-related 4 (PR4). This latter gene encodes a protein with antifungal chitin-binding activity, which is repressed under N-limiting conditions and induced under N-sufficient or higher conditions. In Arabidopsis, this gene has been associated with resistance to necrotrophic pathogens (Catinot et al., 2015) and it is known to be induced by <i>B. cinerea</i> infection (AbuQamar et al., 2006). In addition, genes involved in JA and ET response were also identified. The ACC oxidase gene involved in ET biosynthesis...
was significantly induced during fungal infection in plants grown under N-sufficient conditions, but not at low nitrate concentrations (Table 1). Thus, a differential response of key defense-related genes under contrasting nitrate concentrations might partially explain the differences in susceptibility to B. cinerea in tomato leaves.

Network Analysis Identifies B. cinerea-responsive Regulatory Pathways Associated with the Plant N status

To identify functional relationships and key regulatory TFs among the N/B responsive genes, we performed a gene co-expression network analysis. Due to the limited knowledge of gene interactions in tomato, we decided to incorporate data available from A. thaliana. For ortholog group assignment, tomato sequences (gene models ITAG release 2.4, https://solgenomics.net), matching probe sets on the GeneChip Tomato Genome array, were aligned to the Arabidopsis genome using OrthoMCL (Li et al., 2003). For network inference, we used all Arabidopsis loci identifiers to predict putative protein-DNA interactions, making use of sequence preference information recently obtained for a large number of Arabidopsis TFs (Weirauch et al., 2014) and the gene co-expression database (Obayashi et al., 2014). As shown in Figure 5A, a network comprising 477 genes was obtained. Genes are represented as nodes connected by edges that depict predicted regulatory interactions. In this analysis, 9 co-expression modules were identified using the community cluster (Glay) algorithm in ClusterMaker tools (Morris et al., 2011). This algorithm recognizes functionally related groups and finds densely connected regions in a network (Su et al., 2010). In addition, we found statistically overrepresented biological functions in 7 of these modules using a hypergeometric test in BINGO tools (adjusted p < 0.05 by FDR). Interestingly, network includes functions important for plant defense against necrotrophic fungi, such as stress response, oxido-reduction processes and cell wall and wax biosynthesis processes. Moreover, genes related to ET signaling and the responses to this hormone were also identified as overrepresented in these modules, consistent with

| ID Number | Description | Genome ID | AGI number | Fold change (log2) |
|-----------|-------------|-----------|------------|-------------------|
| **STRESS RESPONSE** | | | | |
| LesAffx.22491.2.A1_at | Flavonoid 3-hydroxylase | Solyc12g042480.1.1 | AT3G07990 | 0.4 3.4 4.1 |
| LesAffx.50270.1.S1_at | Strictosidine synthase-like | Solyc02g082900.2.1 | AT3G1420 | 1.1 4.2 3.8 |
| LesAffx.50270.2.S1_at | Strictosidine synthase-like | Solyc02g082900.2.1 | AT3G1420 | 0.8 4.1 3.7 |
| Les.3652.1.S1_at | Beta-1,3-glucanase | Solyc10g079860.1.1 | AT3G57270 | 0.7 3.5 3.9 |
| Les.3673.1.S1_at | Cytochrome P450 | Solyc12g042480.1.1 | AT4G38620 | –1.4 1.5 2.9 |
| Les.4966.1.S1_at | Response to stress | Solyc09g075070.2.1 | AT1G02850 | –1.6 1.3 2.2 |
| **DEFENSE RESPONSE** | | | | |
| Les.3406.1.S1_at | Chitinase | Solyc10g058800.1.1 | AT3G12500 | –0.2 2.8 2.7 |
| Les.248.1.S1_a_at | Pathogenesis-related 4 | Solyc01g097270.2.1 | AT3G04720 | –0.4 2.7 2.7 |
| Les.3683.1.S1_at | Thaumatin, pathogenesis-related | Solyc08g086200.1.1 | AT4G11650 | 1.1 1.1 2.5 |
| Les.5035.1.S1_at | LRR receptor-like | Solyc01g005730.2.1 | AT1G47890 | 0 1.9 2.5 |
| Les.22.1.S1_at | Oxophytodienoate reductase 3 (OPR3) | Solyc10g086220.1.1 | AT1G76690 | –1.4 0.4 0.3 |
| **OXIDATION REDUCTION PROCESSES** | | | | |
| LesAffx.71628.1.S1_at | Thioredoxin superfamily protein | Solyc03g112770.2.1 | AT5G63030 | –0.3 0.4 0.4 |
| Les.3172.1.S1_at | Glutathione S-transferase | Solyc01g102660.2.1 | AT2G02390 | –0.4 1.1 1.4 |
| Les.2746.2.A1_at | Glutathione S-transferase | Solyc09g011510.1.2 | ND | 3.1 2.8 0.6 |
| Les.132.1.S1_at | ACC oxidase | Solyc07g049500.2.1 | AT1G05010 | –2.9 2.2 3.3 |
| LesAffx.5010.2.S1_at | Cytochrome b561 | Solyc03g025840.2.1 | AT4G25570 | –0.7 0.1 0.2 |
| Les.3449.1.S1_at | Glutathione synthetase | Solyc01g098610.2.1 | AT5G27380 | –2.0 –0.4 0.1 |
| Les.1925.1.A1_at | Glutathione S-transferase | Solyc05g013950.1.1 | AT1G19570 | –3.1 0.7 0.7 |
| Les.2955.1.A1_at | Glutathione S-transferase | Solyc02g068900.2.1 | ND | –0.9 –0.3 0.4 |
| **HORMONE PATHWAY** | | | | |
| Les.132.1.S1_at | ACO4 ethylene-forming enzyme | Solyc07g049500.2.1 | AT1G05010 | –2.9 2.2 3.3 |
| Les.3465.1.S1_at | Ethylene response regulator | Solyc09g089610.2.1 | AT3G23150 | 0.6 1.2 2.0 |
| Les.3818.1.S1_at | Ethylene responsive transcription factor | Solyc09g089950.1.1 | AT3G23240 | –0.7 2.6 2.2 |
| LesAffx.50351.1.S1_at | Anthranilate synthase | Solyc06g006100.2.1 | AT5G07530 | –0.6 –0.1 0.8 |
| Les.3632.1.S1_at | Lipoxigenase family protein 4 (LOX4) | Solyc03g122340.2.1 | AT1G72520 | 3.5 2.6 0.6 |

*Fold change in gene expression for each gene upon B. cinerea infection are referred to mock-treated samples grown under the same N condition (2, 6, and 12 mM nitrate).
FIGURE 5 | Network analysis of transcriptomics data predicts a B. cinerea-responsive regulatory gene network affected by the plant N-status. (A) Network analysis of genes with significant N/B ANOVA model. Genes are presented as triangles (TFs) and rectangles (target genes). Colors are used to distinguish (Continued)
the finding describing how ET responses are important for *B. cinerea* resistance in tomato leaves (Diaz et al., 2002). In addition, we found several TFs belonging to the ERF (Ethylene Response Factor) family, which have been implicated in the plant defense response to *B. cinerea* (AbuQamar et al., 2006; Windram et al., 2012). Nevertheless, susceptibility of tomato fruit to *B. cinerea* had been also associated in part with ET pathways (Cantu et al., 2009; Blanco-Ulate et al., 2013), suggesting complex and tissue-specific ET regulatory networks in response to fungus infections.

In order to distinguish the effect of the plant’s N-status in the defense response against *B. cinerea* (Figure 5A; N/B ANOVA model) from the general plant response to fungal infection irrespective of its N condition (B ANOVA model), we highlight genes with a significant B (*B. cinerea*) ANOVA model within the mentioned network (Figure 5B). When Figures 5A,B were compared, we noticed that only 40% of the mentioned N/B network responded to the infection as a single (B) factor, supporting the link between fungus infection and N response. Interestingly, the majority of the TFs present in the network also link to this interaction (in Figure 5B, note that most TFs at the center of each module are not highlighted in yellow).

To identify TFs that may control relevant functions in the N status-dependent response to *B. cinerea*, we focused on TFs and those putative targets possessing significant co-expression (Figure 5C). We applied a 90th percentile cut-off in co-expression networks considering the absolute value, such that only the highest scoring 10% of edges were selected. A total of 28 TFs are present in the N/B network, from which 22 were highly connected within its center and might represent important regulators of the N/B interaction. Interestingly, TFs within the network and their respective putative targets are mostly involved in transport, oxide-reduction processes and stress and defense responses. In addition, within this group of regulatory proteins, we also identified overrepresented biological functions related to the response to ET and JA.

**The Plant N Status Modulates the Expression of TFs Belonging to Families Involved in Plant Defense**

The results depicted in Figure 5 support the importance of plant N metabolism in plant defense pathways. To validate this prediction, we analyzed the expression levels of 18 TF-encoding genes in leaves or fruits infected by *B. cinerea*, from tomato plants grown under all nitrate concentrations used throughout this work (Table S5). An RT-qPCR analysis of selected TFs supported microarray results and network analysis (Figure 6). Hierarchical clustering analysis of the RT-qPCR data revealed 6 clusters. Cluster #1 (Figure 6, in gray), composed of four TF-encoding genes (NAC-like, WRKY15, BIM, and WRKY70), groups genes that are induced by fungal infection under N-sufficient conditions, but repressed in leaves or marginally induced in fruits of plants grown under N-limiting conditions. In Arabidopsis, WRKY 15 and WRKY 70 (Figure 5, Modules 3 and 8, respectively) have been reported as induced when infected with *B. cinerea*. Interestingly, wrky70 mutant plants showed enhanced susceptibility to this fungus, in a SA dependent manner (AbuQamar et al., 2006).

*B. cinerea* also triggered the induction of zinc-finger TFs (ZAT) genes in leaves, under all N-conditions (Figure 5, Module 7; Figure 6, Cluster #4). ZAT genes are induced in abiotic stress conditions and are required for cytosolic ascorbate peroxidase expression during oxidative stress in Arabidopsis (Rizhsky et al., 2004). These regulatory genes may be involved in the oxidative burst triggered by the pathogen or in oxidation-reduction processes to protect the plant.

Infection by *B. cinerea* enhanced the expression of several TFs involved in the ET response (EIN3, ERF5, and ERF13; Figure 5, Module 1; Figure 6, Cluster #3). ERF5 was slightly induced by the fungal infection, most likely in RR fruits. Among the TFs associated with the ET response, several TFs belong to the ERF family, have been associated with stress responses (McGrath et al., 2005) and as modulators of the SA/JA crosstalk (Van der Does et al., 2013). Interestingly, in infected leaves, ERF1 (Ethylene Response Factor 1; Figure 6, Cluster #2) was significantly induced in N-sufficient conditions and repressed in low N conditions. ERF1 has been implicated in JA and ET signaling, and pathogen defense (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). In addition, the Jasmonate-ZIM-Domain 1 gene (*JAZ1*; Figure 5, Module 5; Figure 6, Cluster #3) was also induced by the fungus infection in leaves under all analyzed N-conditions. This gene is involved in JA-signaling and, recently, its role in plant defense has been described (Li et al., 2014). These different patterns of expression of TFs related with hormone responses, suggest the existence of several regulatory pathways interacting in a complex and highly dynamic manner, linking plant N status, and the plant defense response.

**DISCUSSION**

Although, different agronomic reports indicate that the amount of N fertilization can modulate disease severity caused by pathogens in numerous plant species (Snoeijers et al., 2000; Walters and Bingham, 2007; Dordas, 2008; Fagard et al., 2014), the underlying mechanisms are poorly characterized. In general terms, high N supply decreases the severity...
Despite these general trends, several reports show contradictory evidence, suggesting a complex relationship linking N metabolism and disease infection processes. In this report, we investigated the role of plant N nutrition in the outcome of the plant-pathogen interaction, employing two important agronomic models: *B. cinerea* and *S. lycopersicum*.

We showed that tomato plants grown under contrasting nitrate conditions exhibit differential susceptibility to fungal infection. We used nitrate as a nitrogen source, since it is the most important N source for plants in agricultural soils (von Wirén et al., 2000) and it plays an important role as the major nitrogen resource in tomato plants (Wang et al., 2001; Fu et al., 2015). In agreement with previous studies, tomato plants grown under N-sufficient or higher conditions showed a significantly increased susceptibility to *B. cinerea* (Hoffland et al., 1999; Lecompte et al., 2010). We extended previous results to include fruits and showed that there is reduced susceptibility to *B. cinerea* in fruits at two developmental stages (MG and RR) from plants grown under N-sufficient conditions, suggesting that the nitrogen/disease connection might extend beyond vegetative tissues.

The induction of the plant defense response is an energetically costly process (Berger et al., 2007). As such, different studies have suggested that the major role of the plant’s primary metabolism during the plant-pathogen interaction is to support the increased energy requirements brought about by the defense response (Bolton, 2009; Kangasjärvi et al., 2012), and some pathogens in early steps of infections trigger a nutrient-limiting environment (Mathioni et al., 2011). In this scenario, it is reasonable to hypothesize that “well nourished” plants may defend better, as observed in tomato grown under N-sufficient conditions. This observation sharply differs from crop plants that present less susceptibility under low nitrogen availability (Davidson et al., 2007; Lecompte et al., 2013). Notably, as observed from our global gene expression profiling, plant metabolic processes were significantly affected by *B. cinerea*, irrespective of the nitrogen condition used to grow the plants. This finding is consistent with previous reports showing that the plant-pathogen interaction regulates plant metabolism (Ward et al., 2010; Rojas et al., 2014). In order to establish a favorable energy balance for defense, it has been suggested that the up-regulation of defense-related genes is compensated by the down-regulation of genes involved in other metabolic pathways (Berger et al., 2007; Massad et al., 2012). In agreement with what has been reported for *B. cinerea* infections in *A. thaliana* (AbuQamar et al., 2006; Windram et al., 2012), several genes involved in photosynthesis were down regulated in our tomato experiments in response to the fungus infection, under all N conditions.

In the context of plant-pathogens interactions, the nutrition of the plant can have an effect on secondary metabolite production. Plant N-status modifies amino acid accumulation involved in the biosynthesis of defense-associated secondary metabolites, such as flavonoids and phenylpropanoids (Ward et al., 2010; Fagard et al., 2014). Phenylpropanoid pathways regulate the resistance against *B. cinerea* in the *sitiens* tomato mutant (Seifi et al., 2013). Accordingly, we found several genes involved in this pathway up-regulated by the fungus infections in N-sufficient conditions. In addition, the amino acid content and its relative concentration are modified during the plant disease process, suggesting that amino acid metabolism can impact plant-pathogen interactions. Recently, reports show that genes
involved in amino acid metabolism play a role in plant defense responses to pathogens (Hwang et al., 2011; Seifi et al., 2014). N-metabolism and amino acid homeostasis can modulate the plant redox status affecting the plant disease response (Liu et al., 2010). These results suggest that N metabolism could modulate—at least in part—plant defense responses affecting cellular redox status as well as secondary metabolite pathways.

The relevance of N and amino acid metabolism has been highlighted before in the context of plant-pathogen interactions (Hwang et al., 2011). For instance, several studies have shown that N-related gene expression is altered by pathogens (Pageau et al., 2006; Masclaux-Daubresse and Daniel-Vedele, 2010). Interestingly, the Arabidopsis lht1 (lysine histidine transport 1) mutant is more resistant to a large spectrum of pathogens, an observation that is associated with an altered redox status (Liu et al., 2010). In this regard, we found several genes associated with redox status, N-metabolism and transporters affected by B. cinerea infections, depending on plant N-conditions. NRT2, a putative high-affinity nitrate transporter, was induced only in N-sufficient and higher conditions. Interestingly, in Arabidopsis, two NRT2s have been reported as involved in plant defense (Camañes et al., 2012; Dechorgnat et al., 2012) and the Arabidopsis nrt2.6 mutant exhibits higher sensitivity to the necrotrophic bacterium Erwinia amylovora. The susceptibility of this mutant appears to be related to reduced ROS accumulation (Dechorgnat et al., 2012), again linking redox status, N metabolism and defense. Similarly, we found that oxidation-reduction processes were over-represented among the biological functions responding to the fungal infection, under N-sufficient and higher conditions. Recently, nitrate reallocation in plants has been proposed as a regulator of the trade-off between plant growth and environmental adaptation (Zhang et al., 2014). All these results suggest that nitrate metabolism and transport have an impact on the plant’s defense response, and that the nitrogen-defense connection goes beyond a direct metabolic relationship. Whether, this can be explained by N-derived alterations in the redox status of the plant requires further investigation.

Different plant hormones may provide an additional layer of regulation, underlying—at least in part—the complex relationship between nitrogen metabolism and plant defense response. It has been described that plant hormones play a role in the defense against B. cinerea (AbuQamar et al., 2006; Windram et al., 2012). Specifically, genetic studies on Arabidopsis and tomato indicate that JA and ET are key regulators of this defense response. Treatments with ET in tomato plants increased resistance to this fungus in leaves, while mutants in JA biosynthesis showed increased susceptibility to B. cinerea in leaves (Díaz et al., 2002; AbuQamar et al., 2008). Consistent with these prior findings, genes involved in ET response were identified by the GO term analysis in our network of B. cinerea-responsive genes associated with the plant N status. Likewise, several TFs associated with the JA/ET response were identified, including ERF1 and ERF5. The latter acts as a positive regulator of JA/ET-responsive defense genes against B. cinerea in Arabidopsis (Berrocal-Lobo et al., 2002). Notably, we found that ERF1 was induced in response to B. cinerea under N-sufficient conditions, while repressed in plants grown under N-limited conditions. This TF has been described as a key regulator of JA/ET signaling, activating plant defense responses against B. cinerea in leaves (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003) and necrotrophic Rhizopus nigricans in RR tomato fruit (Pan et al., 2013). Moreover, ERF1 gene expression increases in response to B. cinerea infections in MG tomato fruit but is reduced in infected RR fruit (Blanco-Ulate et al., 2013). MG and RR tomato fruits show different susceptibility responses against this fungus infection, each associated with specific expression profiles of genes involved in ET and others hormone biosynthesis (Blanco-Ulate et al., 2013). More studies are necessary to assess the regulation of this TF in response to infections in different tissues.

Although, the plant hormone SA has been associated with the resistance to biotrophic pathogens, its role in plant defense against B. cinerea is not completely clear. On the one hand, the exogenous application of SA has been reported to result in decreased susceptibility to this fungus in tomato (Audeaert et al., 2002) and Arabidopsis (Ferrari et al., 2003), suggesting that SA affects the resistance against this fungus. On the other hand, the stearoyl-ACP desaturase (sii2) Arabidopsis mutant, which exhibits increased SA levels and higher expression of the PRI1 gene, displayed, increased susceptibility to B. cinerea (Kachroo et al., 2001; Nandi et al., 2005). High resolution temporal transcriptomic analysis of the Arabidopsis defense response against this fungus, revealed complex regulatory networks, with different timings for JA, ET, and SA signaling (Windram et al., 2012) as well as the accumulation of plant phytoalexins and other defense-related proteins and molecules (Van Baarlen et al., 2004; AbuQamar et al., 2006; Scalschi et al., 2015). Our data support a role for these signaling pathways connecting N status and infection. For instance, the silencing of the OPR3 gene enhances susceptibility to B. cinerea by affecting the JA biosynthesis pathway (Scalschi et al., 2015), and we found this gene to be repressed under N-limiting conditions. The WRKY70 TF, which is up-regulated in response to B. cinerea infection, plays a role in SA-JA crosstalk, activating SA-induced genes and repressing JA-responsive genes (Li et al., 2004). Interestingly, the wrky70 mutant is more susceptible to this fungus (AbuQamar et al., 2006), since notably, a gene encoding for a putative WRKY70 TF in tomato is the most repressed gene in response to B. cinerea, as shown here, but only under low N availability, a condition in which higher susceptibility to the fungus was observed.

Taken together, the findings reported here suggest that N metabolism affects different mechanisms, including redox status, secondary metabolites and plant hormones (JA and ET), the latter of which globally alter the expression of defense-related genes. In sum, these mechanisms together modulate disease susceptibility and hence the outcome of the plant-pathogen interaction under different N conditions.

MATERIALS AND METHODS

Growth Conditions of Tomato Plants

Solanum lycopersicum cv. MicroTom plants were cultivated under different N growth conditions employing 16:8 h light:dark cycles (150 micromoles/m²/s) at 20°C. Plants seeds were spread...
in plastic pots containing sterilized vermiculite, previously equilibrated with Murashige & Skoog (MS) modified basal salt mixture without nitrogen (PhytoTechnology Laboratories). This nutrient solution was supplemented with different concentrations of nitrate. Six mM nitrate was found to give maximal growth and thus considered as N-sufficient condition, while 2 mM and 4 mM nitrate were used as severe and mild N-limiting conditions, respectively. To evaluate a higher N input, tomato plants were grown using 12 mM nitrate. Four nutrition solutions—each containing the mentioned nitrate concentrations—were prepared and added to each pot every 2 days, employing the same volume (50 ml). Shoot biomass (dry weight) and N concentration (Dumas method) were determined from an average of 8 plants after 4 weeks of growth. Fruits were tagged at 2-day post anthesis (dpa) and harvested at 32 and 40 dpa as mature green and ripening red (MG and RR, respectively). A color chart was employed to confirm ripening stages.

**Botrytis cinerea Growth Conditions and Virulence Assays**

The necrotrophic fungus used in this study was *B. cinerea* strain B05.10. This strain was routinely cultivated in Petri dishes containing potato dextrose agar (PDA, AppliChem) with 10% homogenized bean leaves. Conidia employed for virulence assays were obtained from an agar plug followed by glass-wool filtration using Gamborg B5-2% glucose (Duchefa Biochemie) medium. For virulence assays, 4-week-old tomato plants grown under different nitrate conditions were inoculated in the third and fourth fully expanded leaves in planta as described (Cantu et al., 2008; Canessa et al., 2013). Briefly, conidia were suspended in Gamborg B5-2% glucose medium and adjusted to a final concentration of $5 \times 10^5$ conidia/ml in the same medium supplemented with 10 mM KH$_2$PO$_4$/K$_2$HPO$_4$, pH 6.4. Conidial suspensions ($10 \mu$l) were used to inoculate leaves of six tomato plants obtained from each N condition analyzed. All plants were incubated inside plastic boxes at 20°C employing the growth conditions mentioned above, under humid environment for the indicated periods of time. Lesions were measured using the ImageJ software employing an external calibration scale (Schneider et al., 2012). On the other hand, fruits were inoculated at the day of harvest, as described (Cantu et al., 2008; Canessa et al., 2013). Briefly, fruits were first disinfected using 10% (v/v) bleach, followed by three consecutive water rinses. Thereafter, fruits were carefully punctured (2 mm depth, 1 mm diameter) employing a sterile needle at five sites per tomato. Subsequently, fruits were inoculated with 10 µl of conidia suspension ($5 \times 10^5$ conidia/ml). Tomatoes used as mock material were inoculated with the same volume of Gamborg B5-2% glucose-10 mM KH$_2$PO$_4$/K$_2$HPO$_4$ solution. All fruit samples were incubated at 20°C in high humidity, during the indicated periods of time (3, 4, and 5 dpi). At the end of the infection period, tomato lesions were measured as mentioned above. The evaluation of tomato susceptibility to *B. cinerea* was independently performed three times employing at least 4 fruits for each experimental condition.

For quantification of fungal development on plant tissue, DNA from leaves or fruits for each experimental condition was isolated following a method for genomic DNA described by Edwards et al. (1991). For real time PCR quantification, 25 µl samples were prepared containing 10 µl of DNA solutions and 300 nM of appropriate primers. Real-time PCR reactions were run in triplicates using the Brilliant SYBR Green QPCR Reagents on a StepOnePlus™ Real-Time PCR System (Life Technologies), as described bellow in RT-qPCR section. The sequences of the primers used in this study are detailed in Table S6. Serial dilutions of pure genomic DNA from each species were used to develop a calibration curve, as described (Gachon and Saindrenan, 2004).

**RNA Isolation**

Total RNA was extracted from both mock-treated and fungus-infected tomato leaves and fruits, following a CTAB-spermidine extraction protocol (Reid et al., 2006). In the case of leaves, a total of 12 leaves per N treatment were pulled together. This procedure was repeated three times ($n=3$) and further used in microarray analysis and RT-qPCR experiments (see below).

For fruits, total RNA was prepared from the combined fruit outer pericarp and epidermis, within approximately 0.25 cm radius around the *B. cinerea* inoculation sites. Each biological replicate ($n=3$) consisted of an independent pool of samples from four different fruits. RNA samples were further quantified and analyzed by microfluidic analysis employing the Agilent Technologies’ 2100 Bioanalyzer, following the manufacturer’s instructions.

**Microarray Analysis**

For microarray hybridizations, three mock-inoculated and three fungus-infected biological replicates from tomato plants grown under 2, 6, and 12 mM nitrate were selected for global gene expression analysis. Five hundred nanograms of total RNA was processed for microarray hybridization using the GeneChip one-cycle target-labeling kit (Affymetrix), according to the manufacturer’s instructions. The fragmented aRNA (7.5 µg) was hybridized on a GeneChip Tomato genome array (containing 10038 tomato probe sets for more than 9200 tomato genes) using standard procedures (45°C for 16 h).

The arrays were washed and stained in a Fluidics Station 450 (Affymetrix). Array scanning was carried out with the GeneChip scanner 3000 and image analysis was performed using the GeneChip Operating Software. Thereafter, GeneChip array data were quality assessed using a set of standard quality control steps described in the Affymetrix manual “GeneChip Expression Analysis: Data Analysis Fundamentals.” Array data were processed and normalized with RMA (Robust Multi-Array Average) (Irizarry et al., 2003) using the affy R package (Gautier et al., 2004). Approximately 60–75% of probe sets were significantly detected in all microarray hybridizations. To evaluate array reproducibility, spearman rank coefficients were computed and ranged between 0.97 and 0.99. The raw data for all hybridizations can be found in NCBI’s Gene Expression Omnibus (Edgar et al., 2002).
and are accessible through GEO Series accession number GSE73006.

To determine DEG in response to B. cinerea infection affected by the N-conditions, normalized data were subjected to a Two-way analysis of variance (ANOVA) with adjusted \( p < 0.01 \) by Benjamini and Hochberg false discovery rate correction (Benjamini and Hochberg, 1995). For ANOVA analysis, we used a model with expression \( Y \) of a given gene \( i \) calculated as \( Y_i = \beta_0 + \beta_1B + \beta_2N + \beta_3N/B + \epsilon \), where \( \beta_0 \) is the global mean, and where \( \beta_1, \beta_2, \) and \( \beta_3 \) are the effects of the B. cinerea infection, the N-condition and the interaction between these two factors (N/B), respectively (Table S1). The variable \( \epsilon \) corresponds to the unexplained variance. In addition, to identify DEG between two conditions (e.g., infected and control samples at each N-condition) the Rank-Product method was used (\( p < 0.05 \)) (Breitling et al., 2004; see Tables S2–S4).

### Functional Annotation of Differentially Expressed Genes (DEG)

For the assignment of functional annotations and categorizations for DEG, we employed the easy to use web tool Generic Gene Ontology (GO) Term Finder (Boyle et al., 2004). For this purpose, we constructed a custom-made GO database employing InterProScan 5 (Jones et al., 2014) and all predicted protein coding genes in the S. lycopersicum genome database (gene models ITAG release 2.4, https://solgenomics.net).

### Gene Network Analysis

Genes possessing a significant Nitrogen/B. cinerea (N/B) interaction factor were selected to carry out a gene network construction (Table S1). First, we perform an analysis of ortholog group assignment between tomato and A. thaliana. For this purpose, tomato sequences (gene models ITAG release 2.4), matching to probe sets on the array were aligned to the Arabidopsis genome, using OrthoMCL (Li et al., 2003) orthologs genes were assigned. Thereafter, with all Arabidopsis loci identifiers, a gene co-expression network was constructed using 11,171 microarray experiments obtained from the ATTED-II database (Obayashi et al., 2014), accessed in March 2015. Co-expression values were calculated using weighted Pearson's correlation coefficient, as described in Obayashi et al. (2014). This database collects gene expression data in Arabidopsis from a wide range of microarray experiments. We included protein–DNA interactions (Weirauch et al., 2014), considering at least one TF binding site in the upstream gene region (1000 bp) and over-representation of the TF binding site (two standard deviations) above the mean occurrence in all the upstream sequences in the genome. To improve the regulatory interaction predictions, the protein–DNA interactions were filtered to include only transcription factor/target pairs for which co-expression values are highly (cut-off above the 90th percentile considering absolute value of gene co-expression) (Obayashi et al., 2014) and significantly correlated (\( p < 0.05 \)) in our microarray experiment. The resulting network was visualized using CYTOSCAPE (Data Sheet S1; Shannon et al., 2003).

### RT-qPCR and Clustering Analysis of Gene Expression Data

cDNA synthesis was carried out using the Improm-II reverse transcriptase according to the manufacturer’s instructions (Promega). RT-qPCR was carried out using the Brilliant SYBR Green QPCR Reagents on a StepOnePlus™ Real-Time PCR System (Life Technologies). For mRNA levels normalization, the PROTEIN PHOSPHATASE 2A catalytic subunit (PP2Ac, SGN-U567355) was used (Løvdal and Lillo, 2009; Dekkers et al., 2012). The expression of this reference gene was stable in our microarray data (CV = 2%) and in microarrays in response to B. cinerea in tomato fruit at two developmental stages (MG and RR, CV = 3% Cantu et al., 2009).

Total RT-qPCR reaction volume was 25 \( \mu l \), containing 2.0 \( \mu l \) of cDNA template and 140 nM of each primer. The reactions were performed under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C, 30 s; 55°C, 30 s; and 72°C, 30 s, followed by a melting curve analysis from 55 to 95°C. Fluorescence values were acquired during the annealing period of the RT-qPCR procedure. All experiments were performed with three biological replicates, with their corresponding three technical replicates. To determine the mRNA levels in response to the B. cinerea infection, infected leaves and fruits from plants grown under 2, 4, 6, and 12 mM nitrate were compared to their corresponding mock-treated control grown under same culture conditions. Genes showing a similar expression pattern were analyzed and visualized using the average-linkage hierarchical clustering performed in Cluster 2.11 software, as described (Eisen et al., 1998).

### AUTHOR CONTRIBUTIONS

PC, GH, IR, AV performed and analyzed virulence assays; GH, AV performed and analyzed microarray experiments; PC and AV conducted the GO analysis of gene expression data; TM, JC, AV conducted bioinformatics analysis for gene network construction; IR, GH, JR, AV, PC completed RT-qPCR analysis; AV, PC, and RG analyzed all data and wrote the paper.

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### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00911

**Figure S1 | Tomato plants grown under contrasting N conditions. (A)** Shoots biomass (average) of MicroTom tomato plants (4-weeks-old) grown under contrasting N conditions. **(B)** N percentage of plants, determined as dry weight (see Materials and Methods; \( n = 4 \)). Different letters indicate significant differences among treatments (\( p < 0.05 \); error bars indicate SEM).
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Audenaert, K., Patter, T., Cornelis, P., and Höfte, M. (2002). In duction of systemic defenses (ITAG2.4, Sol Genomics Network, https://solgenomics.net) and their annotations, Arabidopsis (TAIR, http://arabidopsis.org) or GeneBank accessions, the p-value adjusted by FDR and the log2-fold changes of the comparison between B. cinerea infected and mock-treated sample (B/M) in each N-conditions (2, 6, and 12 mM nitrogen).

Table S1 | Microarray expression data for genes with N/B ANOVA model (adjusted p < 0.01). The table includes the Affymetrix probes, the tomato accessions (ITAG2.4, Sol Genomics Network, https://solgenomics.net) and their annotations, Arabidopsis (TAIR, http://arabidopsis.org) or GeneBank accessions, the log2-fold changes of the comparison between B. cinerea infected and mock-treated sample (B/M) in each N-conditions (2, 6, and 12 mM nitrogen).

Table S2 | Microarray expression data for genes that are either up- or down-regulated by the B. cinerea infection in comparison to mock-inoculated plants grown in N-limiting conditions (2 mM nitrate) (ANOVA; Rank-products, p < 0.05). The table includes the Affymetrix probes, the tomato accessions (ITAG2.4, Sol Genomics Network, https://solgenomics.net) and their annotations, Arabidopsis (TAIR, http://arabidopsis.org) or GeneBank accessions, the p-value, Gene Ontology and the log2-fold changes of the comparison between B. cinerea infected and mock-treated sample (B/M) grown in N-limiting conditions (2 mM nitrate).

Table S3 | Microarray expression data for genes that are either up- or down-regulated by the B. cinerea infection in comparison to mock-inoculated plants grown in N-sufficient conditions (6 mM nitrate) (ANOVA; Rank-products, p < 0.05). The table includes the Affymetrix probes, the tomato accessions (ITAG2.4, Sol Genomics Network, https://solgenomics.net) and their annotations, Arabidopsis (TAIR, http://arabidopsis.org) or GeneBank accessions, the p-value, Gene Ontology and the log2-fold changes of the comparison between B. cinerea infected and mock-treated sample (B/M) grown in N-sufficient conditions (6 mM nitrate).

Table S4 | Microarray expression data for genes that are either up- or down-regulated by the B. cinerea infection in comparison to mock-inoculated plants grown in N-sufficient conditions (12 mM nitrate) (ANOVA; Rank-products, p < 0.05). The table includes the Affymetrix probes, the tomato accessions (ITAG2.4, Sol Genomics Network, https://solgenomics.net) and their annotations, Arabidopsis (TAIR, http://arabidopsis.org) or GeneBank accessions, the p-value, Gene Ontology and the log2-fold changes of the comparison between B. cinerea infected and mock-treated sample (B/M) grown in N-sufficient limiting (12 mM nitrate).

Table S5 | Candidate TF used for RT-qPCR analysis. The table includes the Arabidopsis (TAIR, http://arabidopsis.org) and the tomato accessions (Sol Genomics Network, https://solgenomics.net), the gene names, the module in network analysis and changes in relative expression (log2) between infected and mock-treated samples in leaves and fruits (MG and RR) from plants grown under different N-conditions (2 mM, 4 mM, 6 mM, and 12 mM nitrate).

Table S6 | Primer sequences used for qPCR.

Data Sheet S1 | Gene network in cytoscape.

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